BIOAVAILABILITY AND BIODEGRADABILITY OF DISSOLVED ORGANIC NITROGEN ORIGINATED FROM MUNICIPAL AND ANIMAL WASTEWATER

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Title

BIOAVAILABILITY AND BIODEGRADABILITY OF DISSOLVED ORGANIC NITROGEN ORIGINATED FROM DOMESTIC AND ANIMAL WASTEWATER

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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. In both wastewater sources, ABDON efficiencies (%) 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*. 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.

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LIST OF ABBREVIATIONS

ABDON .....Bioavailable dissolved organic nitrogen

ANPC.....Animal nutrition physiology center

AS .....Activated sludge

B.....Bacteria

BDON .....Biodegradable dissolved organic nitrogen

BNR .....Biological nutrient removal

DDW.....Distilled deionized water

DI .....Deionized

DIN .....Dissolved inorganic nitrogen

DNH_3-N .....Dissolved ammonia nitrogen

DNO_2-N .....Dissolved nitrate nitrogen

DNO_3-N .....Dissolved nitrite nitrogen

DON.....Dissolved organic nitrogen

DON_f.....Final dissolved organic nitrogen after incubation

DON_i .....Initial dissolved organic nitrogen before incubation

MLSS .....Mixed liquor suspended solids

MW .....Molecular weight

NH_3 .....Ammonia

NH_4^+.....Ammonium

NO_2^-.....Nitrite

NO_3^-.....Nitrate

R.....Microalgae *Chlamydomonas reinhardtii*

S .....Microalgae *Selenastrum capricornutum*

SMPs .....Soluble microbial products

TDN .....Total dissolved nitrogen

TF.....Trickling filter

TN .....Total nitrogen

V.....Microalgae *Chlorella vulgaris*

WWTP .....Wastewater treatment plant

CHAPTER 1. INTRODUCTION

1.1. Background

Excess nitrogen in water environment are mainly linked to human activities, including agricultural uses of fertilizer, application of manure, and discharge of municipal wastewater. Nutrient over-enrichment in receiving waters affects ecological systems by stimulating harmful algal blooms. The overabundance of algae in water ecosystems reduces water transparency, creates oxygen-deprived aquatic zones, and ultimately leads to death of dwelling plants and fishes. Dissolved organic nitrogen (DON) is one of the dominant nitrogen forms in surface waters and its natural and/or anthropogenic inputs increases deterioration of the water quality.

DON in aquatic systems originated from both non-point and point sources. Field research and modelling studies suggest that DON in groundwater is mainly released by sandy sediments through digenetic reactions (Gobler and Sanudo-Wilhelmy 2001). Atmospheric DON deposition is another great non-point contributor to watershed and the concentration is greatly affected by human activities and seasonal changes (Zhang et al., 2012). Wastewater effluent from point sources is another significant DON contribution to surface waters.

Current knowledge on the fate and characterization of wastewater derived DON are limited. Biologically enhanced nitrogen removal process largely reduces nitrogen load by transforming dissolved inorganic nitrogen (DIN) to free nitrogen via denitrification process. Thus, DON in advanced wastewater treatment plant (WWTP) effluents becomes the major component in effluent total dissolved nitrogen (TDN). In final effluent of tertiary (advanced) WWTPs, DON concentration ranges between 0.3 and 1.8 mg-N/L which could comprise between 25 and 70% of final effluent TDN (Pehlivanoglu Mantas and Sedlak, 2006; Sattayatewa et al., 2009; Simsek et al., 2012). However, the level of DON from other point and non-point

sources such as wastewaters from animal feeding operations and agricultural runoff are still poorly investigated.

Several studies have been conducted to characterize the compositions and properties of DON in last decade (Pehlivanoglu Mantas and Sedlak. 2008). Nevertheless, identifying the composition of DON at any given time in a treatment plant remains as a great challenge. In primary effluent, proteins are considered as the major group of effluent organic nitrogen (Westgate and Park, 2010). In secondary biological treatment, researchers have confirmed that chelating agents and soluble microbial products (SMPs) can be produced by microorganisms within the process (Parkin et al., 1981; Westgate and Park et, 2010). In treated effluent prior to discharge, Pehlivanoglu Mantas and Sedlak (2008) reported that only about 30% of DON have been identified, which contained organic compounds such as amino acids, dimethylanine (DMA), and ethylenediaminetetraacetic acid (EDTA), while 70% of DON remained unknown. However, organic nitrogen forms in treatment plants effluent are mainly driven by different biological treatment methods. Biological nitrification process can reduce the concentration of hydrophobic portion of DON, while advanced treatment processes, such as powdered activated carbon and soil aquifer treatment, could substantially remove all kinds of organic matters which results in the change of DON composition fraction (Krasner et al., 2009; Chen et al., 2011).

Previous studies evaluated the impact of effluent DON to algal and bacterial species in receiving water environments. Wastewater-derived DON is both biodegradable and bioavailable to bacteria and/or algae in bioassays experiments. Biodegradable DON (BDON) and bioavailable DON (ABDON) have been determined in different stages of wastewater along the treatment train as well as in various biological nutrient removal (BNR) systems, such as activated sludge (AS), anaerobic-anoxic-oxic process (AAO), Bardenpho reactor, trickling filter (TF), and membrane

biological reactor (Urgun-Demirtas et al. 2008; Sattayatewa et al., 2009; Liu et al., 2012; Huo et al., 2013; Simsek et al., 2013; Qin et al., 2015). These studies showed that the BDON and ABDON ranged widely between 28 and 86% of influent DON. Meanwhile, DON became more bioavailable to algae in the presence of bacteria.

Green alga *Selenastrum capricornutum* (*S. capricornutum*) is considered as a test species by the U. S. Environmental Protection Agency (EPA) to examine wastewater nutrients and toxicity. Adopted from EPA Printz Algal Assay Bottle Test, *S. capricornutum* has been commonly applied in bioassay studies to determine the bioavailability of wastewater (Urgun-Demirtas et al. 2008; Sattayatewa et al., 2009; Liu et al., 2012. Simsek et al., 2013). The lack of information on the bioavailability of DON to other algae species limits the complete understanding of ABDON. Therefore, the overall goal of this study was to investigate the bioavailability of DON to three different algal species, which were *S. capricornutum, Chlamydomonas reinhardtii (C. reinhartii)* and *Chlorella vulgaris (C. vulgaris)* with/without bacteria addition in municipal wastewaters and two different algal species, which were *C. reinhartii* and *C. vulgaris* with/without bacteria addition in animal wastewaters.

1.2. Research Objectives

The main scope of this research was to investigate bioavailability of DON to different algal species with/without presence of bacteria. The specific objectives were as follows:

1. To examine DON, ABDON, and BDON in wastewaters collected from a two-stage trickling filter WWTP using three different algal species: *S. capricornutum*, *C.reinhardtii*, and *C. vulgaris* with/without the presence of mixed culture bacteria.

2. To investigate DON, ABDON, and BDON in wastewaters collected from an animal feeding operation center storage tank and from a sheep wastewater storage lagoon system using

two different algal species, which were *C.reinhardtii* and *C. vulgaris* with/without the presence of mixed culture bacteria.

1.3. Hypotheses

The corresponding hypotheses based on the study objectives, are:

1. Bioavailability and biodegradability of DON to three different algal species are different. DON is more bioavailable to algae + bacteria compare to algae only inoculum.

2. DON in agricultural wastewater has similar characterization as domestic wastewater which is bioavailable to algae and/or bacteria.

1.4. Thesis Organization

This thesis contains 5 chapters. Chapter 1 describes the general introduction and overall objectives of the study. Chapter 2 provides an overview of related background and previous literature on DON, ABDON, and BDON. Chapter 3 and 4 describe the ABDON and BDON study in municipal and animal agricultural wastewaters, respectively. Lastly, Chapter 5 provides an overall conclusions and further recommendations.

CHAPTER 2. LITERATURE REVIEW

2.1. Dissolved Organic Nitrogen

2.1.1. Definition, Determination, and Environmental Impact

Nitrogen is an essential nutrient source for plant and animal nutrition and it controls the productivity of aquatic ecosystem. Optimal amount of nitrogen is important to natural surface waters, such as lakes, streams, rivers, estuaries, and oceans; however, in high concentrations it can be a contaminant in the water environment. DON pool in aquatic ecosystem consists of various N-containing functional organic compounds, such as nucleic acids, amino sugars, proteins, and humic substance (Berman and Bronk, 2003). Molecular weight fractionation of DON is an approach to understand the bioavailability of DON. In oceans, about 30% of marine DON is comprised as high molecular weight (HMW) DON which consists of great fraction of amide-N compounds and small portion of humic substances (Repeta et al., 2002; Aluwihare et al., 2005). In municipal wastewater effluent, low molecular weight (LMW) nitrogen compounds that have a molecular size less than 1000 Dalton cutoff, such as free and combined amino acids play a dominant role (50-78%) of the secondary effluent DON (Parkin and McCarty, 1981; Pehlivanoglu-Mantas and Sedlak, 2008).

DON behaves as a dynamic participant in N cycle which is biochemically transformable through ammonification, nitrification, and denitrification by bacteria first to ammonia N (NH_3-N) and nitrate N (NO_3-N), and then back to atmospheric N. In surface waters, the chemical compositions and properties of DON largely depend on its origination from numerous natural and anthropogenic sources, atmospheric deposition, and autochthonous production. Therefore, different DON issues that include DON determination methods, structural composition, sources,

sinks, and environmental impact of DON are main challenges remained, which requires significant consideration.

Since there is not a direct method available to measure DON, one major challenge on DON characterization is the lack of reliable measurement methods to quantify it. Generally, DON is determined indirectly from the mass-balance equation of nitrogen (Equation 2.1.). To increase the accuracy of DON determination, the measurement variance of dissolved ammonia N (DNH_3-N), dissolved nitrite N (DNO_2-N), dissolved nitrate N (DNO_3-N), and TDN must be reduced. TDN is commonly determined using persulfate digestion method that was firstly developed by Delia et al. (1977). In this method, nitrogenous compounds are converted into DNO_3^- with oxidizing reagent under alkaline conditions. In 1992, Crumpton et al. determined DNO_3^- using second derivative UV spectrophotometric methods (SDUS) at the peak of 224 nm. Eckford and Fedorak (2002) further suggested that the SDUS method was reliable to determine DNO_3^- when wastewater contained organic compounds. Sattayatewa et al. (2011) modified and described a simple protocol to measure TDN combining persulfate oxidation and the second-derivative UV spectrometry method. This protocol proved high accuracy on DNO_3^- measurement at very low DON concentration (0.05-3 mg-N/L, Sattayatewa et al., 2011).

$$DON\ (mg\text{-}N/L) = TDN - DNH_3\text{-}N - DNO_2\text{-}N - DON_3\text{-}N \qquad (Eq.\ 2.1.)$$

The accuracy of measuring DON can be further improved by using pretreatment methods that consequently minimize DIN and maximize DON level in wastewater sample. Dialysis pretreatment by membrane (nanofiltration and reverse osmosis) and adsorption process (ion-exchange resin) are considered as the most promising methods for low level DON (in µg-N/L) such as marine water. However, between 4 and 21% of organic matters including DON compounds can be lost during the pretreatment process. In addition, the interference of DOC and

DIN forms becomes another remaining challenge (Crumpton et al., 1992; Vandenbruwane et al., 2007; Xu et al., 2010; Sattayatewa et al., 2011).

2.2. Biodegradable Dissolved Organic Nitrogen (BDON) and Bioavailable Dissolved Organic Nitrogen (ABDON)

2.2.1. Definition, Determination, and Environmental Impact

BDON reveals the portion of DON that can be taken up by bacteria, while ABDON expresses the portion of DON that can be minimized by algae-only or algae + bacteria inocula. The level of wastewater BDON and ABDON rely on different influential factors that include bioassay incubation time, inoculum types, dissolved oxygen level, and the origin of wastewater. Excess amount of BDON and ABDON in surface waters can potentially cause algal growth and dissolved oxygen depletion and ultimately cause eutrophication. These two variables (BDON and ABDON) evaluate the potential environmental effect of wastewater-derived DON to river and estuaries. Therefore, characterizing BDON and ABDON can provide a better understanding for the impact of DON to aquatic environment.

In general, DON from autochthonous sources, such as urban runoff, animal feedlot runoff, and wastewater associated with human activities, are more bioavailable to bacteria and algae, while DON from forest, wetlands, and soil leaching are less bioavailable (Seitzinger and Sanders, 1997 and 1999; Bronk et al., 2010). Certain organic compounds such as urea can be readily converted to ammonium carbonate and it can be found as ammonium instead of urea in aquatic system. Some portions of DON are readily biodegradable and/or bioavailable to bacterial communities in biological treatment systems, while some portions of it remain recalcitrant (Seitzinger and Sanders, 1997; Bushaw-Newton and Moran, 1999; Koopmans and Bronk, 2002; Vahatalo and Zepp, 2005; Pehlivanoglu-Mantas and Sedlak, 2006). In addition, previous

investigations have proved that a great portion (between 50 and 85%) of the refractory DON becomes biodegradable and/or bioavailable to living organisms when the optimum environmental conditions, such as the concentration of initial DON, residence time, type and amount of bacterial and algal communities, DO level, and temperature, are met (Koopmans and Bronk, 2002; Pehlivanoglu and Sedlak, 2004; Vahatalo and Zepp, 2005; Urgun-Demirtras et al., 2008; Sattayatewa et al., 2009; Simsek et al., 2013).

BDON and ABDON determination methods have been developed to evaluate the bioavailability and biodegradability of DON under different experimental conditions (Pehlivanoglu and Sedlak, 2004; Urgun-Demirtas et al. 2008; Khan et al., 2009; Simsek at al., 2012). BDON and ABDON are calculated as the differences of DON values before and after incubation. BDON relies on the changes of initial DON (DON_i) and final DON (DON_f) in bacteria-only (B-only) seeded samples (Equation 2.3.) (Simsek et al., 2012). The ABDON calculation relies on the changes between DON_i and DON_f in algae with/without bacteria inoculated samples before and after the incubation period (Equation 2.2.) (Simsek et al., 2013). Blank corrections (inoculum controls) for either bacteria or algae bioassays and the blank samples are carried throughout the experiments. Final calculations are included blank for DON_i (DON_{bi}) and blank for DON_f (DON_{bf}) (Equations 2.2. and 2.3.).

$$BDON\ (mg\text{-}N/L) = [(DON_i - DON_f) - (DON_{bi} - DON_{bf})] \quad \text{(Eq. 2.2.)}$$

$$ABDON\ (mg\text{-}N/L) = [(DON_i - DON_f) - (DON_{bi} - DON_{bf})] \quad \text{(Eq. 2.3.)}$$

Studies have emphasized on wastewater BDON and ABDON from different aspects of initial wastewater treatment methods, various length of incubation period, and different types of bioassay inoculum (Pehlivanoglu and Sedlak, 2004; Khan et al., 2009; Sattayatewa et al., 2009; Simsek et al., 2013). Prior to determining DON, BDON, and ABDON, the initial samples are

filtered using different pore size of membrane filters including 0.2, 0.45, and 1.2 μm filter sizes depended on the experimental design (Pehlivanoglu and Sedlak, 2004; Urgun-Demirtas et al. 2008; Simsek et al., 2013). Particulate fraction of organic matters that greater than 0.45 μm usually contains various suspended solids, such as algae, protozoa, and bacterial cells, while the portion of DON passed through 0.45 μm is mainly cell fragment and macromolecules including proteins and lipids (Shon et al., 2006). Smaller pore-size filter (such as 0.2 μm) can further eliminate the bacteria, while retaining more organic nitrogen constituents from wastewater samples (Khan et al., 2009).

Different lengths of incubation periods from 14 to 180 days have been applied in bioassay tests (Pehlivanoglu and Sedlak, 2004; Khan et al., 2008). Short incubation periods (5 days), which have been applied in BOD and DOC determination, did not achieve sufficient biodegradation of DON. Then, 14 days (2-week) of incubation period were used to determine BDON and ABDON in the study of Pehlivanoglu and Sedlak (2004) and Urgun-Demirtas et al. (2008). Khan et al. (2008) described a protocol to determine BDON choosing a 20-day incubation period for tertiary treated effluent. They suggested that there was limited BDON exertion after 20 days. Simsek et al. (2012 and 2013) selected 28 days of incubation period to determine wastewater derived DON in primary effluent. Long term incubation such as 60 or 180 days could not contribute to additional biodegradability in bioassay experiment. Nevertheless, long term incubation in the environment may cause the release of DON from phytoplankton and the increase of DON in wastewater (Khan et al., 2009; Sattayatewa et al., 2009).

Both natural inoculum and highly selected bacteria from WWTPs have been applied in BDON and ABDON assay experiments. To represent the real environmental conditions, Sattayatewa et al. (2009) filtered the local river water and used the retentate on the 0.2 μm filter

as natural bacteria. Liu et al. (2012) further selected the bacteria that passed through 1.0 µm pore-size filter and retained on 0.2 µm membrane filter. Moreover, Khan et al. (2009) used mixed liquor suspended solids (MLSS), which contained raw wastewater and activated sludge in their experiments. They investigated different concentrations (30, 60, 120, 240 mg/L) of MLSS in the BDON assay suggesting that MLSS at 240 mg/L was most appropriate for BDON assay. Later, Sattayatewa et al. (2009) and Simsek et al. (2012) further adopted this approach using diluted MLSS and primary wastewater as bacterial inoculum for bioassay.

DON pretreatment of wastewater did not greatly affect the BDON and ABDON assay results. Sattayatewa et al. (2011) prepared both ion-exchange rinsed and untreated wastewater. For pretreated effluent, during BDON and ABDON assay, additional mineral nutrients were added to wastewater to support essential algal growth since nitrate and phosphorus were removed in the ion-exchange resin. BDON and ABDON results were recorded slightly higher in pretreatment of wastewater than they were in untreated wastewater. In terms of degradation, BDON and ABDON showed similar first order degradation rate in both treated and untreated wastewater.

Bioassay is an important approach to study DON and it has been conducted to analyze different nitrogen species in laboratory condition. In the tests, nitrogen uptake by algae largely depended on the bioavailability and energetically expense of different nitrogen forms. In general, ammonium is the most favorable form for algal utilization since it requires less energy and enzyme (He et al., 2013). Lower ammonia concentration (<50 mg/L) is generally preferred by microalgae since high ammonia concentration can inhibit photosynthesis (Abeliovich and Azov, 1976). Moreover, nutrient utilization efficiencies by algae in bioassay experiments are mostly affected by initial nutrient concentration, cell inoculate density, pH, light exposure time and

intensity, and temperature (Cai et al., 2013). The pH value around 7 to 7.5 were most optimal for *C. vulgaris* and *C. reinhardtii* growth, however, *C. reinhardtii* still achieved sufficient growth under pH condition from 6.5 to 9.5 (Kong et al., 2010).

Pehlivanoglu and Sedlak (2004) applied *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 for algae in 14 days.

In ABDON assay tests, algae *S. capricornutum* have been mostly applied to determine wastewater ABDON in previous studies (Pehlivanoglu and Sedlak, 2004; Urgun-Demirtas et al., 2008; Sattayatewa et al., 2009; Simsek et al., 2012). *S. capricornutum* (also called as *Pseudokirchneriella subcapitata; Monraphidium capricornutum; Raphidocelis subcapitata;* and *Ankistrodesmus bibraianus*) is considered as a standard species in the laboratory bioassay.

Culturing *S. capricornutum* is relatively easy under lab condition. In Printz Algal Assay Bottle Test protocol developed by EPA, the recommended *S. capricornutum* culture conditions suggested to control temperature at 25 ± 1℃, light intensity about 4306 lux, and continuous agitation rate at 100 rpm (Miller et al., 1978). However, single test species (*S. capricornutum*) inhibits the comprehensive understanding of the fate and characterization of DON and ABDON.

Other microalgae species such as *C. reinhardtii*, *Scenedesmus obliquus*, and *C. vulgaris* have proved high nutrient removal efficiency and great environmental adaptability in varies wastewaters (An et al., 2003; Kong et al., 2010; Ruiz-Marin et al., 2010). These algae have been used in the studies to remove nitrogen in wastewaters with a wide range of initial concentrations. Both *C. vulgaris* and *C. reinhardtii* showed high TDN removal and were tolerant to high ammonia. *C. vulgaris* removed 23 to 100 % of TDN in wastewater with initial TDN of 13 to 410 mg/L, while *C. reinhardtii* showed 42 to 83% of TDN removal in animal wastewater with an initial TDN of 130 mg/L (Aslan and Kapdan, 2006; Kong et al., 2010). Besides *S. capricornutum*, *C. reinhardtii* have also been used as a test species by EPA in the method, which was a short-term method to estimate the chronic toxicity of effluents and receiving waters to freshwater organisms (Lewis et al., 1994). Those microalgae species can further applied in ABDON assay test.

Bioavailability of DON to *C. vulgaris* and *C. reinhardtii* have not been fully understood; however, studies have demonstrated that when inorganic N (ammonia and nitrate) is limited in the sample, algae species can utilize certain organic nitrogen compounds such as urea and amino acid (Hodson et al., 1969; Happe and Naber, 1993). Hodson et al. (1969) applied 15N labeled tracer experiments and found that *C. vulgaris* metabolized urea and sequentially formed ammonia under dark aerobic condition. Under certain conditions, uptake of compounds, which

occurred via active transport, was caused by the concentration gradient when the organic nitrogen level was high. For large MW compounds, enzyme activates which mainly include amino acid oxidation and peptide hydrolysis, are necessary to breakdown proteins and produce small compounds. Munoz - Blanco et al. (1990) observed that *C. reinhardtii* utilized the L-amino acid directly and further oxidized it into ammonia to support algal growth. In addition, leucine amino peptidase, which is capable of hydrolyzing proteins, have been found on cell wall of *C. reinhardtii* indicating the existence of external proteolytic enzyme activities to hydrolyze peptides (Langheinrich, 1995).

2.3. DON, BDON, and ABDON in Natural Water Ecosystem

DON consists of a great proportion of TDN in natural streams, lakes, and marine ecosystem. The fraction of DON in surface waters is higher than other constituents in the N pools, such as particulate organic nitrogen (PON), ammonium, nitrate, and nitrite (Berman and Bronk, 2003). In general, DON concentrations in rivers and lakes are below 100 μM-N, while the DON to TDN ratio could show a wide range (8 to 83%) (Hopkinson et al., 1998; Seitzinger and Sanders, 1997). Even though DON concentrations in some selected rivers (Parker River, Childs River, Susquehanna River, and Satilla River) in Georgia and Maryland were low that varied between 23 and 56 μM-N, the fraction of DON to TDN was relatively high that varied between 19 and 94% of TDN (Hopkinson et al., 1998).

DON in surface waters can be released from natural inputs, such as forest floor, sediments, and atmospheric deposition. DON can also be naturally released via biological processes of cell lysis and bacterial transformation from soil and sediments (Berman and Bronk, 2003). In some studies, DON is the dominant nitrogen form in leaching of forest floor and soil (Michel and Matzner, 1999; Lapworth et al., 2008). Additionally, human intervention has great

impact on the fraction of DON. Significant amount of DON in rivers and lakes originated from anthropogenic sources which were associated with human activities, such as municipal and industrial discharges, agriculture wastewaters from livestock operations, and irrigation runoff with nitrogen fertilizer application, which may further affect the water quality in estuaries and coastal areas.

Perakis and Hedin (2002) observed that DON contributed about 80% of TDN in the rivers of an unpolluted forest in South America, while DON:TDN ratio was greatly reduced to 20% in polluted forest of North America. Boyer et al. (2006) has observed that riverine DON contributed 33 to 37% of TDN to coastal area, while significant amount of it originated from municipal and agricultural sources. In summer, the increase of surface flow input from municipal and agricultural sources resulted in the change of water salinity and the seasonal variation of DON:TDN ratio in the river of Southeast England as higher DON fraction was observed in summer than in winter (Badr et al., 2008). In addition, studies on fog and rainfall have suggested that DON contributed 20 to 65% of atmospheric TDN, while the majority of DON was produced during fossil fuel combustion processes (Cornell et al., 2003; Boyer et al., 2006).

Transportation and accumulation of DON in downstream surface waters can greatly affect the living organisms in a water body since DON is a primary constituent in aquatic systems. The fate and amount of DON transportation and transformation in surface waters relied on different factors that include the spatial and temporal variations of surface water, residence time of the nutrient and living organisms in the water, light intensity and exposure time, initial DON loading rate, temperature, dissolved oxygen level, and the type of bacterial and algal communities.

DON in marine ecosystems was firstly believed to be largely refectory and unavailable for algae and microorganism uptake. Earlier studies reported that DON had relatively fast turnover cycle and very slow degradation rate (<0.005 day^{-1}μM, Jackson and Williams, 1985). The traditional belief was that if DON was an available N source to phytoplankton or bacteria, the concentration of DON wouldn't have been depleted. Later, with isotopic techniques, which were developed in 1970s, studies showed that DON was regenerated by phytoplankton due to the uptake of inorganic nitrogen (NH_4^+, NO_3^-). Similar DON release rate and uptake rate results in the stable concentration of DON in marine environment over the time and spaces scales (Bronk and Glibert, 1993; Bronk et al., 1994; Berman and Bronk, 2003; Bronk et al., 2010).

In fact, blooms of phytoplankton were observed in the waters that contained high DON and low DIN levels (Keller and Rice 1989; Gobler and Sanudo-Wilhelmy 2001). In long term analyses, researchers noticed that enrichment of DON concentration resulted in the enhanced growth of alga *A. anophagefferens* and caused the destructive brown tides in northeast U.S. embayment (LaRoche et al., 1997; Golber et al, 2002; Gilbert et al., 2007). Studies have found that some phytoplankton can hydrolyze DON substrates directly, while others can utilize DON indirectly by deriving N source from DON pool with extracellular enzyme (Berg et al., 2002; Mulholand et al., 2004).

The bioavailability and biodegradability of DON from different sources of rivers, atmosphere, and estuaries have been investigated (Wiegner and Seitzinger, 2001; Badr et al., 2008). LaRoche et al. (1997) found that relatively high DON level (35 μM) in the Long Island bay stimulated the brown tide blooms (*A. anophagefferens*) in 1980s. By observing decrease of DON and an increase of algal cells, authors suggested that brown tide were more likely to occur in drought year when DON was more readily available than surface water DIN sources.

Furthermore, DON associated with anthropogenic sources tends to be more bioavailable than DON in natural sources. Seitzinger et al. (2002) examined that the bioavailability of DON was observed in sources of urban rainfall runoff, agricultural runoff, and forest, referring that DON in urban runoff was the most bioavailable to phytoplankton.

Some phytoplankton species were found to be able to hydrolyze organic compounds, such as amino acid, urea, peptides, and proteins in natural environment (Berg et al., 2002; Gobler et al., 2002; Glibert et al., 2007). Wiegner et al. (2006) determined bioavailability of DON to phytoplankton in nine rivers of eastern U.S. They applied natural bacteria in river water samples under dark condition for 6 days of incubation. The results showed that 37 to 49% of initial DON was available to bacteria even though DIN existed in the samples. However, it was more common that riverine DON becomes a primary nitrogen sources for algae and microorganisms when inorganic nitrogen was limited. Another in-situ study conducted by McCarthy et al. (1997) investigated the nitrogen preference of phytoplankton in the Chesapeake Bay over a 13 months period. Their result indicated that N uptake sequence of phytoplankton was ammonium > urea > nitrate.

Photochemical decomposition of DON is another pathway to provide N source to algae and bacteria. Under sunlight exposure, low MW compounds (ammonia, nitrate, and dissolved primary amines) were released from effluent organic nitrogen and substantially uptake by algae in a photochemical release assays (Bronk et al., 2010). Labile bioavailable N were produced from biologically recalcitrant DON via photochemical reactions to support N-limited plankton growth (Vähätalo and Järvinen, 2007).

Overall, monitoring the entire large DON pool via the bioassay approach has issues remained. The main difficulty is that bioassay studies can measure only a small portion of DON

uptake while it was difficult to represent the large DON uptake rates and the DON regeneration rate (Bronk et al., 2007). Current available data on BDON and ABDON is not enough to understand the behavior and characterization of BDON and ABDON from point and non-point sources. Further characterization of organic nitrogen should be performed.

2.4. DON, BDON, and ABDON in Anthropogenic Sources

2.4.1. Municipal Wastewater Treatment Plants

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).

The concentration of DON and BDON decreases along the treatment trains. In WWTP influent, DON level ranged between 5.1 and 9.0 mg-N/L, which consisted of 2.3 to 4.4% of dissolved combined amino acids (DCAA), 0.6 to 1.0% of dissolved free amino acids (DFAA), and other organic compounds. Huo et al. (2013) have observed limited DON removal (0 to 10%) in primary clarifiers, while most DON was removed within biological treatment process.

Certain portion of organic matters can be removed during the biological treatment. Studies have investigated the fate of DON and BDON in different operational systems to understand removal and biodegradability of wastewater-derived DON (Sattayatewa et al., 2009; Simsek et al., 2012; Huo et al., 2013). Simsek et al. (2012) determined the fate of DON and

BDON removal in a trickling filter plant. During the treatment process in the plant, about 62% of influent DON and 72% of influent BDON were removed mainly in the BOD and nitrification treatment units. BDON in treatment train found between 51 and 69% of DON, which were high enough to be consider as critical for the stringent TDN discharge limits. Moreover, Huo et al. (2013) examined DON and BDON level in an anaerobic, anoxic, and oxic treatment plant. About 78% of influent DON was removed in the study, while the major DON reduction (70%) was achieved in anaerobic process. They also observed 84% of DFAA and 48% of DCAA removal referring that low molecular weight (MW) DON, such as urea and amino acid, were effectively removed during the biological process (Huo et al., 2013).

Based on previous studies, DON removal efficiency ranging from 60 to 80% indicates that a portion of DON is refractory and remains resistant to degrade during the biological wastewater treatment process (Simsek et al., 2012; Huo et al., 2013). A portion of refractory DON (0.1 to 0.2 mg-N/L) in wastewater treatment plants originated from drinking water sources (Lee et al., 2006). As treated drinking water was used during human activities, DON remained and further sent to WWTPs. Pehlivanoglu-Mantas and Sedlak (2006) reported that around 10% of DON in wastewater effluent was originated as disinfection by-product N-nitrosodimethylamine (NDMA), which was produced from water treatment plants. Results showed that more than 50% of NDMA was biodegraded after 30 days of incubation in the bioassay study. They further suggested that during the BDON assay a portion of refractory DON in effluent can be minimized by microbial activity. In addition, Westgate and Park (2010) confirmed the existence of refractory DON by measuring protein concentration and protein profiles in effluent along the treatment trains. They found that some high MW proteins (50 to

150 kDa) remained in both primary and secondary effluents that were considered as recalcitrant DON.

Moreover, some researchers observed that some portion of DON can be newly generated in different treatment units of the BNR process. In secondary biological treatment, with the appearance of low protein bands (25 to 50 kDa), Westgate and Park (2010) indicated that soluble microbial products (SMPs), which consisted of mainly amino acids and proteins, were produced via microbial activities. In the aerobic process of an activated sludge reactor, Czerwionka et al. (2012) found that $DON_{0.1\,\mu M}$ was released by anoxic microorganisms during the nitrification process. However, Sattayatewa et al. (2010) observed an increase of DON in primary anoxic zone which may resulted from anoxic microbial metabolism. In the BNR effluent, around 90% of DON was produced in biological treatment process (Pehlivanoglu-Mantas and Sedlak, 2006). To control effluent DON concentration, it is necessary to investigate the optimal operating conditions for microbial processes to reduce SMPs concentration and minimize the release of DON.

Chen et al. (2011) evaluated the transformation of effluent DON in a river water that receives wastewater effluent from a WWTP, and found that DON concentration decreased along the length of the river. They determined 17 and 35% of DON removal in summer and winter, respectively, within the 14.3 miles length of the river. Finding higher biodegradability of DON in winter was unexpected by the authors, since bacterial activity must be reduced during the winter compare to summer. However, it was discovered that 3.9 mg/L free chlorine was applied to the wastewater effluent in summer but not in winter. They speculated that formation of chloramines during the chlorination altered the biodegradation of DON. Overall, they concluded that water

quality changes in an effluent dominated river Chen et al. (2011). The fate and behavior of effluent DON and its impact to the natural ecosystems requires further studies on DON.

2.4.2. Animal Wastewater

Livestock wastewaters generated from concentrated animal feeding operations, such as dairy, poultry, swine, and beef feedlots, are crucial agricultural point sources. TDN level in animal wastewater can reach to thousands in mg-N/L, while N mainly existed in the form of ammonia and organic nitrogen. The organic load from animal wastewater is much higher than domestic sewage wastewater.

Anaerobic digestion process is commonly considered as an effective way to treat animal wastewater organic matters which can stabilize organics and produce methane gas. Hydrolysis is the first step in anaerobic digestion. During hydrolysis, organic polymers (proteins and lipids) are breakdown to mono and oligomers (sugars, amino acids, peptides) which become available to other bacteria. Enzymatic and chemical pre-treatment during digestion can help improve the biodegradation degree and rate of bacteria. For example, in swine wastewater, the level of dissolved organics was increased by 26.6 and 2.5% by adding cellulose (*Trichoderma reesie*) and protease enzymes (*Bacillus licheniforms,* Lee et al., 2008). Acidic (HCl) and alkaline (NaOH) treatment of swine wastewater can also promotes the breakdown of soluble organic matters. However, most studies focus on reduction of organics and production of methane gas. Further studies are necessary to investigate the effects of animal wastewater DON to aquatic and soil environment.

CHAPTER 3. BIOAVAILABILITY OF DISSOLVED ORGANIC NITROGEN TO GREEN MICROALGAE *SELENASTRUM CAPRICORNUTUM*, *CHLAMYDOMONAS REINHARDTII*, *CHLORELLA VULGARIS* AND BACTERIA IN MUNICIPAL WASTEWATER

3.1. Introduction

Biological availability of DON to bacterial and/or algal species in aquatic ecosystems accelerates DON transformation into highly soluble inorganic nitrogen forms include ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-). Some algal species preferred to utilize organic nitrogen species over nitrate (Berman and Chava, 1999; Mulholland et al., 2004). Hence, excessive concentrations of DON accumulation increase the readily bioavailable and biodegradable primary limiting nutrients in aquatic systems (Paeral et al., 1997; Seizinger and Sanders, 1997; Gobler et al., 2002, 2005). Lack of removing organic nitrogen remains potential for eutrophication that can result in algal blooms in receiving water. From mid-2000, brown tides in northeast U.S. estuaries were found correlated to DON level in receiving waters from modeling and long-term observations (MacIntyre et al., 2004; Trice et al., 2004; Glibert et al., 2007). The algal blooms had great impact to local marine ecosystems, fisheries, and public health (Cape et al., 2011). Therefore, removing wastewater derived DON is crucial to control the cumulative amount of nitrogen in surface waters.

Due to the complex structure of DON, reducing DON (either from influent wastewater or generated during the biological process) has not been successful. Around 20% of DON consists of compounds such as DCAA, DFAA, protein, urea, and ethylenediaminetetraacetic acids (EDTA), while the major portion of DON structure remains unknown (Berman and Bronk et al., 2003; Pehlivanoglu-Mantas and Sedlak, 2008; Huo et al., 2013). Researchers have confirmed

that some other compounds including chelating agents and soluble microbial products were produced by organisms during biological treatment process (Parkin et al., 1981; Westgate and Park, 2010). Therefore, understanding the composition and characterization of DON at any given time in treatment plant is still a great challenge.

ABDON examines the portion of DON which can be minimized by algae-only or algae + bacteria inocula. 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, 2006; 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 to grow in the laboratory conditions and has high efficiency to utilize the primary nutrients. *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 (van der Heever 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 of hazard organics (Staples et al., 2002) to quantify pollutants bioavailability. In this study, three different algal species, *S. capricornutum, Chlamydomonas reinhardtii* and *Chlorella vulgaris* and their combination with bacteria were used to obtain DON, ABDON, and BDON data in the samples collected from three different locations along the two-stage TF WWTP. The results were analyzed and compared to investigate if *C. reinhardtii* and *C. vulgaris* were also suitable to use as control species in aquatic environment.

3.2. Material and Methods

3.2.1. Samples Source, Collection, and Preparation

Wastewater samples were collected from the City of Fargo WWTP. The plant has a two-stage tricking filter process, which are biochemical oxygen demand (BOD) TFs and nitrification TFs, with a peak pumping capacity of 29 MGD and an average flow of 11-15 MGD. The facility consists of an influent pumping station, screening, grit removal, two pre-aeration channels, seven primary clarifiers, three BOD TFs, two intermediate clarifiers, two nitrification TFs, one final clarifier, chlorination, and de-chlorination units. The treated wastewater from the plant is discharged continuously to the Red River. The samples were collected from three different locations, which were after primary clarifier, after BOD TF, and after nitrification TF 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 about one hour after collection.

3.2.2 Algal and Bacterial Bioassay Preparation

Algal and/or bacterial inoculum were used to inoculate the wastewater samples. Three different algal species, which were *S. capricornutum, C. reinhardtii,* and *C. vulgaris* were used. The algae strains were obtained from UTEX (University of Texas Culture Collection of Algae, Austin, TX) and cultured in the laboratory as needed. The 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. Stock *S. capricornutum, C. reinhardtii,* and *C. vulgaris* strains were cultivated in 500 ml clear bottles at 25℃ under aerobic conditions. Algae were illuminated under fluorescent lamp (5400 Lm) for 12 hours light / dark cycle. All the glassware, media, and double de-ionized water (DDI) were autoclaved at 121℃ for 30 min before used in

each experiment. Stock *S. capricornutum, C. reinhardtii*, and *C. vulgaris* strains were cultivated in 500 ml clear bottles at 25℃ under aerobic conditions. Algae were illuminated under fluorescent lamp (around 5400 lm) for a 12 hrs. light/dark cycle. Cultured algal bioassay were harvested by centrifuging at 3000 rpm for 5 min and washed with DDI water twice before inoculation. Initial cell density in each sample was controlled around 1×10^5 cells/mL to achieve supplemental growth. Bacterial bioassay was prepared from influent (raw wastewater) of the city of Fargo WWTP, which contained returned bacteria from intermediate clarifier. Bacterial bioassay was also centrifuged at 3000 rpm for 5 min and rinsed with DDI water before using. *S. capricornutum, C. reinhardtii*, and *C. vulgaris*, bacteria were abbreviated as S, R, V, and B, respectively, in entire study.

3.2.3. Analytical Methods, ABDON and BDON Procedures

About 50 ml filtered samples were used to analyze the initial parameters, which were DNH_3-N, DNO_2-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. TDN was converted to nitrate after digestion and measured with SDUS method using UV-Visible spectrophotometer (APHA et al., 2005).

After determining initial parameters, all the samples were placed in 250 ml bottles for 14 and 21 days of consecutive incubation using algae-only, algae + algae, algae + bacteria, and bacteria-only seeds. Amber bottles were used to incubate the samples using bacterial-only inoculum, while clear bottles were used to inoculate the samples using algae-only, algae + algae, or algae + bacteria inoculum. The same parameters as in initial samples were measured and

finally ABDON and BDON were determined for both 14 and 21 days of incubation periods, respectively. The ABDON and BDON calculations relied on the change between initial DON (DON_i, DON before incubation) and final DON (DON_f, DON after incubation) values. The details of the ABDON and BDON methods were explained elsewhere (Simsek et al., 2012, 2013).

ABDON experiments in this study were divided into 8 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. BDON experiment was presented in only one portion, which was bacteria-only seeded sample. For the inoculation, 1.5 ml of algae and/or 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 one 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 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.

Cell density was measured during by using algae counting method during the incubation to evaluate algal growth. Samples were observed by ZEISS LSM microscope using 1ml haemacytometer chamber.

3.2.4. Statistical Analyses

Minitab 17 was used in this study for all statistical analyses. Sample means and standard derivations were calculated from the duplication for each treatment. One way analysis of

variance (ANOVA) table was conducted at $p \leq 0.05$ to evaluate the effects caused by three algal species with the presence and absence of bacteria.

3.3. Results and Discussions

Initial dissolved inorganic nitrogen, initial TDN, and initial DON were determined in the samples collected from all three locations in Fargo WWTP. Afterward, all the samples were seeded and incubated to determine final DON, ABDON, and BDON and all the results were presented in the Figures 4.1 to 4.4.

3.3.1. After Primary Clarifier

3.3.1.1. Dissolved Inorganic Nitrogen and TDN

The samples collected from after primary clarifier contained 33.74 mg-N/L of DNH_3-N (77% of TDN) and less than 0.5 mg-N/L of DNO_2-N + DNO_3-N (<3% of TDN). Average initial TDN measured as 42.53 mg-N/L. After 14 days of incubation, about 4.23 mg-N/L ammonia remained in the samples seeded with algae S while about 14.10 and 9.22 mg-N/L ammonia remained in the samples seeded with R and V, respectively. After 21 days of incubation, more than 99% of ammonia in all the samples were nitrified. In addition to ammonia already exist in the primary effluent samples, some portion of ammonia released through ammonification of DON and this portion of ammonia was also consequently nitrified. Figure 1a and b expressed that the big portion of ammonia was converted to nitrite and a certain portion of it was converted to following nitrate in algae and/or bacteria seeded samples.

Dissolved nitrite after 14 and 21 days of incubation was high in all algae and/or bacteria seeded samples (Figure 3.1a). Nitrite in all the samples after 14-day of incubation was varied between 21.55 and 35.00 mg-N/L, while it reduced to between 15.40 and 26.00 mg-N/L after 21-day of incubation. High nitrite accumulation in the samples possible occurred due to lack of air

during the incubation. González 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.

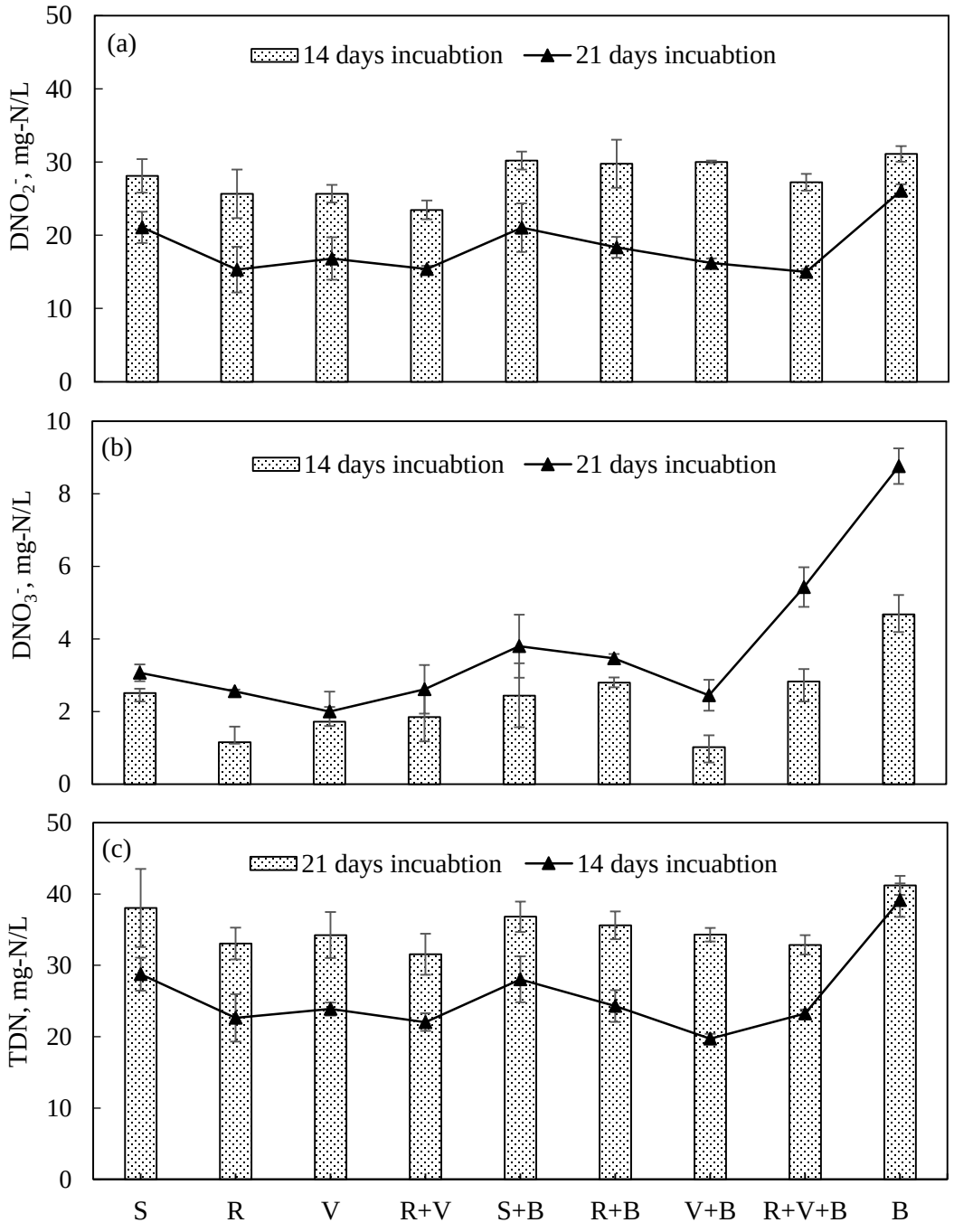

Figure 3.1. Algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B) inoculum for: (a) DNO_2-N, (b) DNO_3-N, (c) TDN, (d) DON in Fargo WWTP primary effluent after 14-day and 21-day incubation.

Additional experiments were conducted to monitor air influence on the partial nitrification by diluting the influent samples about 50% to reduce nitrogen loading and remained same volume/air ratio in the incubation bottle. The ammonia and TDN concentrations in these diluted samples were measured as average 15.6 and 23.41 mg-N/L, respectively. Diluted samples were incubated for 21 days and the results showed that between 72 and 91% of ammonia was nitrified into nitrate, while the nitrite level is extremely low (NO_2-N < 0.50 mg-N/L). This outcome proved that lower ammonia concentration required less air and mitigated the effect of partial nitrification. Furthermore, results showed that high nitrite concentration in the samples did not affect either algal growth or ABDON and BDON levels (Simsek et al., 2013).

Dissolved nitrate was low in all the samples seeded with algae and/or bacteria for both 14 and 21 days of incubation because of high nitrite level in the samples (Figure 3.1b). The highest nitrate value was recorded on the sample seeded with R+V+B, which was less than 3.0 mg-N/L. TDN levels were reduced in all the samples compare to the initial TDN value indicating that that algae and/or bacteria utilized nitrogen for their growth. (Figure 3.1c). However, this reduction was minimal for bacteria-only seeded samples (about 2 mg-N/L reduction). Previous studies also proved that algal-bloom intensity were declined in watershed ecosystem by reducing nitrogen load (Nuzzi and Waters, 2004; Gobler et al., 2005). 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). These results showed that algae *C. reinhardtii* and *C. vulgaris* could be used as a test species similar to algae *S. capricornutum*.

3.3.1.2. DON, ABDON, and BDON

Initial DON after primary clarifier sample was 8.96 mg-N/L, which comprised of 20% of initial TDN. After incubation, DON concentration was reduced in all the samples (Figure 3.2a). DON reductions in algae-only (S, R, and V) and algae + algae (R + V) seeded samples were not significant and the reduction was varied between 20.5 and 35.3% of initial DON. However, DON was reduced more in algae + bacteria (A+B, R+B, V+B, and S+R+V+B) seeded samples, 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. The magnitude of DON reduction in the samples after 14 and 21 days of incubation was similar in algae + bacteria seeded samples indicating that algae and bacteria interactions were essentially shorten the incubation period (Sattayatewa, et al., 2009; Simsek et al, 2013). DON reduction in bacteria-only seeded sample was recorded as 50.9%, which was higher compare to the same reduction in algae-only seeded samples indicating that bacteria-only seed reduced at least 15.6% more DON than algae-only seed did.

Bioavailable and biodegradable DON for 14 and 21 days of incubations were presented in Figure 3.2b. Bioavailability of DON was low in pure cultured algae *S. capricornutum, C. reinhardtii,* and *C. vulgaris*, varied between 3.5 and 5.9 mg-N/L. However, when those algae mixed with bacteria (S + B, R + B, and V + B), ABDON was increased significantly ($P \leq 0.05$) because of symbiotic relationship between algae and bacteria. In those algae + bacteria data, *S. capricornutum* + bacteria had the lowest ABDON value (6.5 mg-N/L) compare to other two types of algae, even though statistically was not different. The maximum attainable ABDON value could be the value of influent DON (average 8.96 mg-N/L). However, none of the results in Figure 2b achieved this ABDON value explained that a certain portion of DON remained as

recalcitrant DON in the sample. The highest ABDON value in Figure 3.2b observed in R + V + B inoculated sample as 8.27 mg-N/L, which was very close to the maximum average initial DON values of 8.96 mg-N/L. 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 maximum (100%) ABDON production through algae + bacteria was not attainable during the 14, 21, or 28 days of incubation period by using algae *S. capricornutum* as a test species. Other studies were 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; Pehlivanoglu-Mantas and Sedlak, 2006; Sattayatewa et al., 2009; Simsek et al., 2013). Furthermore, Figure 2b proved that the ABDON results for bacteria involved 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. BDON result (biodegradability to bacteria-only) after 14 and 21 days of incubation showed that about 66.3 and 77.3% of initial DON was biodegradable to bacteria, which was significantly higher than algae-only inoculated sample. On the contrary, BDON was lower than ABDON seeded algae + bacteria explained that certain portion of DON was degraded by bacteria and subsequently used by algae.

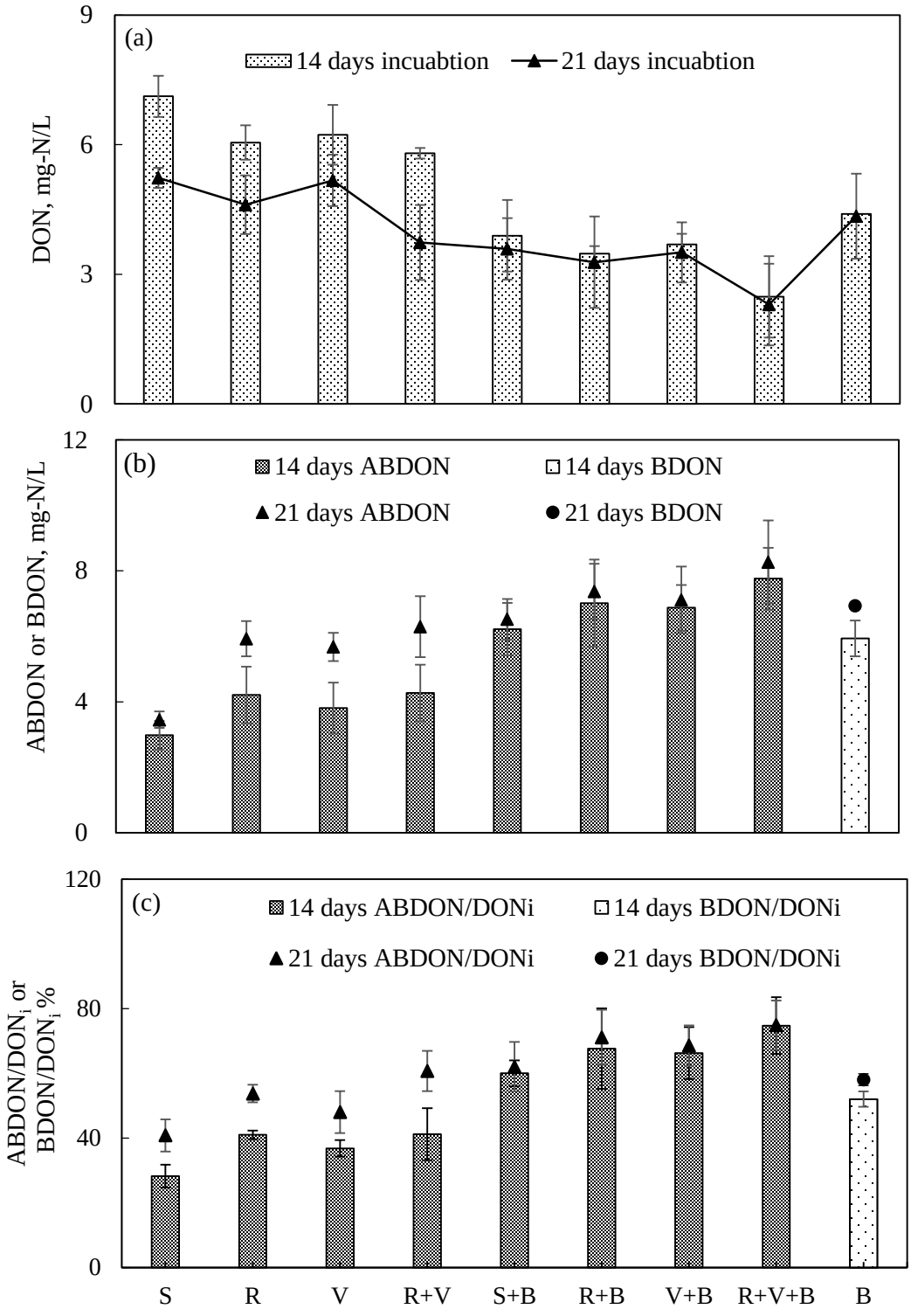

Figure 3.2. (a) DON, (b) ABDON and BDON, and (c) ABDON or BDON as a fraction of DON_i after 14 and 21 days of incubation using algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B) inoculum in Fargo WWTP primary effluent.

Initial DON fraction of ABDON and BDON were presented in the Figure 3.2c. 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 for this location. These results expressed that 14 days of incubation for algae + bacteria is appropriate to attain the maximum ABDON level (Urgun-Demirtas et al, 2008; Pehlivanoglu, E. and Sedlak, D.L., 2004), while 21 days of incubation is more appropriate for algae-only seeded samples. Algae + bacteria results showed that 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). For bacteria-only seeded sample, around 52 and 58% of DON were biodegradable to bacteria in 14 and 21 days of incubation, respectively. These results showed that, even though 14-day of incubation for algae + bacteria seeded sample was sufficient to utilize the certain amount of DON, this incubation time was not sufficient for bacteria-only seeded samples.

3.3.2. After BOD Trickling Filter

3.3.2.1. Inorganic Nitrogen and TDN

Wastewater samples collected after BOD TF location consisted of average: 20.5 mg-N/L of initial DNH_3-N, 0.16 mg-N/L of initial DNO_2-N, and 8.65 mg-N/L of initial DNO_3-N. In this location, about 40% of ammonia from primary effluent (average 33.74 mg-N/L) was nitrified into nitrite and subsequently to nitrate in the WWTP. Results from after 21-day incubation showed that, more than 97% of ammonia was either nitrified or used by algae and bacteria to

support algal and bacterial growth. Nitrite concentrations after 14-day of incubation varied regardless of the type of inoculum between 10.08 and 21.58 mg-N/L in all the samples. For 21-day of incubation, all the nitrite values reduced and varied between 9.48 and 16.08 mg-N/L except nitrite value in bacteria-only seeded sample, which was recorded as 0.25 mg-L/N. On the contrary to high nitrite value in after primary clarifier location, the bacteria-only seeded samples in after BOD TF location had sufficient dissolved oxygen to complete nitrification in the samples (Figure 3.3a).

Nitrate values varied between 5.01 and 27.88 mg-N/L after 14 and 21 days of incubation and the values were more or less similar for 14 and 21 days of incubations (Figure 3.3b). Nitrate in the sample might be originated from nitrification of nitrite and ammonium or ammonification of DON. Nitrate in algae-only inoculated samples were lower (<9.45 mg-N/L), compare to bacteria-only inoculated samples (27.88 mg-N/L) explained that algae utilized dissolved nitrate to support their growth, while bacteria was mostly responsible to convert ammonia and nitrite to nitrate (Figure 3.3b). Previous studies showed that more nitrates were utilized by algae compare to nitrate utilized by bacteria (Sattayatewa et al., 2009; Simsek et al., 2013). 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. Bacteria-only inoculated samples showed that all ammonia was nitrified into nitrate after 21 days of incubation.

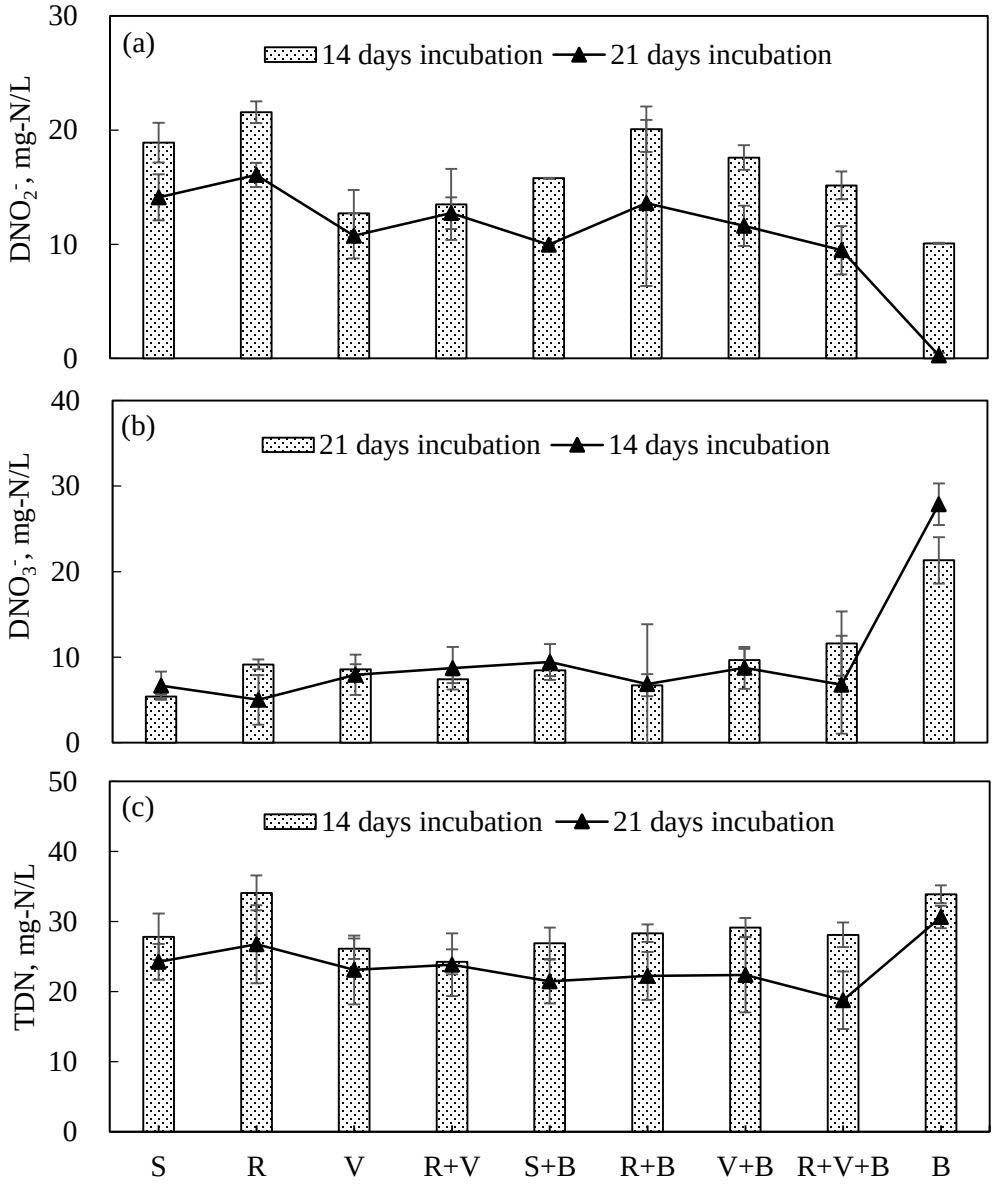

Figure 3.3. (a) DNO_2-N, (b) DNO_3-N, and (c) TDN after 14 and 21 days of incubation using algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B) inoculum in Fargo WWTP BOD trickling filter effluent.

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 14 and 21 days of incubation, the trend for TDN after BOD TF samples (Figure 3.3c) were 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. In bacteria-only inoculated sample, TDN before and after incubation was quite similar since only about 6.5% reduction in TDN was observed.

3.3.2.2 DON, ABDON, and BDON

Average initial DON after BOD TF samples was recorded as 6.59 mg-N/L (Figure 3.4a) which was lower than DON after primary effluent (36.5% reduction in the treatment plant). However, DON/TDN ratio in BOD TF samples (18.23%) was comparable to DON/TDN ratio in primary effluent location (20.24%). 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 and 29%. These results indicated that the organic fraction of the TDN in the effluent were quite high and 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 becoming increasingly important.

After 14 and 21 days of incubation, DON reduction in all the samples showed that some portion of DON was bioavailable to algae-only and bacteria-only seeds while some portion of DON was bioavailable to both algae + bacteria seeds (Figure 3.4a). Furthermore, some portion

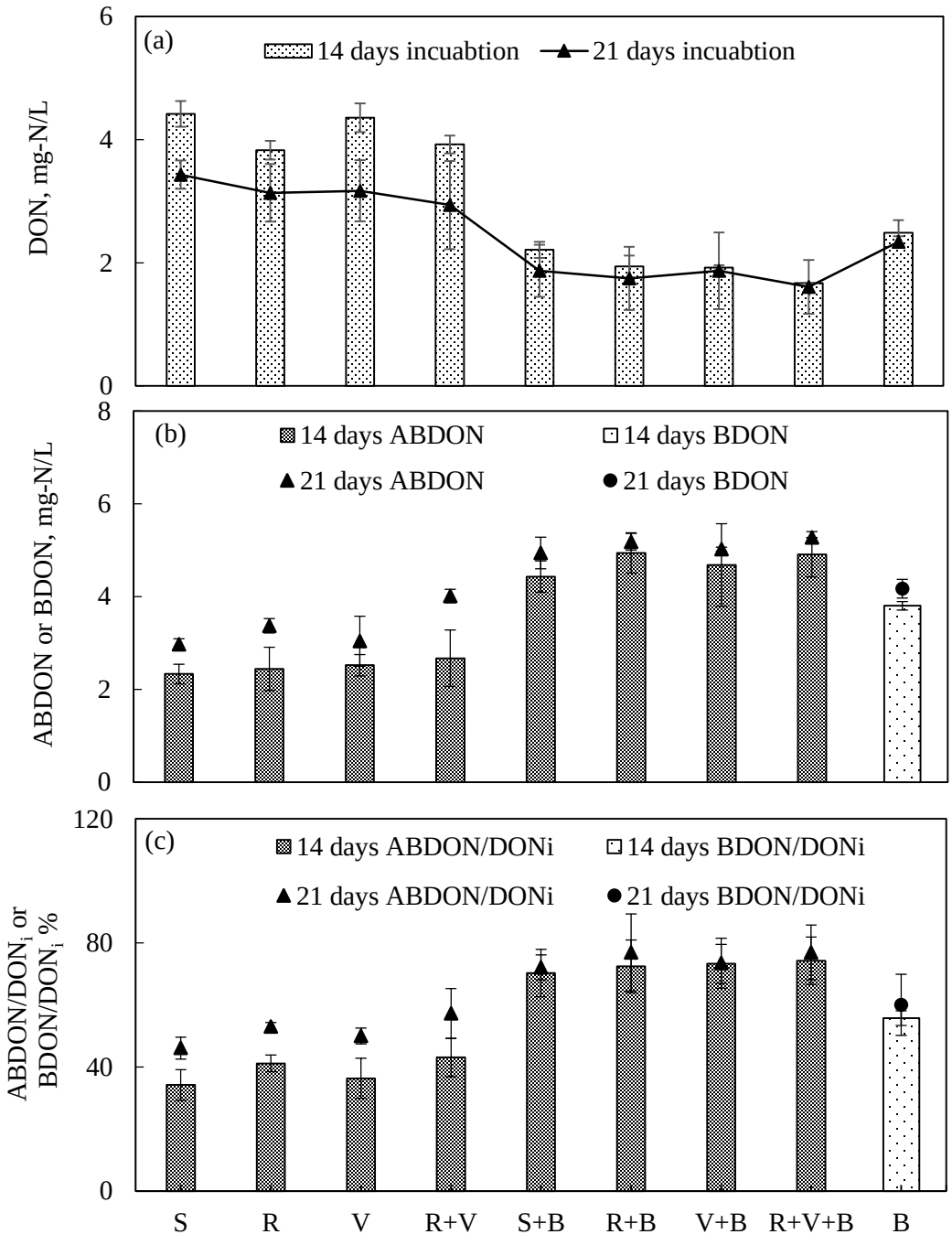

Figure 3.4. (a) DON, (b) ABDON and BDON, and (c) ABDON or BDON as a fraction of DON_i after 14 and 21 days of incubation using algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B) inoculum in Fargo WWTP BOD trickling filter effluent.

of DON neither bioavailable to algae nor bacteria seeds, which was considered as non-bioavailable or recalcitrant DON. 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.

ABDON and BDON data were presented in Figure 3.4b. For pure algae samples, ABDON for S, R, and V after 21 days incubation were ranged from 2.97 to 3.37 mg-N/L, which were lower than ABDON level in primary effluent. ABDON and BDON in BOD TF effluent showed similar trends with previous location (after primary clarifier) that bacteria addition was always increased DON degradability and availability. To have more insight on ABDON, initial DON fraction of ABDON and BDON were calculated and presented in Figure 3.4c 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 was 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. The decreased level of refractory DON indicated that SMPs was not produced during the BOD TF treatment. Released from the dead cells, SMPs is more likely to produce under anoxic and anaerobic conditions (Sattayatewa et al., 2009).

3.3.3. After Nitrification Trickling Filter

3.3.3.1. Inorganic Nitrogen and TDN

Initial DNH_3-N, DNO_2-N, and DNO_3-N after nitrification trickling filter were 1.19, 0.21, and 31.90 mg-N/L, respectively. About 95% of ammonia was transformed into nitrite and following to nitrate in the treatment train of the plant itself. Therefore, ammonia values after incubation in all the samples were under detection limit. Similarly, nitrite values after 14 and 21 days of incubation were under 0.96 mg-N/L in all the samples (Figure 3.5a). Nitrite was increasing in the samples from 14 to 21 days of incubation and the highest nitrite increment was observed in bacteria-only seeded sample with 89% of increment. This outcomes indicated that 14 days of incubation was not adequate for bacteria to complete biological degradation of nitrogen (Khan et al., 2009, Sattayatewa et al., 2009; Simsek et al., 2012).

Initial nitrate after 14 and 21 days of incubation was reduced in algae and algae + bacteria seeded samples while it was more or less the same in bacteria-only seeded samples (only slight reduction, 6.20%, was observed) (Figure 3.5b). 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 on nitrate compared to the initial nitrate. This means algae + bacteria can be able to remove 39.9% of nitrate from treated wastewater without advanced treatment (denitrification) application. Initial TDN was measured as 36.35 mg-N/L, which was reduced in all the inoculum conditions except TDN in bacteria-only seeded sample (33.87 mg-N/L). TDN in bacteria-only seeded samples was almost in balance with TDN before incubation (Figure 3.5c). 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.

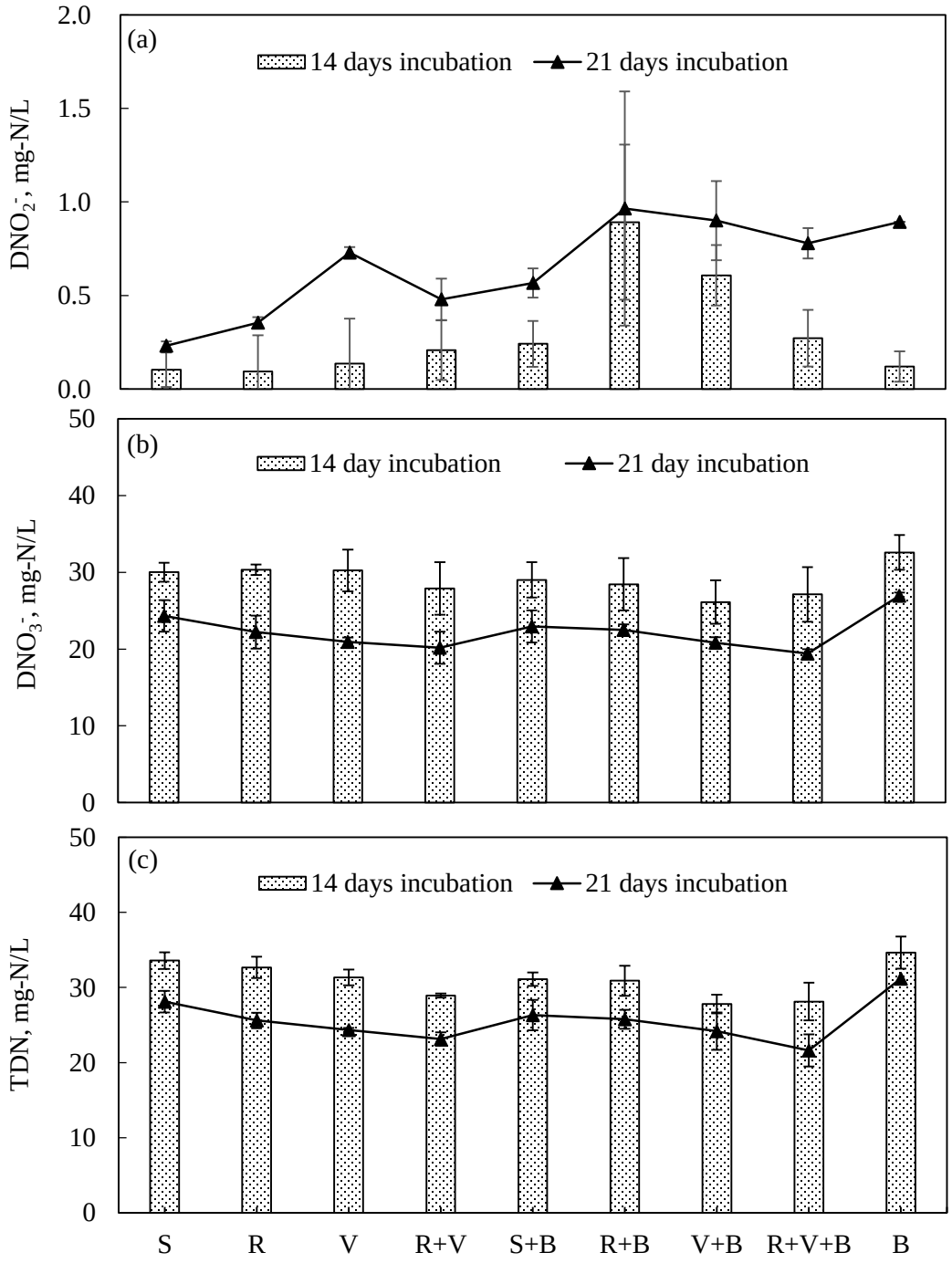

Figure 3.5. (a) DNO_2-N, (b) DNO_3-N, and (c) TDN after 14 and 21 days of incubation using algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B) inoculum in Fargo WWTP nitrification trickling filter effluent.

3.3.3.2. DON, ABDON, and BDON

Average initial DON (before incubation) in after nitrification was recorded as 3.76 mg-N/L, which comprised of 8.7% of initial TDN in after nitrification location. 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 3.6a). 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 and bacteria-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.

ABDON and BDON data was presented in Figure 3.6b. ABDON or BDON to DON_i ratio were presented in Figure 3.6c. ABDON was low in algae-only seeded samples compare to ABDON in algae + bacteria seeded samples, which was the similar trends were 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 and BDON trends were similar. All these results indicated that the differences in DIN levels in three locations did not significantly affect the bioavailability and biodegradability of DON. The average ABDON level for *C. reinhartti* was slightly higher (statistically not 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

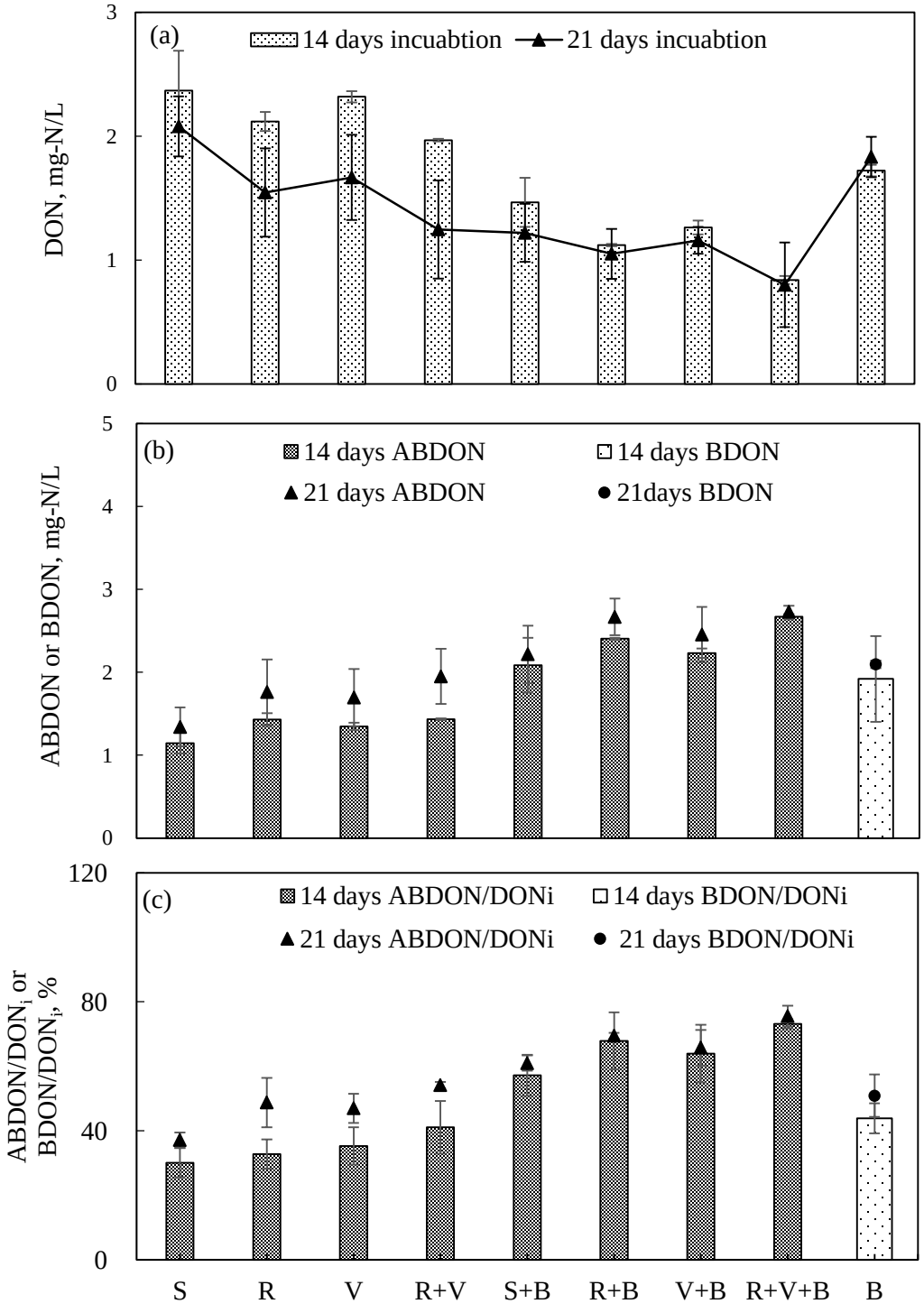

Figure 3.6. (a) DON, (b) ABDON and BDON, and (c) ABDON or BDON as a fraction of DON_i after 14 and 21 days of incubation using algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B) inoculum in Fargo WWTP nitrification trickling filter effluent.

experiment. Additionally, a strong correlation between organic N and protein molecules such as peptides were investigated (Langheinrich, 1995; Westgate and Park. 2010).

Similar ABDON/DON_i and BDON/DON_i trends were observed in after nitrification TF location compare to two previous locations (after primary and BOD TFs). 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. This results explained that 26.9% of initial DON in after nitrification location was recalcitrant DON, which was not removed in this study using algae *C. reinhardtii*, *C. Vulgaris* and mixed culture bacteria. Overall, R + V + B inoculated samples demonstrated the maximum bioavailability of DON in all three locations. The magnitude of BDON was less than ABDON in this location and about 50.9% of initial DON was recorder as BDON. This result confirmed that algae addition in the sample increased DON utilization.

3.4. Summary

This study provides important insight on bioavailability of DON using three different algal species (*S. capricornutum*, *C. reinhartti* and *C. vulgaris*) with/without bacteria addition in wastewater samples collected from three different locations in a two-stage TF WWTP.

For the overall treatment train, the initial ABDON was around 6.48 mg-N/L and the final ABDON was reduced to 2.84 mg-N/L indicating that 64% of ABDON was reduced in the biological treatment. Similarly, around 66% of bioavailable BDON was removed in TF.

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 it was 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. 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.

CHAPTER 4. DISSOLVED ORGANIC NITROGEN IN ANIMAL WASTEWATER: BIODEGRADABILITY AND BIOAVAILABILITY

4.1. Introduction

Nutrient enrichment originated from livestock operations in aquatic ecosystems stimulates overabundance of algal growth and causes 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 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, pathogen, 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 cause contamination and may responsible abnormalities (alteration of endocrine function) in aquatic organisms. Proper wastewater management should be implemented to protect human and environmental health from exposure of these chemicals originated from natural and synthetic steroidal hormones (Purdom et al., 1994; Khan et al., 2008).

Livestock wastewaters have high nutrient (particularly nitrogen and phosphorous) values compare to domestic wastewaters because of high concentrations of animals in a very limited

area. Some parameters in livestock wastewaters are several times higher than in domestic wastewater. For instance, some parameters in the piggery wastewaters can be ranged of: biochemical oxygen demand (BOD) 500-8000, volatile solids (VS) 5000-8000, total nitrogen (TN) is 900-2000, and total phosphorous (TP) is 80-400 as mg/L. (Cronk et al., 1996; Gonzalez et al., 2008; Prajapati et al., 2014). Ammonia-N values can be reached up to 8000 mg/L in piggery wastewaters depending on the size and operational characteristics of the feedlots (Bernet et al., 1996).

Animal wastewater has been considerable used as a growth medium of algal biomass 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 to treat piggery wastewaters and to determine their biodiesel production capacity. They expressed that microalgal-based treatment systems can significantly reduce nutrient concentrations in piggery wastewater at a minimal cost when the optimum conditions are met. *C. Vulgaris* was one of the species they used as a potential microalgal species to remove nutrients from piggery wastewater and quantify biomass production in their study. After 20 days of *C. Vulgaris* cultivation, TN was reduced from 53 to 27 mg/L. They concluded that TN can be utilized as NO_3^-, NO_2^-, NH_4, and N_2, however they overlooked organic nitrogen in their study (Abou-Shanab et al., 2013).

Prajapati et al., (2014) used four different algal strains to determine biomass production potential of dairy cattle wastewaters. Along with the other physiochemical parameters, they measured nitrate-nitrogen and total ammonia nitrogen before and after algal treatment. DON has not been evaluated in their study. Initial value of total ammonia and nitrate nitrogen were recorded as about 160 and 75 mg/L, respectively. After algal treatment using four different algae,

nitrate-N removal efficiency varied between 78 and 83% while ammonia-N removal efficiency varied between 74 and 98%. These high removal efficiencies showed that animal dairy cattle wastewater is an important nutrient sources for algal species.

Even though total nitrogen (TN) and inorganic nitrogen determination are well documented in livestock wastewaters, data for DON is limited. Obaja et al., (2003) determined TN (1700 mg/L) and inorganic species (NH_4^+-N = 1650 mg/L, NO_3^--N and NO_2^--N were not detected) in piggery wastewater, however they did not mention about organic nitrogen (ON) in their study though from the mass balance equation, ON can be calculated as 50 mg/L, which is quite high.

Previous studies indicate that some portions of DON in aquatic system is biodegradable and bioavailable to bacteria and algae over the time scale and it is an important nutrient source in nitrogen limited surface waters (Liu et al., 2011, Wiegnet et al., 2006). Biodegradable DON (BDON) is a portion of DON that is mineralized by bacteria. Bioavailable DON (ABDON) is a portion of DON that is utilized by bacteria and/or algae. There is no method to measure BDON and ABDON directly; however, they can be calculated from the difference between initial and final DON values using bioassay procedures (Khan et al., 2009, Simsek et al., 2012, 2013). Removing BDON and ABDON in livestock wastewaters before final discharging could reduce eutrophication potential.

In this study, DON, BDON, and ABDON were evaluated in livestock wastewaters that collected from an animal feedlot and a sheep wastewater storage lagoon. For BDON and 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 *Chlamydomonas reinhardtii* (*C. reinhardtii*) and *Chlorella vulgaris* (C. *vulgaris*)

have demonstrated their ability to remove nitrogen species in domestic wastewater, however these two species have not been used to test DON bioavailability in livestock wastewaters (Kim et al., 2007; Kong et al., 2010; Lee et al., 2006).

4.2. Material and Methods

4.2.1. Sample Preparation and Sampling Locations

Grab animal wastewater samples were collected from two different sources: Animal Nutrition and Physiology Center (ANPC) and ii) a sheep wastewater storage lagoon. Wastewater samples were filtered first through 1.2 μm pore-size glass fiber filters and subsequently filtered through 0.45 μm pore-size glass fiber filters (Whatman Inc. Kent, UK) within one hour after the collection. Six sets of samples were collected from April 2014 to October, 2014.

4.2.1.1. Wastewater Samples Collected from Animal Feedlot

In the first part of the study, the animal wastewater samples were collected from animal feedlot, which is an animal research facility belongs to North Dakota State University (NDSU) in Fargo, North Dakota. Varies types of animals have been raised in this facility and the number and types of animals at a certain time varies depends on the research need. During the sample collection time, there were sheep, pig, and mostly cattle (about 100-head cattle) in the feedlot. About 60,000 gal/day wastewater was generated in the facility. The wastewater flows through a solid separator unit for hay separation and then the liquid portion (wastewater) flows 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.

4.2.1.2. Wastewater Samples from Sheep Wastewater Storage Lagoon

In the second part of the study, the animal wastewater samples were collected from a storage lagoon that receives animal wastewater and runoff from a sheep research feedlots, which

is also belongs to NDSU. There were about 200 head of sheep available during the sample collection period. The lagoon received rain water as well in some of the sampling time frame. When it was needed, the lagoon wastewater was pumped out to nearby crop field. The wastewater samples were collected at 0.4 m depth from the lagoon surface.

4.2.2. Algal and Bacterial Inoculum Preparation

Two algal species, *C. reinhardtii* and *C. vulgaris*, were used in this study to inoculate the wastewater samples. Both 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. Both algae were cultured in 500 ml clear bottles with continuous aeration at 20℃. 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 *C. reinhardtii* and *C. vulgaris* solutions were centrifuged at 3000 rpm for 5 min and rinsed with DI water twice before inoculated in the samples. The initial algal cell density was measured around 10^5 cells mL^{-1} by using a ZEISS LSM microscope to achieve supplemental growth during ABDON incubation. 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). In this study, *C. reinhardtii*, *C. vulgaris*, and bacteria were abbreviated as R, V, and B, respectively. All the glassware, including double de-ionized water (DDI) were sterilized by autoclaving at 121℃ for 20 - 30 min before conducting any experiment.

4.2.3. Experimental Design

All the samples were inoculated using algae and/or bacteria and incubated for 21 days to determine DON, ABDON and BDON. The 21 days of incubation period in the experiment was

proceeded from previous studies (Pehlivanoglu and Sedlak, 2004; Urgun-Demirtas et al., 2008; Sattayatewa et al., 2010; Simsek et al., 2012) and preliminary results of this study. To determine ABDON, the samples were inoculated using algae-only (R-only or V-only), algae + algae (R + V), algae + bacteria [(R-only or V-only) + B], or algae + algae + bacteria (R + V + B) inoculum. To determine BDON, the samples were inoculated using bacteria-only (B) inoculum.

For each experiment, a 100 ml of wastewater sample was placed in a 250 ml of clear bottle and seeded with 1.5 ml pure culture algal species and 1.5 ml of bacteria based on design of the experiment. The sample volume to air volume in the bottle did not exceed to 50% ratio to ensure sufficient air contact to maintain oxygen level (Miller et al., 1978). Control samples were prepared for each bioassay by adding the algae and/or bacterial inoculum to DDI water and treating it the same way as the sample. Bacteria seeded samples were incubated in amber bottle in dark while algae seeded samples were placed in clear bottles with 12hr dark/ light cycle of artificial light illumination. All the experiments were conducted on a continuous shaker (VWR orbital standard shaker) at the rate of 100 rpm to maintain the complete mixing during the incubation. After 21 days incubation, wastewater samples were centrifuged at 3000 rpm for 5 min to separate algae and/or bacteria from the sample before measurement. After 21-day of incubation, algal growth in each algal and/or bacterial bioassays were evaluated by measuring dry cell weight (Miller et al., 1978). Based on the procedure, 50 ml samples were filtered through 0.2μm micro pore filter (Pall Life Scientific), while the retentate on the filter (algal biomass) was dried at 80 ℃ for 24 hr.

4.2.4. Determination of DON, ABDON, and BDON

There is not a method available to measure DON directly. Therefore, DON was calculated as the difference between TDN and total dissolved inorganic nitrogen (TDIN). The

details of inorganic nitrogen and TDN measurements and following DON, BDON, and ABDON determinations were explained elsewhere in detail (Simsek et al., 2012, 2013).

4.2.5. Statistical Analyses

Minitab 17 was used in this study for all the statistical analyses. Sample means and standard derivations were calculated from the duplication or triplication of each treatment. One way analysis of variance (ANOVA) was performed at $P \leq 0.05$ to evaluate the statistical difference between BDON or ABDON under different inoculation conditions.

4.3. Result and Discussion

Animal feedlot and storage lagoon samples were analyzed before and after incubation to determine dissolved nitrite, dissolved nitrate, and dissolved ammonia, TDN, DON, ABDON, and BDON. The results were presented in the Figures 1 through 4.

4.3.1. Animal Feedlot Wastewaters

4.3.1.1. Inorganic Nitrogen and TDN

Average values of inorganic nitrogen concentrations before incubation (raw wastewater) and following 21 days of incubation results for animal feedlot samples were presented in Figure 4.1. After 21-day of 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 available to algae + bacteria inoculum, respectively. Furthermore, about 99% of ammonia was removed in bacteria-only seeded sample. 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.

Nitrate after incubation was low in algae-only seeded samples while it was quite high in bacteria and bacteria-only seeded samples. The similar results were obtained in previous studies

(Urgun-Demritas et al., 2008; Simsek et al., 2013) that nitrate was high in bacteria involved samples while it was low in algae-only seeded samples.

Nitrate after incubation was low in all the samples regardless of the type of the seeds except it was high in bacteria-only seeded samples. These results indicated that algae utilize nitrate for their growth while bacteria was increasing the nitrate through nitrification process. In general, either ammonia or nitrite were quite high in all inoculum conditions after incubation for animal feedlot samples (Figure 4.1). This outcome expressed that partial nitrification was 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). To understand the effect of high nitrite accumulation in the samples, the pH values were monitored every three days during the incubation. It was recorded that pH increased to 8.13- 8.30 in algae-only seeded samples on 3rd incubation day, compare to initial (before incubation) pH values (ranged from 7.40 to 7.69). In algae + bacteria seeded samples, pH reached to the highest value (9.04 -9.47) at 11^{th} day of incubation. However, the high pH values were not affected the life of algae and/or bacteria. Similarly, additional two sets of experiments were conducted to address oxygen depletion effect on nitritation by diluting the initial wastewater by 1:3 portions to reduce nutrient loading in the samples. TDN became about 15 mg-N/L in diluted animal feedlot and all other parameters in the samples reduced proportionally. More than 99% of ammonia (<0.5 mg-N/L left) was nitrified all the way to nitrate after 21 days of incubation in both algae + bacteria seeded samples. These results indicated that higher initial N loading needs extra oxygen to support algae and/or bacteria for their assimilation and metabolism activities.

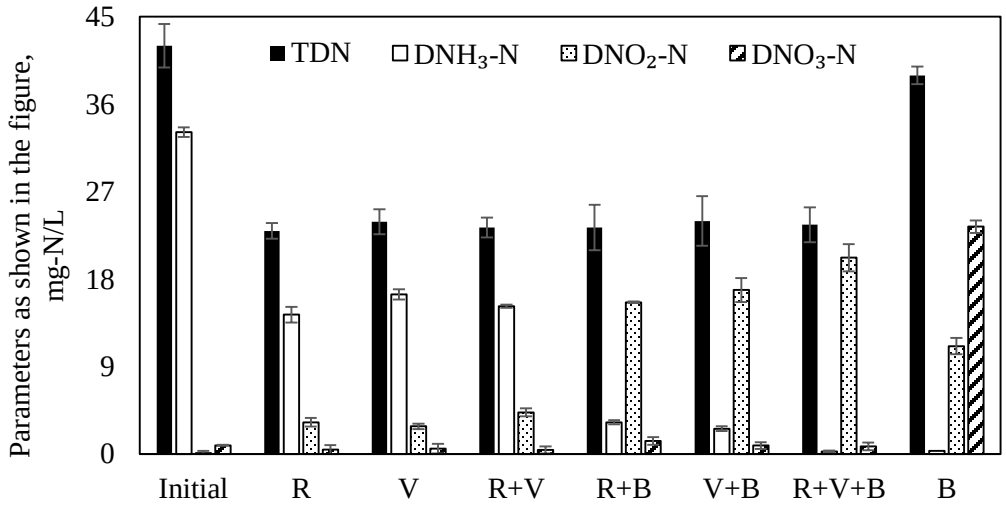

Figure 4.1. TDN, NH_3-N, NO_2-N, and NO_3-N in animal feedlot samples for different algae and/or bacteria inoculum: Algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B).

Average initial TDN (before incubation) mostly constituted of ammonia and DON since the wastewater sample from 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 TDN before incubation since algae utilized nitrogen species (mainly ammonia) for their growth. The TDN values after incubation reduced to 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. TDN before and after incubation in bacteria-only seeded ample was in balance and only minor TDN reduction (9%) was recorded after incubation. The similar reduction in TDN in bacteria-only seeded sample was observed in a previous study conducted by Simsek et al. (2012).

4.3.1.2. DON, BDON, and ABDON

Average DON concentration before incubation was recorded as 7.71 ± 0.18 mg-N/L, which comprised of about 18.4% of TDN before incubation (Figure 4.2a). The magnitude of DON in ANPC effluent and typical DON in domestic wastewater (raw wastewater) were quite similar. DON after incubation in algae and/or bacteria seeded samples was varied between 1.63 and 4.54 mg-N/L. These results showed that between 21.1 and 58.9% of initial DON was utilized by either algae and/or bacteria. DON after incubation (DON residue in the sample) to initial TND ratio was calculated and results showed that between 3.9 and 10.8% of DON in initial TDN were remained in this samples, which consider as refractory (unbiodegradable and unbioavailable) DON (Figure 4.2a). DON reduction was the lowest in *C. reinhardtii* seeded samples while it was the highest in algae + algae + bacteria seeded sample. The highest reduction in DON after incubation to initial TDN was observed in *C. reinhardtii* + *C. vulgaris* + bacteria seeded sample. 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. This indicated that while DON was reducing in each bioassay sample, TDN was also reducing because of algal and bacterial utilization of nitrogen species.

BDON results showed that, average 3.97 mg-N/L of initial DON, which was 54.8% of initial DON was biodegradable to bacteria-only inoculum. Similar results were obtained in previous studies (43-65% of BDON removal efficiency) for domestic wastewaters (Urgun-Demirtas et al., 2008; Simsek et al., 2013).

Bioavailability of DON in both algae *C. reinhardtii* and *C. vulgaris* and their combination with bacteria was determined and presented in Figure 4.2b. DON bioavailability was significantly higher ($P \leq 0.05$) in *C. reinhardtii* inoculum compare to in *C. vulgaris*

inoculum. As a result of symbiotic relationship between algae and bacteria, the bioavailability of DON increased in both types of algal species with bacteria addition. 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 two algal species.

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 B_{12}, and organic carbon source (Santos and Reis. 2014).

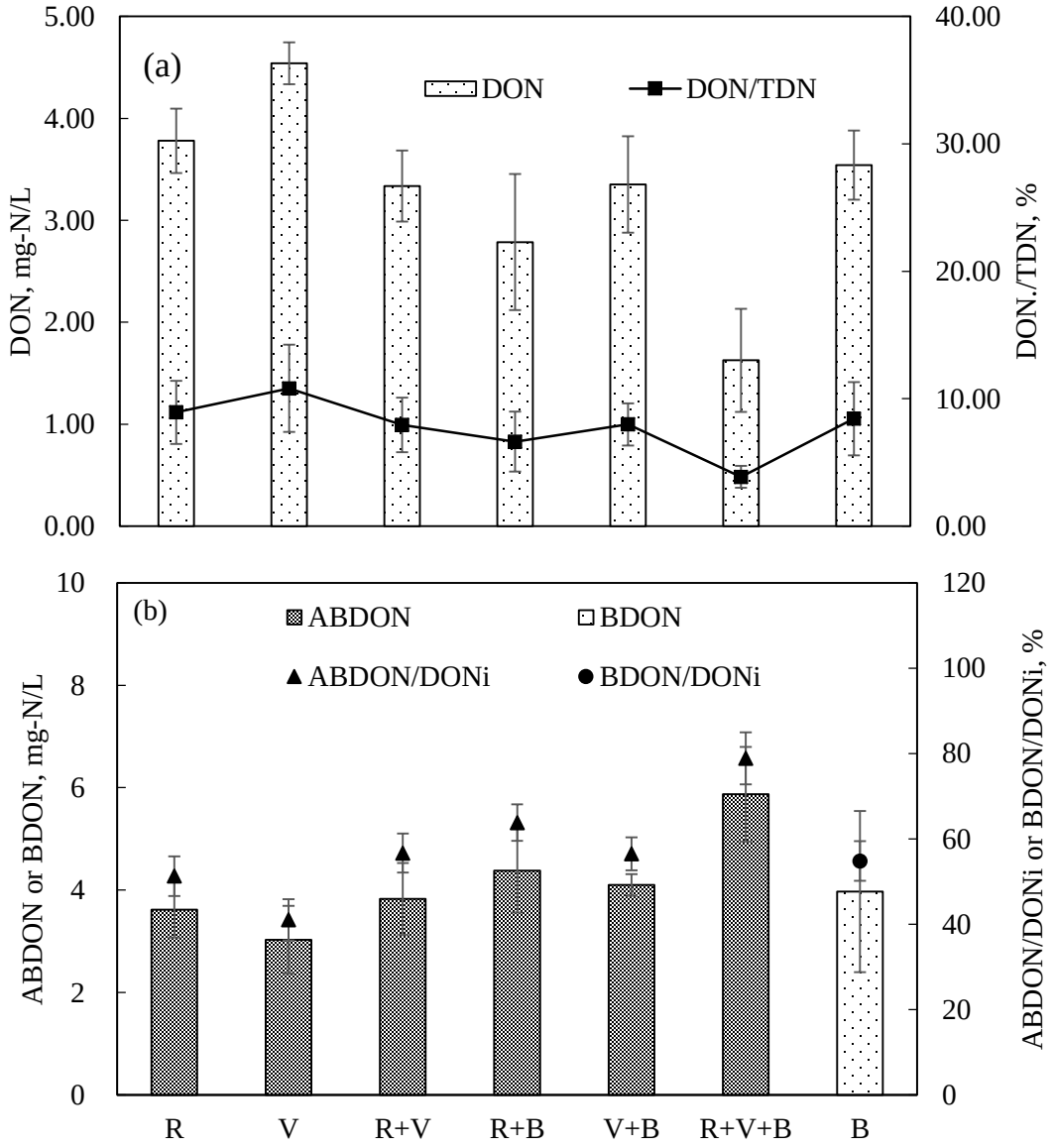

Figure 4.2. (a) DON and DON/TDN, (b) BDON, ABDON, and their initial DON fraction in animal feedlot samples for different algae and/or bacteria inoculum: Algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B).

The fractions of $BDON/DON_i$ and $ABDON/DON_i$ represent the biodegradability and 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 ANPC effluent (Figure 4.2b).

As explained earlier, two sets of samples were diluted to investigate nutrient loading and DO relationship. Therefore, BDON and ABDON was determined in 1:3 portion of diluted sample to observe oxygen deficiency effect on DON. Results showed that biodegradability and bioavailability of DON was not changed significantly ($P < 0.05$) between non-diluted and 1:3 diluted samples from animal feedlot, 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.

Overall results from both types of algae and/or bacteria inoculum showed that some portions of DON was both biodegradable and bioavailable, which was overlapping, while some portions of DON was only bioavailable or only biodegradable. In other words, some portions of DON were determined as refractory to both algae and/or bacteria. The overlapping portion of DON is more critical in aquatic systems since there is a high possibility to breakdown DON by either algae or bacteria to increase nutrient availability to algal and microbial species.

4.3.2. Lagoon Wastewater

4.3.2.1. Inorganic Nitrogen and TDN

Dissolved nitrate and TDN results before and after incubation for the samples collected from the sheep feedlot lagoon are presented in Figure 4.3. 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 TDIN was about 2.86 mg-N/L, which was quite lower than TDIN in animal feedlot sample. The reason for low inorganic nitrogen in the lagoon is 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/or 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 was about 12.00 mg-N/L, which consisted of mostly DON since TDIN was very low in the samples. After incubation, ammonia and nitrite were completely removed in all the samples (Figure 4.3). 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. 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 was mainly responsible for nitrification in these samples.

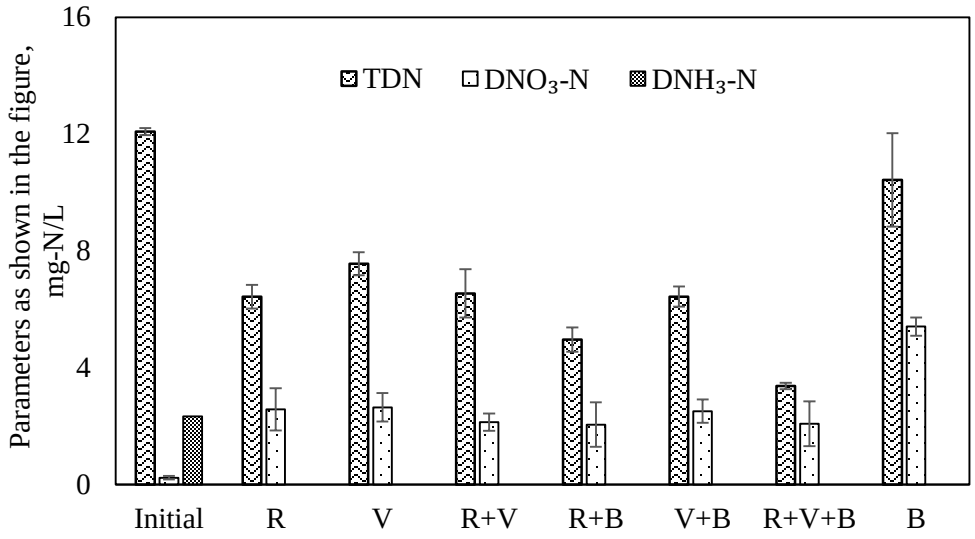

Figure 4.3. TDN, NH_3-N, NO_2-N, and NO_3-N in storage lagoon samples for different algae and/or bacteria inoculum: Algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B).

4.3.2.2. DON, BDON, and ABDON

DON was an important component of the sheep feedlot lagoon samples, which was measured as 8.50 mg-N/L 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 4.4a).

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. *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, BDON, and their ration to initial DON data are presented in Figure 4.4b. 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 was 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 ration showed similar trend with ABDON data in all the inoculum condition.

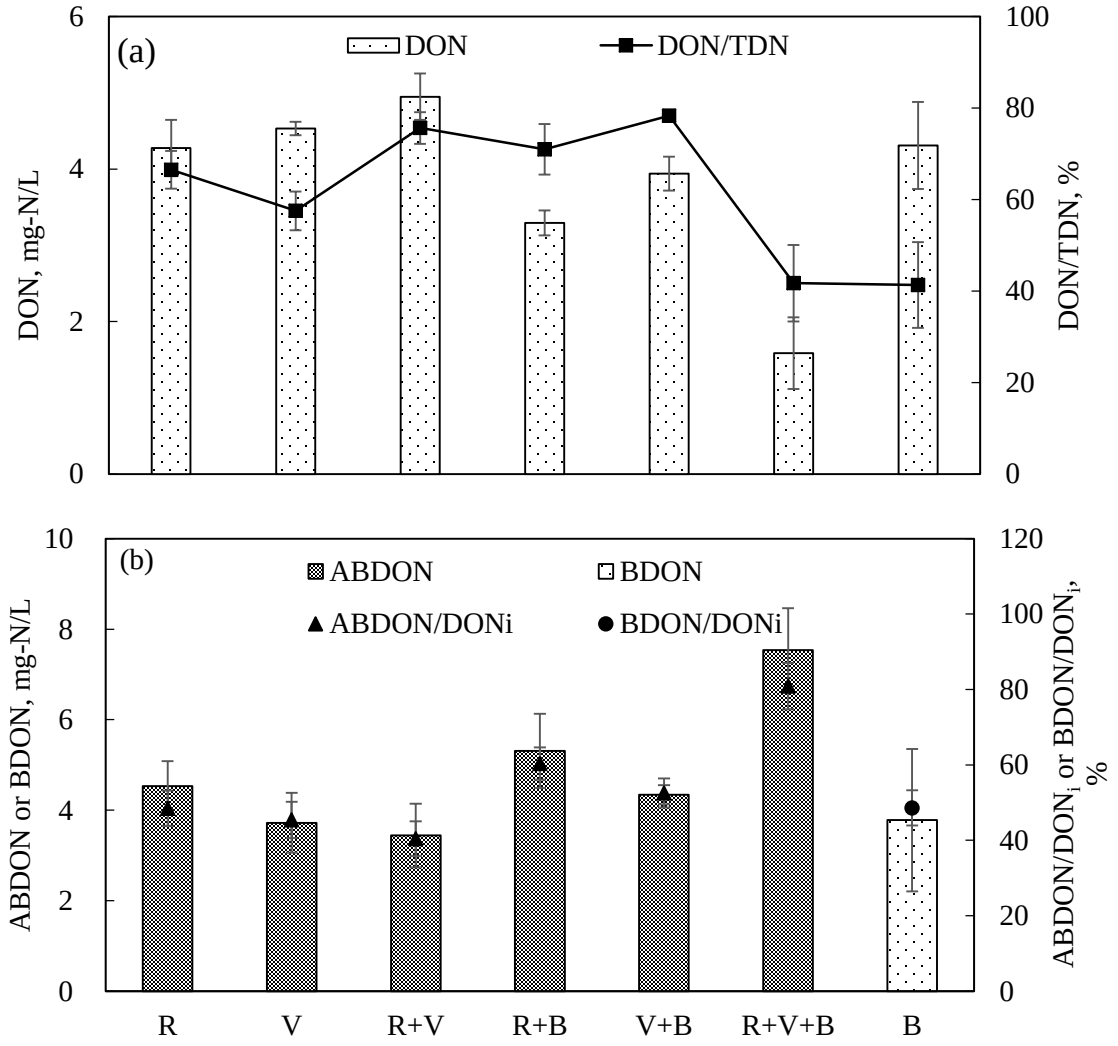

Figure 4.4. (a) DON and DON/TDN, (b) BDON, ABDON, and their initial DON fraction in storage lagoon samples for different algae and/or bacteria inoculum: Algae [*S. capricornutum* (S), *C. reinhardtii* (R), and *C. vulgaris* (V)] and/or bacteria (B).

BDON incubation results showed that average 3.78 mg-N/L of DON was degraded by bacteria in the lagoon sample. Algae and algae + bacteria data showed that some portion of this BDON was utilized by algae. Furthermore, the BDON to initial DON ratio proved that about 48.6% of DON was biodegraded to lower molecular weight compounds by bacteria. Even though some portions of DON was biodegraded by bacteria, some portions of it remained as recalcitrant, which was non-biodegradable in the sample.

4.3.3. Dry Cell Weight in ANPC and Lagoon Samples

Measuring and quantifying dry cell weight of algal and bacterial biomasses in the samples is critically important to monitor algae-nutrient relationship and furthermore to evaluate ABDON and BDON potential in the samples. Dry cell weight in both type of animal wastewater was measured after 21 days of incubation period to examine how the presence of bacteria and N concentration affected the growth of *C. reinhardtii* and *C. vulgaris* species. Results showed that *C. reinhardtii* + bacteria inoculum demonstrated the highest biomass productivity in wastewater from animal feedlot (Table 4.1). With the presence of bacteria + algae, more biomass was produced than either inoculated algae-only or bacteria-only samples, indicating that bacteria involvement promoted algal growth in both wastewater samples collected from animal feedlot and lagoon. ABDON and BDON incubation experiments in this study supported this outcome since algae + bacteria samples always reduced DON concentration in the samples. The biomass production inoculated with bacteria was only slightly increased in animal feedlot wastewater contained higher TDN than wastewater from lagoon, while it was significantly increased inoculated with algae or algae + bacteria.

Table 4.1. Biomass density in animal feedlot and lagoon samples inoculated using different combination of algal and/or bacterial inoculum.

ANPC samples	
Type of inoculum	Biomass Density, g/L
R	0.48 ± 0.04
V	0.37 ± 0.05
R+B	0.77 ± 0.08
V+B	0.62 ± 0.02
B	0.12 ± 0.02
Lagoon samples	
Type of inoculum	Biomass Density, g/L
R	0.15 ± 0.02
V	0.12 ± 0.01
R+B	0.30 ± 0.03
V+B	0.25 ± 0.02
B	0.09 ± 0.02

4.4. Summary

The vast majority of the available information in animal wastewater studies are not explain biodegradability and bioavailability of DON and its impact on natural environment. DON, BDON, and ABDON data were collected from two different animal wastewaters sources, which were animal feedlot and sheep lagoon. Samples were inoculated using *C. reinhardtii* and *C. vulgaris* and bacteria (MLSS). The results showed that from 3.21 to 5.87 mg-N/L of DON (comprised about 51.3% to 78.9% of initial DON) from ANPC effluent and from 3.44 to 7.54 mg-N/L of DON (comprised about 40.5% to 80.9% of initial DON) from lagoon samples were bioavailable to any combination of algae and bacteria. ABDON and BDON trends in both types of wastewater sources were similar. *C. reinhardtii* + *C. vulgaris*+ bacteria seeded samples utilized initial DON more than other combination of algae and/or bacteria. 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, which could be in receiving water.

CHAPTER 5. CONCLUSIONS

5.1. Conclusions

This research was conducted under batch conditions with controlled temperature, aeration, and illumination to investigate bioavailability and biodegradability of DON. The study reveals important findings and provides information to increase the quality of receiving waters. In municipal wastewater, ABDON efficiencies for all three algae were not significantly different, which indicated that *C. reinhardtii* and *C. vulgaris* can be used as a test species for nitrogen determination similar to *S. capricornutum*. Short incubation period (14-day) was adequate to complete ABDON exertion for algae + bacteria inoculum. However, DON was still available for algae after 14 days (until 21 days) which can imply that effluent DON in aquatic system have more profound environmental impacts over the time scale.

BDON and ABDON in two different animal wastewater sources were thoroughly investigated in this study. Along with the algae, bacteria addition into the samples produced high ABDON value indicated that there was a symbiotic relationship between bacteria and all three types of algae. In an animal operation center and animal waste lagoon, DON comprised about 18.4 and 70.8% of TDN, respectively. High ABDON level (40 to 81%) in animal wastewater indicated the potential need to remove animal DON before discharge. Overall, results from this study showed that DON from anthropogenic sources is highly bioavailable to algae and bacteria. Future works are required to analyze the characterization of DON.

5.2. Recommendations

To have a comprehensive understanding on the research, there are topics that can be further addressed:

- It is necessary to investigate different operational conditions, such as in continuous stirred tank reactors or pilot scale reactors, to promote DON degradation rate and enhance ABDON removal in a shorter incubation period.
- A proteomic analysis of protein profiles can be conducted before and after bioassay tests to provide more information on molecular weight and properties of bioavailable, recalcitrant, and newly generated DON (if any).
- Detecting extracellular and intracellular enzyme activity of algae and bacteria are recommended to understand the mechanism of the symbolic relationship of algae and bacteria to remove DON.
- Levels of different forms of DON, such as urea and amino acids, in municipal wastewater and animal wastewater can be determined via chemical analysis before and after incubation to understand the bioavailability and biodegradability of specific nitrogen forms.
- Further experiments can be conducted by incubating river water and animal wastewater samples to monitor the effects of ABDON in receiving waters.

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