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

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ABSTRACT

Effluent dissolved organic nitrogen (DON) is problematic in nutrient sensitive surface waters and needs to be reduced to meet demanding total dissolved nitrogen discharge limits. Bioavailable DON (ABDON) is a portion of DON utilized by algae or algae + bacteria, while biodegradable DON (BDON) is a portion of DON decomposable by bacteria. ABDON and BDON in a two-stage trickling filter (TF) wastewater treatment plant was evaluated using three different microalgal species, *Selenastrum capricornutum*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris* and mixed cultured bacteria. Results showed that up to 80% of DON was bioavailable to algae or algae + bacteria inoculum while up to 60% of DON was biodegradable in all the samples. Results showed that *C. reinhardtii* and *C. vulgaris* can be used as a test species the same as *S. capricornutum* since there were no significant differences among these three algae species based on their ability to remove nitrogen species.

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Introduction

Biological availability of dissolved organic nitrogen (DON) to bacterial and/or algal species in aquatic ecosystems accelerates DON transformation into highly soluble inorganic nitrogen forms including ammonia, nitrite, and nitrate. Hence, excessive concentrations of DON accumulation ultimately increase the eutrophication in aquatic systems (Paerl, 1997; Seitzinger and Sanders, 1997; Gobler et al., 2005). Algal blooms caused by excess total nitrogen loads in coastal areas have a great impact on marine ecosystems, fisheries, and public health. Managing inorganic nitrogen discharges into receiving waters helps to reduce the frequency of algal blooms.

However, previous studies confirmed that some algal species preferred to utilize organic nitrogen species in the lack of inorganic nitrogen in aquatic systems (Berman and Chava, 1999; Mulholland et al., 2004). Similarly, from mid-2000, brown tides in northeast U.S. were found significantly correlated to DON level in receiving waters (MacIntyre et al., 2004; Trice et al., 2004; Glibert et al., 2007).

Sources of DON in aquatic ecosystems originated from both nonpoint and point sources including groundwater seepage, livestock excrement, residential and urban runoff, and domestic wastewater treatment plants (WWTPs) (Cape et al., 2011). Recent advances on treatment technologies in WWTPs mostly help to remove inorganic nitrogen through

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nitrification and denitrification processes and DON in influent largely remain unchanged throughout the plant. Chen et al. (2011) explained that effluent DON is essentially biodegradable and further bioavailable to aquatic species. Urgun-Demirtas et al. (2008) investigated the DON bioavailability in plot-scale nitrification plant and a laboratory-scale nitrogen removal reactor. They found that 18% to 61% of initial DON (total nitrogen was 4 to 5 mg/L) was bioavailable to algae and bacteria. Another study was conducted to investigate the fate of organic nitrogen in four different Bardenpho biological nitrogen removal WWTP effluent. DON concentration varied between 0.5 to 2.0 mg-N/L in those four plants even though there was a large variation observed in the influent DON concentration (Sattayatewa et al., 2010). They concluded that the fate of DON in the plant depended on plant operating conditions (solids retention time, influent flow, wastewater temperature). A plant that worked longer solids retention time (23–25 days) produced low effluent DON to soluble nitrogen ratio (Sattayatewa et al., 2010).

Removing wastewater derived DON is crucial to control the cumulative amount of nitrogen in surface waters. However, reducing DON (either from raw wastewater or generated during the biological process) has not been successful because of the complex structure of DON. The major portion of wastewater derived DON structure is currently unknown and about 20% of it consists of dissolved combined amino acids (DCAA), dissolved free amino acids (DFAA), proteins, urea, and ethylenediaminetetraacetic acids (EDTA) (Berman and Bronk, 2003; Pehlivanoglu-Mantas and Sedlak, 2008; Huo et al., 2013). Researchers have confirmed that some compounds including chelating agents and soluble microbial products (SMPs) were produced by organisms during biological treatment process (Parkin and McCarty, 1981; Westgate and Park, 2010). Nevertheless, understanding the composition of DON at any given time/process in treatment plant is still a great challenge.

Bioavailable DON (ABDON) is a portion of DON that is bioavailable to algae directly or after bacterial degradation (Urgun-Demirtas et al., 2008; Simsek et al., 2013). Biodegradable DON (BDON) is a portion of DON mineralized by bacteria in dark conditions (without algae) (Sattayatewa et al., 2009; Simsek et al., 2013). Wastewater effluent ABDON and BDON is important for controlling excess nutrients in receiving waters. The bioavailability of DON to algae and bacteria in receiving waters depends on various environmental conditions such as residence time, temperature, dissolved oxygen (DO) level, pH, and type of living organisms. Although many studies exist on the ABDON from natural and anthropogenic sources (Bushaw et al., 1996; Seitzinger and Sanders, 1997; Vähätalo and Zepp, 2005; Bronk et al., 2007) limited studies are available on the ABDON in domestic wastewaters (Pehlivanoglu and Sedlak, 2004; Urgun-Demirtas et al., 2008; Xu et al., 2010; Simsek et al., 2013).

A majority of previous studies used a unicellular green microalgae *Selenastrum capricornutum* (also known as *Raphidocelis subcapitata*) to investigate bioavailability of nitrogen since *S. capricornutum* has some advantages, including easily grown in laboratory conditions and high efficiency to utilize the primary nutrients. Moreover, *S. capricornutum* has been used and suggested by United States Environmental Protection Agency as a test species of water quality and fresh

water algae toxicity studies (Vanderheever and Grobbelaar, 1998). It has been widely applied in toxicity studies of ionic liquids (Pham et al., 2010), metal oxide nanoparticles (Kahru et al., 2008), and propylene glycol ethers (Staples and Davis, 2002) to quantify pollutants bioavailability. In this study, three different algal species, *S. capricornutum*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris* and their combinations with bacteria were used to obtain DON, ABDON, and BDON data in the samples collected from primary and secondary treatment locations along the two-stage trickling filter (TF) WWTP. The results were analyzed and compared to investigate if *C. reinhardtii* and *C. vulgaris* were also suitable to use as control species the same as *S. capricornutum* in wastewaters.

1. Material and methods

1.1. Samples source, collection, and preparation

Wastewater grab samples were collected from the City of Fargo, North Dakota WWTP. The plant has a two-stage TF process, which are biochemical oxygen demand (BOD) TFs and nitrification TFs, with a peak pumping capacity of 110,000 m^3/day and an average flow of 57,000 m^3/day. 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; (1) after primary clarifier location (location 1), (2) after BOD TF location (location 2), and (3) after nitrification TF location (location 3) along the WWTP. The schematic diagram of sample collection locations is presented Appendix A Fig. S1. A total of six sets of samples were collected from May 2013 to December 2014 and average values were presented in this study. Before performing the experiments, all the wastewater samples were filtered first through 1.2 μm pore-size glass fiber filters (GF/C, Whatman Inc. Kent, UK) and subsequently filtered through 0.2 μm fiber filter (Pull Scientific, USA) approximately 1 hr after collection.

1.2. Algal and bacterial bioassay preparation

Algal and/or bacterial inoculum were used to inoculate the wastewater samples. Three different algal species, *S. capricornutum*, *C. reinhardtii*, and *C. vulgaris*, were used as test species to test if those algae would be utilized the nitrogen in the samples to support their growth. 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 mmol/L NaNO$_3$, 0.17 mmol/L CaCl$_2$·2H$_2$O, 0.3 mmol/L MgSO$_4$·7H$_2$O, 0.43 mmol/L K$_2$HPO$_4$, 1.29 mmol/L KH$_2$PO$_4$, and 0.43 mmol/L NaCl. Stock *S. capricornutum*, *C. reinhardtii*, and *C. vulgaris* strains were cultivated in 500 mL clear bottles at 25°C under aerobic conditions. Algae were illuminated under a fluorescent lamp (5400 lm) using a 12-hr light/dark cycle. All the glassware, media, and double de-ionized water (DDI) were

autoclaved at 121°C for 30 min and 15 psi before used in each experiment. Cultured algal bioassays were harvested by centrifuging the certain amount of cultured algae at 3000 r/min for 5 min and washed with DDI water twice before use. The washed algal culture was re-suspended in DDI water to form a concentrated algal suspension with an initial cell density of 1×10^5 cells/mL to achieve efficient growth. Bacterial bioassay was prepared from influent (raw wastewater) of the City of Fargo WWTP since it consisted of returned bacteria from intermediate clarifier. The bacterial bioassay was also centrifuged at 3000 r/min for 5 min and rinsed with DDI water before using. *S. capricornutum*, *C. reinhardtii*, and *C. vulgaris* were abbreviated as S, R, and V, respectively, in the entire study.

1.3. Analytical methods, ABDON and BDON procedures

Filtered samples (50 mL) were used to analyze the initial parameters, which were dissolved ammonia N (DNH_3-N), dissolved nitrite N (DNO_2-N), dissolved nitrate N (DNO_3-N), and total dissolved nitrogen (TDN). DON was calculated from the mass balance equation (Simsek et al., 2013). All the measurements were carried out in duplicate or triplicate for each sample. The diazotization, second derivative ultraviolet (UV) spectrophotometric (SDUS) method, and salicylate method were used to test nitrite, nitrate, and ammonia, respectively (Table 1). TDN was converted to nitrate after digestion and measured with SDUS method using UV–Visible spectrophotometer (APHA, 2005) (Table 1).

After determining initial parameters, 150 mL of the wastewater 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 inoculum. These 14 and 21 days of incubation periods were determined by analyzing the 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. Amber bottles were used to incubate the samples using bacteria-only inoculum, while clear bottles were used to inoculate the samples using algae-only, algae + algae, or algae + bacteria inoculum. After the incubation, the same parameters as in the initial samples were measured and finally ABDON and BDON were determined for both 14 and 21 days of incubation periods. The ABDON (mg-N/L) and BDON (mg-N/L) calculations relied on the changes between initial DON (DON_i, DON before incubation) and final DON (DON_f, DON after incubation) values (Eqs. (1) and (2)). DON_{bi}

and DON_{bf} are DON before and after incubation for control. Details of the ABDON and BDON methods are previously described (Simsek et al., 2012, 2013).

$$BDON = [(DON_i - DON_f) - (DON_{bi} - DON_{bf})] \tag{1}$$

$$ABDON = [(DON_i - DON_f) - (DON_{bi} - DON_{bf})] \tag{2}$$

ABDON experiments in this study were divided into 8 portions based on the type of inoculum; 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 inoculated sample. Algae (1.5 mL) and/or bacteria (1.5 mL) inoculum were used to inoculate all the bottles and agitated continuously on an orbital shaker at 100 r/min (VWR standard orbital shaker) with caps that were tightly closed. However, all the bottles were aerated daily by opening the caps one or twice a day for 3–4 min during the incubations to maintain the oxygen in the samples. The aeration was appropriate for algal and bacterial growth (Urgun-Demirtas et al., 2008; Simsek et al., 2012, 2013). After the incubation, wastewater samples were centrifuged with 3000 r/min for 5 min to separate either algae and/or bacteria from the samples before measurements. Control samples were carried out throughout the experiments for each bioassay by adding each inoculum to distilled deionized water. All the necessary corrections were made as shown in Eqs. (1) and (2) using the results obtained from control samples.

Cell density was measured during the incubation by using algae counting method to evaluate algal growth, which was determined by adding 0.1 mL Lugol's solution to the samples and preserving them in dark. Samples were observed by a laser scanning microscope (ZEISS, LSM 700) using 1 mL hemocytometer chamber to ensure sufficient algal cells at the beginning of the ABDON incubation (10^5 cells/mL).

1.4. Statistical analyses

Minitab 17 (Minitab Inc., 2016) was used for all the statistical analyses. Sample means and standard derivations were calculated from the triplication of each treatment. One-way analysis of variance (ANOVA) was used at $p \leq 0.05$ to evaluate the variation in DON, BDON, and ABDON caused by three algal species with the presence and absence of bacteria.

2. Results and discussions

Initial dissolved inorganic nitrogen, initial TDN, and initial DON were determined in the samples collected from all three locations in the City of Fargo WWTP. All the samples were seeded using algae and/or bacteria inocula and incubated to determine final DON, ABDON, and BDON. The results are presented in the Figs. 1 to 5.

2.1. DON and TDN

The initial concentrations of DON and TDN in location 1 (after primary clarifier), location 2 (after BOD TF), and location 3

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

Parameter	Testing method	Detection limit (mg/L)	Instrument
Ammonia	Salicylate	0.02–2.5	Hach DR 6000 spect.
Nitrite	Diazotization	0.003–0.5	Hach DR 6000 spect.
Nitrate	SDUS	0.01–3.0	Varian Cary 50 UV–V spect.
TDN	SDUS	0.01–3.0	Varian Cary 50 UV–V spect.

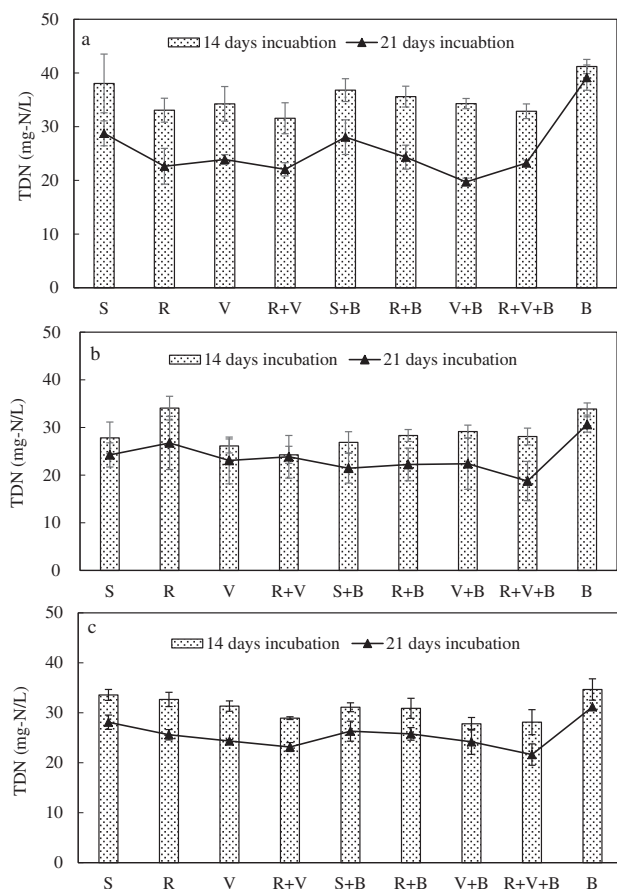TDN: Total dissolved nitrogen; SDUS: second derivative ultraviolet spectrophotometric.

Fig. 1 – TDN (a) after primary clarifier, (b) after biochemical oxygen demand trickling filter, and (c) after nitrification trickling filter locations in the samples collected from the City of Fargo Wastewater Treatment Plant. TDN: Total dissolved nitrogen; S: *S. capricornutum*; R: *C. reinhardtii*; V: *C. vulgaris*; and B: Bacteria.
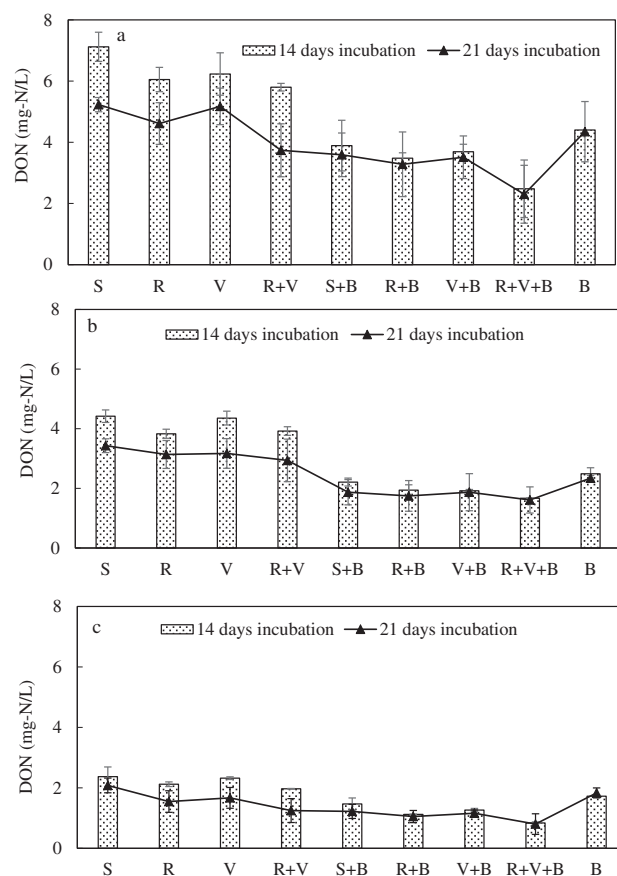

Fig. 2 – DON (a) after primary clarifier, (b) after biochemical oxygen demand trickling filter, and (c) after nitrification trickling filter locations in the samples collected from the City of Fargo Wastewater Treatment Plant. DON: Dissolved organic nitrogen; S: *S. capricornutum*; R: *C. reinhardtii*; V: *C. vulgaris*; and B: Bacteria.

(after nitrification TF) are presented in Table 2. A large portion of ammonia in the samples was converted to nitrite and a certain portion of it was converted to following nitrate in algae and/or bacteria seeded samples during the 14-day incubation in location 1 (Appendix A Figs. S2 and S3). However, complete nitrification was not achieved in any of the samples in this location during 14 days of incubation. After 21 days of incubation, more than 99% of ammonia were either nitrified to nitrate or utilized by algae. There were two sources of ammonia available in the samples. In addition to free ammonia in the samples, an additional portion of ammonia was released through ammonification of DON and this portion of ammonia was also consequently nitrified or utilized during the incubation. Similarly, after 21 days of incubation in locations 2 and 3, more than 95% of ammonia were either nitrified or taken up by algae and bacteria to support algal and bacterial growth. All these results showed that, the ammonia values after 21 days of incubation in these three locations were under detection limit.

Initial dissolved nitrite values were low (mg-N/L ≤ 0.26) in all three locations (Table 2). After 14-day of incubation dissolved nitrite values in locations 1 and 2 were quite high (varied between 10.08 and 35.00 mg-N/L) regardless of the

type of inoculum in all the samples (Appendix A Fig. S2a and b). After 21-day of incubation, all nitrite values decreased in both locations to between 9.48 and 26.00 mg-N/L except nitrite in only one sample (bacteria-only seeded sample in location 2) decreased to 0.25 mg-N/L (98% reduction). Presumable, high nitrite accumulation in the samples occurred due to lack of DO during the incubation while sufficient amount of DO was provided in bacteria-only seeded samples in location 2 (Appendix A Fig. S2b). Gonzalez et al. (2008) conducted a study in wastewater samples 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 in lagoon or pond systems.

In this study, additional experiments (parallel to original experiments) were conducted to monitor DO influence on the partial nitrification (or high nitrite accumulation) issues in the first two locations. Primary clarifier location samples were diluted about 50% to reduce nitrogen loading in the samples. The ammonia and TDN concentrations in these diluted samples were measured as 15.6 and 23.41 mg-N/L, respectively. The samples were incubated for 21 days and the results showed that between 72% and 91% of ammonia was nitrified
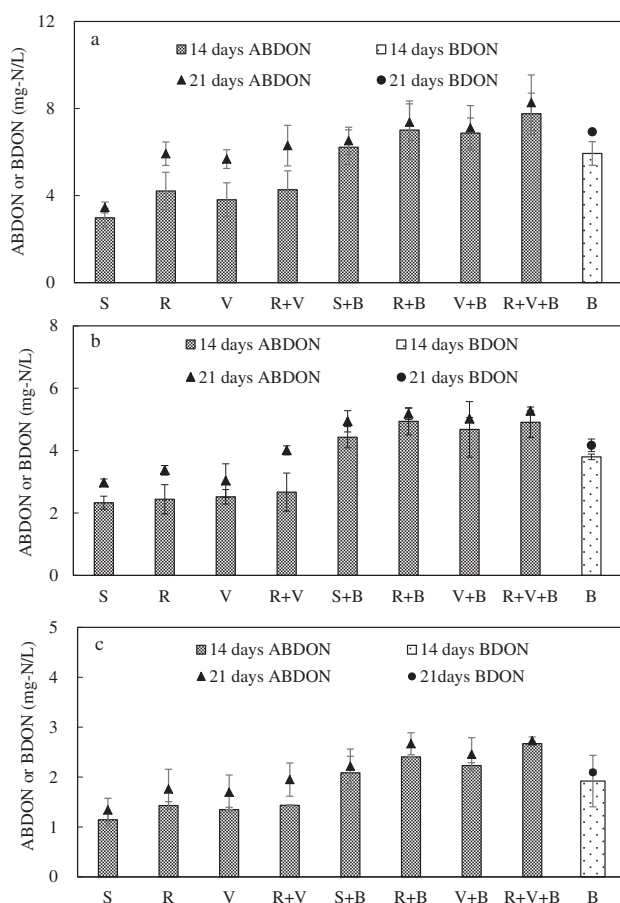
Fig. 3 – ABDON or BDON (a) after primary clarifier, (b) after biochemical oxygen demand trickling filter, and (c) after nitrification trickling filter locations in the samples collected from the City of Fargo Wastewater Treatment Plant. ABDON: Bioavailable dissolved organic nitrogen; BDON: Biodegradable dissolved organic nitrogen; S: _S. capricornutum_; R: _C. reinhardt_ii; V: _C. vulgaris_; and B: Bacteria.

Fig. 4 – ABDON/DON$_i$ and BDON/DON$_i$ (a) after primary clarifier, (b) after biochemical oxygen demand trickling filter, and (c) after nitrification trickling filter locations in the samples collected from the City of Fargo Wastewater Treatment Plant. ABDON: Bioavailable dissolved organic nitrogen; DON: Dissolve organic nitrogen; BDON: Biodegradable dissolved organic nitrogen; S: _S. capricornutum_; R: _C. reinhardt_ii; V: _C. vulgaris_; and B: Bacteria.

into nitrate and therefore, remaining nitrite level in the sample was extremely low (NO_2-N < 0.50 mg-N/L). This outcome proved that high nitrogen loading required high DO supply to complete the nitrification and the samples needed to be aerated often during the incubation. In fact, overall results showed that high nitrite concentration in the samples did not affect either algal growth or ABDON and BDON levels (Simsek et al., 2013).

Nitrite values in location 3 for 14 and 21 days of incubation were under 0.96 mg-N/L in all the samples (Appendix A Fig. S2c). Nitrite was increasing in all the samples through 14 to 21 days of incubation in this location and the highest nitrite increment was observed in bacteria-only seeded sample (89% of increase). This outcome indicated that 14 days of incubation was not adequate for algae and/or bacteria to complete biological degradation of nitrogen (Khan et al., 2009, Sattayatewa et al., 2009; Simsek et al., 2012).

Dissolved nitrate in location 1 was under 4.67 mg-N/L in all the samples seeded with algae and/or bacteria for both 14 and 21 days of incubation (Appendix A Fig. S3a). Low nitrate values were recorded since, as explained earlier, the nitrite values in
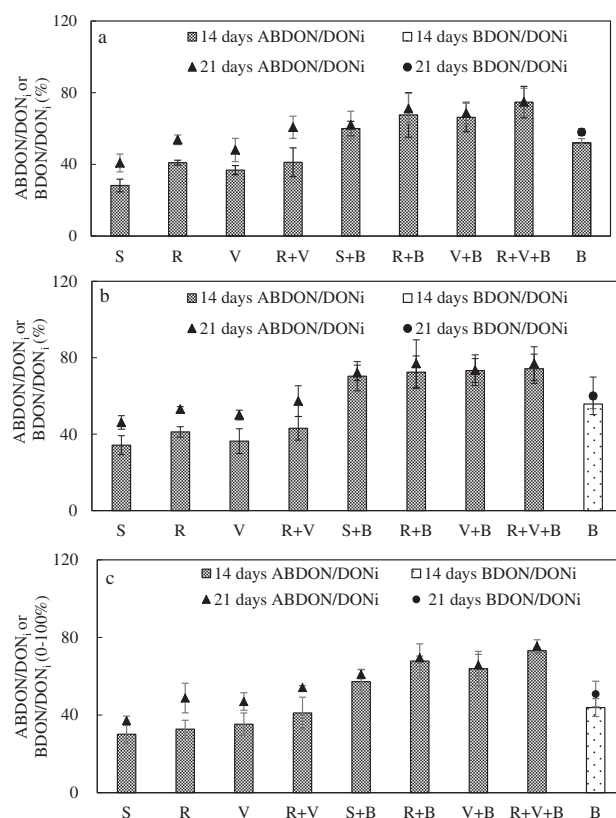
each sample in this location were very high. In location 2, nitrate values after 14 and 21 days of incubation were more or less the same in algae and algae + bacteria seeded samples while about 38% increment was observed in bacteria-only seeded sample. Nitrate in algae and algae + bacteria inoculated samples in 21 days of incubation were low (<3.80 mg-N/L) while nitrate in bacteria-only inoculated sample was high (27.88 mg-N/L). These results showed that algae consumed dissolved nitrate to support their growth while bacteria were mostly responsible to convert ammonia and nitrite to nitrate (Appendix A Fig. S3b). All ammonia in bacteria-only inoculated samples were nitrified into nitrate after 21 days of incubation. Previous studies showed that more nitrates were utilized by algae compared to nitrate utilized by bacteria (Sattayatewa et al., 2009; Simsek et al., 2013). As shown in Table 2, the initial samples from the after nitrification TF location had high ammonia and nitrate and very low nitrite values. Therefore, algae and bacteria in these samples have abilities to use either ammonia or nitrate. Cai et al. (2013) addressed that ammonia was more favorable to algae during algal assimilation process when ammonia, nitrite, and nitrate were all existed in the water ecosystem. They concluded that utilization of ammonia requires less enzyme and energy.
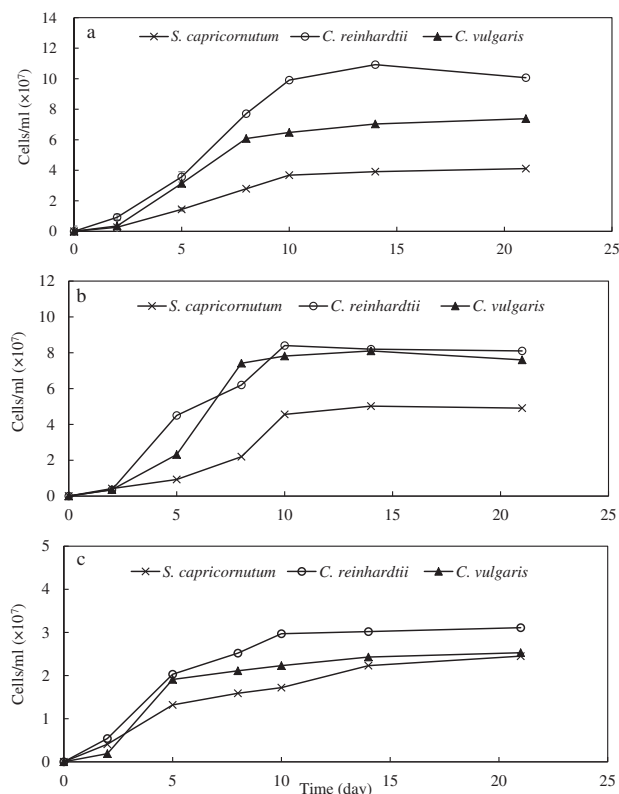
Fig. 5 – Growth curves of S. *capricornutum*, C. *reinhardtii*, and C. *vulgaris*: (a) after primary clarifier, (b) after biochemical oxygen demand trickling filter, and (c) after nitrification trickling filter locations in City of Fargo Wastewater Treatment Plant.

Initial nitrate values in location 3 were slightly reduced in all the samples after 14 days of incubation and this reduction increased after 21 days of incubation (Appendix A Fig. S3c). Out of three algal species, the highest nitrate reduction was observed in C. *vulgaris* seeded sample (34.4% of initial nitrate). The lowest nitrate in location 3 was measured in C. *reinhardtii* + C. *vulgaris* + bacteria seeded samples as 19.43 mg/L, which expressed 39.9% reduction of initial nitrate. This explained that algae + bacteria consortia can be able to remove at most 39.9% of nitrate from treated wastewater in the given conditions.

TDN after incubation (14 or 21 days) in all three locations was reduced in all the samples compared to the initial TDN values and these reductions were minimal for bacteria-only seeded samples (9% or lower) (Fig. 1). TDN values continued to reduce from 14 to 21 days of incubation; however, this reduction did not change beyond 21 days of incubation (data not shown beyond 21-day of incubation). Therefore, 21 days

of incubation were sufficient for either bacteria, algae, or algae + bacteria inocula (Simsek et al., 2012, 2013). TDN reduction and biomass accumulation in the samples showed that algae and algae + bacteria utilized nitrogen for their growth. Previous studies also proved that algal bloom intensity was declined in watershed ecosystem with the reduction of nitrogen load (Nuzzi and Waters, 2004; Gobler et al., 2005). Average initial TDN (before incubation) in location 2 was recorded as 36.27 mg-N/L (Table 2), which was lower than initial TDN in location 1 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 TDNs in location 2 (Fig. 1c) was similar to the TDNs in location 1. TDN reduction in algae inoculated samples increased with the presence of bacteria in all three locations. Initial TDN was more or less the same in BOD and nitrification TF locations expressed that TDN did not change between these two locations.

After 21 days of incubation, initial TDN reduction in alga-only inoculated samples, which were S, R, and V in all three locations was 33.32%, 47.53%, and 44.63% in location 1, respectively; 33.17%, 26.25%, and 36.37% in location 2, respectively; and finally 23.18%, 30.04%, and 33.48% in location 3, respectively. S. *capricornutum* had the lowest TDN reduction while C. *reinhardtii* and C. *vulgaris* achieved higher TDN reduction in locations 1 and 3, respectively. Ammonia and nitrate were dominant in primary clarifier and nitrification TF samples, respectively (Table 2). These results showed that algae C. *reinhardtii* utilized ammonia more than other two algal species while C. *vulgaris* utilize nitrate more than other two algal species. High ammonia and nitrate utilization of algae C. *reinhardtii* and C. *vulgaris* showed that these two species grow well in domestic wastewaters and they might be used as a test species as well as the accepted standard strain algae S. *capricornutum*. Further studies might be conducted to analyze inorganic nitrogen utilization of S, R, and V by using synthetic wastewater.

2.2. DON, ABDON, and BDON

Average initial DON values for all three locations are presented in Table 2. After incubation, DON concentrations reduced in all the samples regardless of the type of inoculum (Fig. 2). There was not a significant difference on DON reductions among the algae-only and algae + algae seeded samples for 21 days of incubation in all the samples from all three locations. DON reduction in algae + bacteria (A + B, R + B, V + B, and S + R + V + B) seeded samples was high in all the samples. DON in algae-only seeded samples in location 1 varied between 4.61 and 5.23 mg-N/L while DON in algae + bacteria seeded samples in the same location varied between 2.30 and 3.59 mg-N/L (Fig. 2a). Similar trends were

Table 2 – Average initial concentrations (before incubation) of nitrogen species in three different locations.					
Sampling location	NH$_3$-N (mg-N/L)	NO$_2$-N (mg-N/L)	NO$_3$-N (mg-N/L)	TDN (mg-N/L)	DON (mg-N/L)
Primary clarifier	33.74	0.26	0.27	43.13	8.96
BOD trickling filter	20.50	0.16	8.65	36.27	6.59
Nitrification trickling filter	1.19	0.21	31.90	36.59	3.76
TDN: total dissolved nitrogen; DON: dissolved organic nitrogen; BOD: biochemical oxygen demand.					

observed in locations 2 and 3 (Fig. 2b and c). These results proved that symbiotic relationship between algae and bacteria enhanced DON biodegradability and following bioavailability. Similarly, the percentage of DON reduction in the samples after 14 and 21 days of incubation was more or less the same in algae + bacteria seeded samples in all three locations and expressed that algae and bacteria interactions were essentially shortening the incubation period, which is an advantage on the recycling of nitrogen substrate in natural environment (Sattayatewa et al., 2009; Simsek et al., 2013).

Average initial DON in location 2 was lower than DON in location 1 and expressed that a portion of DON was removed in BOD TF location (36.5% reduction in DON). DON/TDN ratio in location 2 (18.23%) was comparable to DON/TDN ratio in location 1 (20.24%). Westgate and Park (2010) determined DON/TDN ratio after secondary clarifier locations in five different WWTPs (employed either activated sludge with diffused or mechanical aeration process or the Ludzack–Ettinger process) and found those ratios between 7% to 29%, which was comparable to the results obtained in this study. All these results indicated that the organic fraction of the TDN in the effluent was quite high and in some critical areas regulatory agencies may require WWTPs to remove DON in order to reduce TDN discharge concentration. After 21 days of incubation, the lowest DON was determined in R + V + B seeded sample as average 1.33 mg-N/L (recalcitrant DON), which was about 20% of initial DON.

Average initial DON in location 3 was recorded as 3.76 mg-N/L, which comprised of 8.7% of initial TDN. In fact, this DON value was the same as the final effluent DON value in the plant 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.

For nitrification TF location, algae + bacteria seeded samples for all three types of algae reduced DON under 1.12 mg-N/L. The highest DON reduction was observed in R + V + B seeded samples, 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. Overall, after 14 and 21 days of incubation, DON reduction in all the samples showed that some portions of DON were bioavailable to algae-only and bacteria-only seeds while some portions of DON were bioavailable to algae + bacteria seeds. Furthermore, some portions of DON were neither bioavailable to algae nor bacteria seeds, which were considered as non-bioavailable (recalcitrant) DON.

ABDON and BDON for 14 and 21 days of incubations are presented in Fig. 3 and initial DON fraction of ABDON and BDON for 14 and 21 days of incubations are presented in Fig. 4 for all the samples collected from three locations. Bioavailability of DON in location 1 was low in algae-only (S. capricornutum, C. reinhardtii, and C. vulgaris) seeded samples, varied between 3.5 and 5.9 mg-N/L (Fig. 3a). However, in algae + bacteria seeded samples (S + B, R + B, and V + B), ABDON was increased significantly ($p \leq 0.05$), which proves the symbiotic relationship

between algae and bacteria. S. capricornutum + bacteria seeded samples achieved the lowest ABDON value (6.5 mg-N/L) compared to other two types of algae + bacteria; however, this outcome was not statistically different.

The maximum attainable ABDON value could be the value of influent DON (average 8.96 mg-N/L). Therefore, none of the results in Fig. 3a achieved this ABDON value. It can be explained that a certain portion of DON remained in the samples as recalcitrant DON. The highest ABDON value in Fig. 3a was observed in R + V + B inoculated sample as 8.27 mg-N/L, which was very close to the maximum average initial DON value. These results showed that about 92% of DON was possible to be bioavailable to algae + bacteria in primary effluent samples when the optimum conditions were met. Previous studies also explained that achieving maximum (100%) ABDON value in algae + bacteria seeded sample was not attainable during the 14, 21, or 28 days of incubation periods by using algae S. capricornutum + bacteria as test species (Pehlivanoglu and Sedlak, 2004; Sattayatewa et al., 2009; Westgate and Park, 2010; Simsek et al., 2013). The same studies give explanation 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. Furthermore, Fig. 3a proved that the ABDON results for bacteria added samples (algae + bacteria and algae + algae + bacteria) were not very different from 14 to 21 days of incubation results even though 21 days of incubation results were always slightly higher (<2%) than 14 days of incubation results in all the locations. These outcomes indicated that 14 days of incubation period was actually sufficient to reach more than 90% of attainable ABDON values for algae + bacteria inocula. BDON result (biodegradability to bacteria-only) after 14 and 21 days of incubation showed that 66.3% and 77.3% of initial DON were biodegradable to bacteria, which was significantly higher than algae-only inoculated sample. On the contrary, BDON was lower than ABDON, indicating that a certain portion of DON was degraded by bacteria and subsequently used by algae.

Initial DON fraction of ABDON and BDON (ABDON/DON$_i$ and BDON/DON$_i$) in location 1 are presented in the Fig. 4a. For the algae-only seeded samples, C. reinhardtii achieved the highest bioavailability (ABDON/DON$_i$) (53.7%) while S. capricornutum achieved the lowest bioavailability (40.8%) after 21 days of incubation. In general, the bioavailability of DON to pure culture algae (S, R, or V) increased about 13% from 14-day to 21-day of incubation. However, algae + bacteria seeded samples showed a very minimal increment (1%–2%) between 14 and 21 days of incubation in this location. These results expressed that 14-day incubation for algae + bacteria seed was appropriate to attain the maximum ABDON level (Urgun-Demirtas et al., 2008; Pehlivanoglu and Sedlak, 2004), while a 21-day incubation was more appropriate for algae-only seeded samples. Algae + bacteria results showed that about 20% to 31% more ABDON was produced than in algae-only seeded samples because of a symbiotic relationship between algae and bacteria (Simsek et al., 2013; Huo et al., 2013). For bacteria-only seeded samples, around 52% and 58% of DON were biodegradable to bacteria (BDON) in 14 and 21 days of incubation, respectively (Fig. 4a), indicating that even though 14-days 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.

ABDON and BDON in location 2 are presented in Fig. 3b. ABDON and BDON in BOD TF effluent showed similar trends with after primary clarifier location. In the algae-only seeded samples, ABDON values for S, R, and V after 21 days of incubation ranged from 2.97 to 3.37 mg-N/L, which were lower than ABDON levels in algae-only seeded samples in location 1. To have more insight on bioavailability and biodegradability of DON in location 2, initial DON fractions of ABDON and BDON were calculated and presented in Fig. 4b. DON_i fraction of ABDON for algae-only inoculated samples ranged from 46% to 53% for all three types of algae, while the same fraction in algae + bacteria seeded samples ranged from 72% to 77% (in 21 days of incubation). These results showed that bioavailability of DON in location 2 in both algae-only and algae + bacteria inoculated samples was high compare to bioavailability of DON in location 1 indicating that DON became more bioavailable to algae and bacteria 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 prior to a biological system, such as by adsorption process (Sattayatewa et al., 2009; Liu et al., 2012). Soluble microbial product is a portion of refractory DON which generally is considered to resist to degradation (non-bioavailable/non-biodegradable) during bioassay (Jin et al., 2011; Kunacheva and Stuckey, 2014). The decreased level of refractory DON indicated that SMP was not produced during the BOD TF treatment. Released from the dead cells, SMP is more likely to be produced under anoxic and anaerobic conditions (Sattayatewa et al., 2009).

ABDON and BDON data collected in location 3 are presented in Fig. 3c and ABDON or BDON to DON_i ratio is presented in Fig. 4c. ABDON was low in algae-only seeded samples compared to ABDON in algae + bacteria seeded samples in this location. The similar trend was observed in locations 1 and 2. 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. reinhardtii* was slightly higher (not statistically significant) than other two algal species in all three locations. Previous studies explained that on the cell wall of *C. reinhardtii*, aminopeptidase (apase) enzyme was found to work functionally to hydrolyze proteins and peptides which can best explain the phenomenon of higher ABDON in the bioassay experiment. Additionally, a strong correlation between organic N and protein molecules such as peptides was found (Langheinrich, 1995; Westgate and Park, 2010).

ABDON/DON_i and BDON/DON_i trends were similar in location 3 compare to two previous locations (Fig. 4c). The highest ABDON after 21 days of incubation was observed in R + V + B seeded samples as 76% of initial DON in this location. These results explained that at most (minimal DON release through end of 21-day incubation might be considered) 24% of initial DON after nitrification TF location was recalcitrant DON, which was not removed in this study using algae *S. capricornutum*, *C. reinhardtii*, *C. vulgaris* and mixed culture bacteria. Overall, R + V + B inoculated samples demonstrated the maximum bioavailability of DON in all three locations. The magnitude of BDON was less than ABDON in this location and about 50.9% of initial DON was recorded as BDON. All these results confirmed that algae + bacteria seeded samples increased DON utilization.

2.3. Algal growth in different sampling locations

Cell densities of algae-only seeded samples were measured to evaluate the growth of each algal species (Fig. 5). Growth rate could be varied by experimental conditions such as initial nutrient concentration, light exposure time and intensity, and temperature during the cultivation stage. Samples collected from after primary clarifier location with higher ammonia concentration (33–35 mg-N/L) provided rapid algal growth for all three algal species, while algal growth rates after nitrification TFs were the slowest. The maximum cell number of three algae; *S. capricornutum*, *C. vulgaris*, and *C. reinhardtii* reached to 4.11×10^7, 10.06×10^7, and 7.38×10^7 colony forming units (CFU), respectively. Higher cell density may potentially produce a self-shading effect, reduces the amount of light penetrating through the bioreactor, and inhibits the growth of algae (Tam and Wong, 2000; Ruiz-Marin et al., 2010). Even though some microalgae species in a previous study were observed to have limited growth when ammonium ranged from 25 μmol/L NH_4^+-N to 1000 μmol/L NH_4^+-N, this repressive effect did not occur in this study (Collos and Berges, 2004). Overall, *C. reinhardtii* showed higher cell density than *S. capricornutum* and *C. vulgaris* in all three sampling locations. *C. vulgaris* in all the samples had shorter lag phase (8 days) indicating greater adaption ability than *C. reinhardtii* and *S. capricornutum* (10 days). Smaller cell diameter of *C. vulgaris* (2.7 μm for *C. vulgaris*, 10 μm for *C. reinhardtii*. 2.7 μm for *S. capricornutum*) with greater disparity during the incubation didn't show higher growth rate indicating that the cell size did not affect the growth of algae and its nitrogen removal efficiency.

3. Conclusion

This study provides important insight on bioavailability of DON using three different algal species (*S. capricornutum*, *C. reinhardtii* and *C. vulgaris*) with/without bacteria addition and biodegradability of DON using bacteria-only in wastewater samples collected from three different locations in a two-stage TF WWTP. In all the locations, about 70% to 80% of DON was bioavailable to mix-cultured algae + bacteria system. From this study, it can be concluded that *C. reinhardtii* and *C. vulgaris* can be selected as a standard test species over *S. capricornutum* because of their ability to remove nitrogen species from wastewaters.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.12.017.

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