Environmental Science & Technology

Article

Cite This: Environ. Sci. Technol. XXXX, XXX, XXX-XXX

pubs.acs.org/est

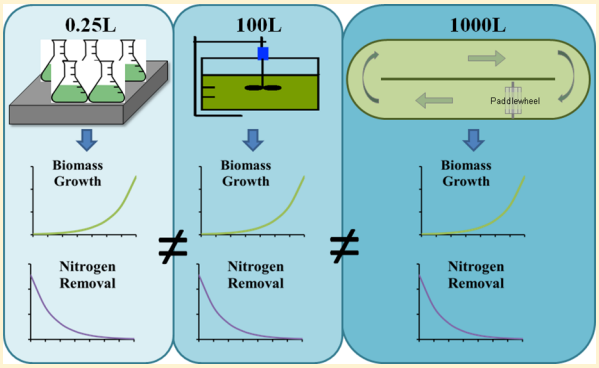
Influence of Scale on Biomass Growth and Nutrient Removal in an Algal−Bacterial Leachate Treatment System

Kaitlyn D. Sniffen, Jacob R. Price, Christopher M. Sales, and Mira S. Olson*

Drexel University, 3141 Chestnut St., Philadelphia, Pennsylvania 19104, United States

Supporting Information

ABSTRACT: Data collected from experiments conducted at a flask scale are regularly used as input data for life cycle assessments and techno-economic analyses for predicting the potential productivities of large-scale commercial facilities. This study measures and compares nitrogen removal and biomass growth rates in treatment systems that utilize an algae−bacteria consortium to remediate landfill leachate at three scales: small (0.25 L), medium (100 L), and large (1000 L). The medium- and large-scale vessels were run for 52 consecutive weeks as semibatch reactors under variable environmental conditions. The small-scale experiments were conducted in flasks as batch experiments under controlled environmental conditions. Kolomogov−Smirnov statistical tests, which compare the distributions of entire data sets, were used to determine if the ammonia removal, total nitrogen removal, and biomass growth rates at each scale were statistically different. Results from the Kolmogov−Smirnov comparison indicate that there is a significant difference between all rates determined in the large-scale vessels compared to those in the small-scale vessels. These results suggest that small-scale experiments may not be appropriate as input data in predictive analyses of full scale algal processes. The accumulation of nitrite and nitrate within the reactor, observed midway through the experimental process, is attributed to high relative abundances of ammonia- and nitrite-oxidizing bacteria, identified via metagenomic analysis.



INTRODUCTION

Algae are frequently investigated for use in nutrient removal from wastewater sources, as the presence of nutrients can cause costly economic and ecological damage to receiving water bodies.[1,2] While it is most commonly suggested that algae-based nutrient removal be utilized in domestic wastewater treatment, the direct treatment of landfill leachate may also be favorable. Landfill leachate is a high strength liquid waste, containing concentrations of ammonia regularly exceeding 800 mgN/L and is most commonly trucked to and treated at domestic wastewater treatment facilities, costing landfills hundreds of thousands to millions of dollars each year. Treating the leachate directly may reduce these costs to landfills. Few studies have examined the treatment of landfill leachate by algae, and even fewer have done so on a long-term and large-scale basis.[3−9]

Data from long-term, large-scale studies should be used as input for predictive analyses such as life cycle assessments (LCAs) and techno-economic analyses (TEAs) which are used to evaluate if a commercial investment in a technology are environmentally favorable and economically feasible, respectively. However, many of these assessment tools utilize input data generated from small-scale studies, carried out under conditions that are significantly different than those that occur on a commercial scale.[10−14] Studies conducted by Moody et al. and Quinn et al. have shown that the wildly variable results from LCAs and TEAs are due to inconsistent input data and system boundaries, which define the processes included in the assessments. These studies emphasize that it is crucial to use input data from studies that mimic the environmental conditions and type of growth vessel of the proposed commercial-scale process in these predictive analyses.[15,16]

It has repeatedly been shown in studies using algae,[15,17] *E. coli*[18−21] and yeast[20,22] that production efficiencies from small-scale studies using microorganisms do not scale proportionally to those occurring on a commercial scale. For example, changes in vessel characteristics, specifically surface area to volume ratios,[20,23,24] mixing dynamics (such as types of mixing used),[20,24,25] and environmental conditions (e.g., temperature, light exposure)[26−28] can significantly affect the activity of microorganisms.[20,23,24] Despite this discrepancy between results from small- and large-scale studies, the vast majority of LCAs and TEAs for evaluating the overall environmental impact and technical feasibility of large-scale algae production systems use algal growth rates and densities from small-scale studies as input values for their estimation of algal growth technologies.[15,16,29]

Received: August 2, 2017
Revised: October 11, 2017
Accepted: October 20, 2017
Published: October 20, 2017

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Figure 1. Large-, medium-, and small-scale vessels used in this study. This study used 100 L aquarium tanks as the medium-scale vessels (A), 1000 L raceway ponds as the large-scale vessels (B), and 0.25 L flasks as the small-scale vessels (C).

The study presented here uses a system that couples algae biomass production with the treatment of landfill leachate. Specifically, this study compares algal growth and nitrogen removal using untreated leachate as a nutrient source in liquid cultures of a mixed algae/bacteria consortium at three different-scaled systems: 0.25 L, 100 L, and 1000 L. Algal growth and nitrogen removal rates measured in small-scale (0.25 L) systems are statistically compared to rates to observed in medium- (100 L) and large-scales (1000 L) systems to determine if results from small-scale experiments are representative of larger-scale systems.

The small-scale (0.25 L) vessels were operated as seven-day batch experiments[30−32] in an environmental chamber under controlled laboratory conditions. The flask scale and controlled growth conditions were deliberately chosen as the comparison scale for this study as this experimental set up is similar to those most frequently used to generate input data for LCAs and TEAs. In contrast, the medium- (100 L) and large- (1000 L) scale vessels were operated on a seven-day semibatch cycle but under the uncontrolled environmental conditions of a greenhouse. These operating conditions were chosen as the environmental conditions of a full-scale outdoor system will not be controlled.

MATERIALS AND METHODS

Nutrient Feed and Inoculum. The nutrient feed used in this study was raw, untreated landfill leachate collected from the Sandtown Landfill in Felton, DE. Leachate was refrigerated at 2 °C from collection until use. Over the duration of the study, the ammonia content and pH of the leachate ranged from 320 to 935 mgN/L and 6.48 to 7.56, respectively. A more detailed analysis of the leachate composition at each collection time point, data provided by the Sandtown Landfill, is described in Table S1 in the Supporting Information. The algae−bacteria culture used in this study was first collected from a fish pond on the University of Pennsylvania campus. The inoculant was grown in the urban greenhouse using leachate as a nutrient feed.

Sampling, Preparation, and Analysis of Metagenome. The microbial population's composition and structure determines a bioreactor's kinetic behavior, which is commonly observed through chemical analysis. To assist in understanding these behaviors, metagenomic sequencing was applied to a sample collected from one of the growth vessels. Total DNA was extracted from four biological replicates collected prior to the beginning of the experimental period of this study. DNA extraction was carried out immediately after sample collection following the methods described in detail within Price et al.[33] The four replicate DNA extractions were then assessed for quality and concentration via NanoDrop 2000 and QuBit 2.0 and then pooled and submitted for 2 × 250 paired-end sequencing on an Illumina HiSeq sequencer. The Metagenomics RAST pipeline[34] was used to analyze the taxonomic and functional composition of the resulting sequences. Raw reads were uploaded to the MG-RAST server, and paired-end reads were joined using the join-paired-ends function. Quality control measures were applied including the removal of artificially induced sequencing artifacts,[35] the removal of sequences derived from *H. sapiens*,[36] and the removal of low quality sequences (minimum Phred score of 15, and all sequences were trimmed such that they contained a maximum of five low quality base calls).[37] Metagenome identification and quality control statistics are provided in Table S2 in the Supporting Information.

Medium- and Large-Scale Experimental Design. *Setup.* This set of experiments used two 100 L Plexiglas aquarium tanks (ATs) and two 1000 L raceway ponds (RWPs) from MicroBio Engineering (San Luis Obispo, CA). The 100 L

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

ATs and the 1000 L RWPs are referred to herein as medium- and large-scale vessels, respectively. The working volumes of the ATs and RWPs were 60 L and 600 L, respectively. Examples of the medium- and large-scale vessels used in this study can be found in Figure 1A and B.[38] All vessels were inoculated with an algae−bacteria culture, at the beginning of this study in February 2016, and housed in a greenhouse on the roof of an academic building at Drexel University in Philadelphia, PA.[5,38] These vessels were exposed to semi-ambient conditions; no additional lighting or heating/cooling was provided. Daylight hours ranged from 9 h:20 min to 15 h:1 min throughout the year, though the actual amount of light to reach the cultures in the greenhouse was substantially less due to weather and urban obstructions. Temperatures of cultures ranged from 7.8 to 41.7 °C.

Operation. The medium- and large-scale vessels were operated as semibatch reactors, on a seven-day batch cycle. Initial samples were taken at the beginning, and final samples were taken at the end of each weekly cycle, where ammonia-N, nitrate-N, nitrite-N, and biomass density were measured and analyzed. To prepare the system for the next weekly cycle, the entire volume of the medium-scale AT was pumped into the large-scale RWP. After mixing, one-third of the liquid and biomass volume was removed and then replaced with water and leachate. A portion of this mixture was then transferred back into the AT and the next week's cycle would begin. The volumes of the medium- and large-scale vessels were mixed together in between each week in order to maintain equivalent initial nutrient and biological conditions at the two scales. The volume of leachate added each week was increased slowly throughout the study, ranging from 5 to 40 L per week. Exact weekly leachate additions can be found in Figure S1 of the Supporting Information. These vessels were operated for 52 consecutive weeks, February 2016−February 2017. A more detailed description of this method can be found in Sniffen et al.[38]

Environmental Monitoring. Water temperature, pH, and dissolved oxygen within the tanks and raceway ponds were collected at 5 min intervals throughout the year-long study using Neptune System's APEX controller and probes (Morgan Hill, CA). Weekly maxima, minima, and averages were calculated for each of these parameters.

Oxygen Production Analysis. The total suspended solids contained in the weekly samples was made up of live and dead cellular biomass as well as particulates from the leachate additions. The rate of oxygen production was measured to determine the oxygen production activity relative to the total suspended solids density.[39,40] Oxygen production rates of the biomass samples were measured at the beginning and end of each week. Rates from each sample were measured using 450 mm Unisense O_2 probes (Aarhus N, Denmark) over 10 min intervals, which were run in biological triplicates. Each sample was run at 100% and 25% dilution of the initial samples. The 25% dilution samples were found to give a better representative oxygen production rate than samples with high biomass densities which may have experienced significant self-shading (data not shown).

Mixing Dynamics. The flow rate through the raceway pond was determined using the EPA float method.[41] The mixing efficiency of the ponds was determined using a salt tracer test. Five liters of a 250 g/L sodium chloride salt tracer solution was poured into the raceway pond. Water samples were taken at six locations around the pond at 10-s intervals. The conductivity of these samples was measured, and the concentration of salt tracer was determined using a standard curve.

Small-Scale Experimental Design. Flask-scale studies were performed using the same algae culture and leachate as those used in the medium- and large-scale vessels. The ranges of nutrient and biomass concentrations used in these small-scale studies mimicked those used in the medium- and large-scale studies. These small-scale studies used 250 mL flasks with a 100 mL working volume, shaken continuously in an Innova 44 environmental chamber (Eppendorf, Hamburg, Germany), under constant 25 °C temperatures and continuous light, as depicted in Figure 1C. Each nutrient and biomass concentration condition was tested using biological triplicates. The duration of all small-scale experiments was 7 days. Ammonia-N, nitrate-N, nitrite-N, and biomass density were measured and analyzed at the beginning and end of each of the experiments.

Analytical Methods. Samples collected from experiments run at all scales were analyzed using the following analytical methods: ammonia-N, nitrate-N, and nitrite-N were measured using Hach test methods 10031, 10020, and 8153, respectively, with a DR2400 spectrophotometer. Removal rates of each nitrogen species along with total dissolved nitrogen were calculated using eqs 1 and 2. In eqs 1 and 2, C_{oN} and C_{fN} are the initial and final concentrations of the nitrogen species [mgN/L], respectively, where t is time [days],[5] N_T is the total inorganic nitrogen concentration [mgN/L], and R_i is the removal rate of species i [mgN/L/day] for each batch period. These equations were developed based on preliminary studies which monitored nitrogen removal and biomass growth every 1 to 2 days during week-long studies. These preliminary studies found that the removal and growth rates were linear throughout the week.

$$N_T = C_{NH3} + C_{NO2-} + C_{NO3-} \quad (1)$$

$$R_i = (C_{oN} - C_{fN})/t \quad (2)$$

Biomass density was measured at the beginning and end of each week by standard total suspended solid protocol using a 0.45 μm filter and 20 mL of sample.[42] Biomass density samples were run using biological duplicates. Weekly biomass growth rates were calculated using eq 3, where $C_{o,\mathrm{Biomass}}$ and $C_{f,\mathrm{Biomass}}$ are the initial and final biomass concentrations [g/L], R_B is the biomass growth rate [g/L/day], and t is time [days].

$$R_B = (C_{f,\mathrm{Biomass}} - C_{o,\mathrm{Biomass}})/\mathrm{t} \quad (3)$$

Statistical Analysis. Weekly rates (nutrient removal and biomass growth) and environmental data were pooled for regression and correlation analysis to identify general trends. Weekly nutrient removal and biomass growth rates were then binned by scale for rate comparisons. Kolmogov−Smirnov statistical tests are used to determine if the distributions of two data sets are significantly different. Data binned by scale was compared using Kolmogov−Smirnov tests to determine if the weekly rates calculated at each scale were statistically different. Regressions and correlation data categories included initial and final weekly ammonia-N, nitrate-N, and nitrite-N concentrations and removal rates; initial and final weekly biomass concentrations and growth rate; and initial and final weekly oxygen production rates; as well as maximum, minimum, and average pH, water temperature, dissolved oxygen, and number of daylight hours. SPSS 24[43] and Microsoft Excel were used to analyze this data.

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

RESULTS AND DISCUSSION

Environmental Conditions. Seasonal trends of the temperature, pH, and dissolved oxygen (DO) in the tanks and raceway ponds, over the course of the year-long study, are shown in Figure 2A–C. Over the course of a day, the pH, temperature, and dissolved oxygen in the tanks and raceway ponds rise and fall on a diurnal cycle. This daily trend is shown over a representative two-week span, depicted in Figure 3. As confirmed by observed data, the ATs and RWPs were subjected to identical environmental conditions due to their colocation.

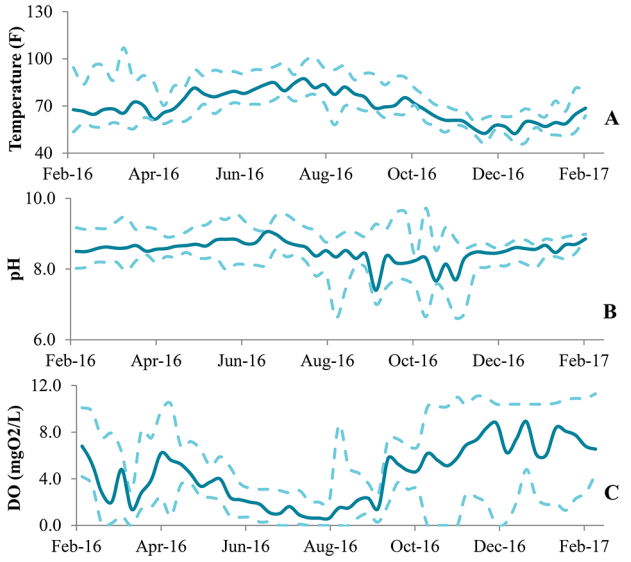


Figure 2. Weekly environmental conditions of the medium- and large-scale vessels. The weekly average temperature (A), pH (B), and dissolved oxygen (C) over the course of the year-long experimental period are shown. In each panel, the weekly maximum and minimum values are shown as dashed lines. The solid line represents the weekly average value of each parameter.

Medium- and Large-Scale Experimental Results. The ranges in initial ammonia, total nitrogen, and biomass concentrations over the course of the study were 0.2–161 mgN/L, 8.12–187.8 mgN/L, and 40–1780 mgBiomass/L, respectively. A breakdown of initial conditions by scale is presented in Table S3 in the Supporting Information. Leachate input volumes were approximately 20 L/week during the first half of this study, while the input volumes during the second half were approximately 30–40L/week. During times of high nitrite concentrations in the systems, no leachate was added. Weekly leachate input volumes during this study can be found in Figure S1 of the Supporting Information.

The dissolved nitrogen content of the leachate was 99% in the form of ammonia. Ammonia and total nitrogen removal rates along with biomass growth rates varied over the course of the study; the average ± standard deviation of these rates for the large-, medium-, and small-scales are presented in Table 1. The ammonia and total nitrogen removal rates for each of the vessels are presented in Figure 4A and B.

The maximum biomass density measured over the course of this study was 2100 mg/L. Biomass growth rates ranged from −106 to 199 mg/L/day. A negative growth rate means that there was biomass loss over the course of the week. Biomass loss was seen in 70 out of 205 weeks' worth of data from all vessels. Negative biomass growth rates did not statistically favor any scale, season, or environmental condition. Detailed discussion of this point can be found in Sniffen et al. (2017, submitted). When accounting for only weeks with positive growth rates, the average and standard deviation was 28 ± 32 mg/L/day. This range of growth rate is similar to rates reported for algae growth fed with domestic wastewater.[44−47] Weekly biomass growth rates for all vessels can be found in Figure S2 of the Supporting Information.

Metagenomics Results. Alpha diversity within the metagenomic sample was estimated to be 376 taxa. Annotations from the NCBI Reference Sequence Database[48−50] and the M5nr protein sequence database,[51] acquired via the MG-RAST interface, were used to analyze and interpret the taxonomic composition of the microbial community. The vast majority of reads were attributed to the domains of Bacteria and Eukaryota (Table S4, Supporting Information). Proteobacteria and Bacteroidetes were the first and second most abundant phyla respectively (Table S5, Supporting Information). Under the annotations for both reference databases, Chlorophyta and Cyanobacteria comprised about 2% of the annotated reads (Table S5). *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Scenedesmus obliquus* accounted for the bulk of the reads attributed to Chlorophyta (Table S6, Supporting Information). The distribution of reads among species falling within the phyla Cyanobacteria was much more uniform at the species level (Table S7, Supporting Information); agglomerating taxa by Genus indicates that the genus *Synechococcus* accounts for roughly 30% of the reads within Cyanobacteria, followed by *Cyanothece spp.* at 19%, and *Nostoc spp.* at 12% within this

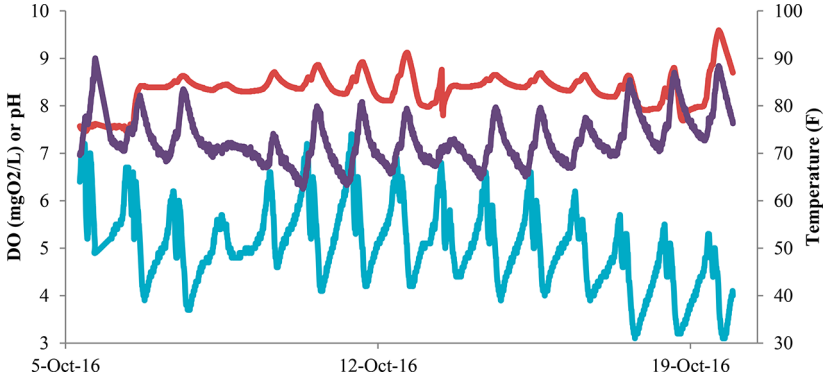


Figure 3. Diurnal cycle of vessel conditions. A two-week sample of the daily fluctuations of pH, dissolved oxygen, and temperature of one large-scale vessel are shown. pH is shown in red, DO in blue, and temperature in purple.

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 1. Average ± Standard Deviation of Ammonia Removal, Total Nitrogen Removal, and Biomass Growth Rates, at Each Scale

	Large scale	Medium scale	Small scale
Total N Removal Rate (mgN/L/day)	1.63 ± 2.95	1.82 ± 3.97	3.16 ± 2.73
Ammonia Removal Rate (mgN/L/day)	2.33 ± 3.05	2.68 ± 3.90	2.96 ± 2.71
Net Biomass Growth Rate (mg/L/day)	12 ± 24	4 ± 38	18 ± 21

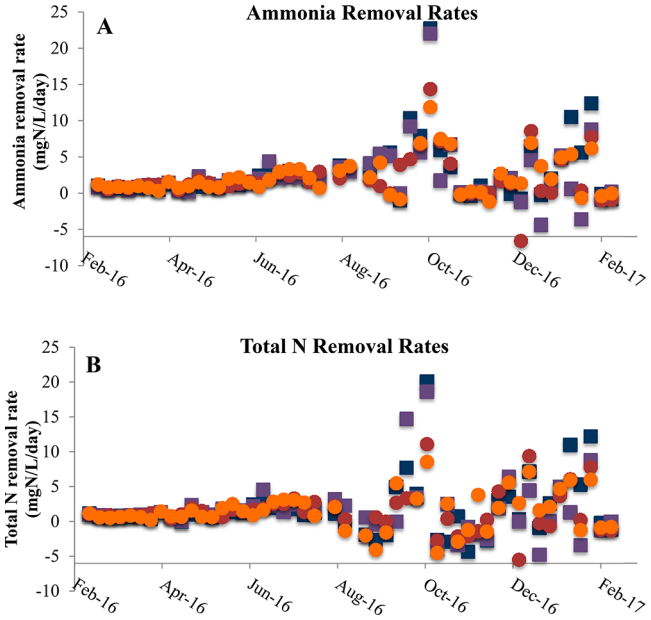


Figure 4. Ammonia (A) and total nitrogen (B) removal rates from medium- and large-scale vessels. The ammonia and total nitrogen removal rates over the year-long study are shown. Rates from large-scale vessels are shown in red and orange circles. Rates from medium-scale vessels are shown in blue and purple squares.

phyla. AOB and NOB were observed to comprise roughly 12% and 1% of the total microbial population (Table S8, Supporting Information). The anammox bacteria *Candidatus kueneia* was also discovered in the M5nr annotations. This indicates that anammox may be a potential nitrogen transformation pathway within this system (Table S8).

Low abundances for eukaryotes, such as Chlorophyta, within this study, are common in metagenomic analyses, arising from their being underrepresented within reference databases, including those used by MG-RAST.[52] This induces bias toward the identification and annotation of bacterial sequences, while sequences from eukaryotic sources may fail to be annotated. Because of this, the abundances of Chlorophyta reported here should be viewed as a floor or minimum and should not be directly compared to the relative abundance of the kingdom Bacteria or the taxa therein.

Statistical Analysis. A Pearson's correlation matrix was calculated for all of the categories listed in the statistical Methods section. Strong, statistically significant correlations were found between initial ammonia concentration and ammonia removal rate (r = 0.854, p < 0.0005) and initial ammonia concentration and total nitrogen removal rate (r = 0.703, p < 0.0005). Moderate, but statically significant, correlations were found between biomass growth rate and total nitrogen removal rate (r = 0.196, p < 0.006), biomass growth rate and maximum pH (r = 0.267, p < 0.0005), total nitrogen removal rate and maximum pH (r = 0.226, p = 0.001), and ammonia removal rate and maximum pH (r = 0.197, p = 0.006). The Pearson's correlation matrix calculated that biomass growth rate, total nitrogen removal rate, and ammonia removal rate were all moderately, but significantly, positively correlated to the weekly maximum pH.

Stepwise regressions were evaluated for ammonia removal, total nitrogen removal, and biomass growth rates. The best fit, statistically significant regressions with corresponding parameters and standardized beta values are presented in Table 2; nonstandardized values for these regressions can be found in Table S9 of the Supporting Information.

Water temperature influenced the regressions of the total nitrogen removal and biomass growth rates. The maximum weekly water temperature had a positive effect on biomass growth, and the minimum weekly water temperature had a negative effect on total nitrogen removal. This appears to show that both nitrogen removal and biomass growth rates increase with warmer temperatures. This effect is expected as nitrogen consumption and biomass growth are dependent on many

Table 2. Statistically Significant Regression Models

Dependent Variable	Model Components	R^2	ANOVA Regression Sig.	Standardized β Coefficients	Sig of Coefficients
Ammonia Removal Rate	Weekly Initial NH_3 conc.	0.837	p < 0.0005	0.909	p < 0.0005
	Weekly Avg DO			−0.188	p < 0.0005
	Weekly Max pH			0.18	p < 0.0005
	Weekly Min pH			−0.125	p < 0.0005
Total N Removal Rate	Weekly Initial NH_3 conc.	0.62	p < 0.0005	0.802	p < 0.0005
	Weekly Max pH			0.246	p < 0.0005
	Weekly Min water temp			−0.348	p < 0.0005
	Weekly Avg Daylight hrs			0.343	p < 0.0005
Biomass Growth Rate	Weekly Initial biomass conc.	0.395	p < 0.0005	−0.946	p < 0.0005
	Weekly Avg Daylight hrs			0.509	p < 0.0005
	Weekly Max water temp			0.196	0.002
	Weekly Min DO			−0.14	0.021

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

cellular enzymes and chemical reactions, many of which are highly affected by temperature.

The weekly average dissolved oxygen significantly affects the regressions of the ammonia removal rate and biomass growth rate. Fluctuations in environmental conditions are expected when culturing outdoors. Accounting for the changing environmental influences is important when making predictions about large-scale, outdoor productivities.

The Kolmogov−Smirnov tests were used to compare the weekly rates from the large-, medium-, and small-scales to determine if the sets of rates from these experiments were statistically different. The ammonia removal, total nitrogen removal, and biomass growth rates found from the small-scale flasks were compared to those from the medium-scale ATs and large-scale RWPs. The significance of the comparison between these growth vessels can be found in Table 3.

Table 3. Significance of Kolmogov−Smirnov comparisons between rates from small-, medium-, and large-scale experiments[a]

Kolmogov−Smirnov comparisons	Ammonia Removal Rate *p* value	Total N Removal Rate *p* value	Net Biomass Growth Rate *p* value
Large scale vs medium scale	0.614	0.838	**0.008**
Large scale vs small scale	**0.020**	**<0.0005**	**<0.0005**
Medium scale vs small scale	**0.039**	**0.002**	**0.001**

[a]Large-scale n = 97, medium-scale n = 99, small scale n = 60.

The results of the Kolmogov−Smirnov tests indicate that in terms of ammonia and total nitrogen removal there is a statistically significant difference between the rates that were measured at the large- and medium-scales to those that were measured at the small scale. The comparison of biomass growth rates showed that there is a significant difference among the biomass growth rates in systems run at all scales. The growth of photosynthetic algae is highly influenced by exposure to light, which is dependent on the characteristics of the growth vessel. An increase in scale can significantly change growth vessel characteristics including surface area to volume ratio, mixing dynamics, environmental conditions, and exposure to the inoculation of wild organisms.[20,23]

Surface Area to Volume Ratio. The vessels at each scale have different surface area to volume ratios. Since photosynthetic algae are influenced by light exposure, the light-exposed surface areas (LE-SA) of all vessels are of interest and are compared in Table 4. The surface area of the medium-scale ATs is only 0.22 m^2; however, light can enter the transparent sides of the tanks, which increases the sunlight-exposed area to 1.16 m^2. The LE-SA:V ratio of the aquarium tank is approximately 12 m^{-1}; if only the top surface area of the tank is accounted for, then the SA:V of the aquarium tank is approximately 3 m^{-1}. Due to the angle of incidence between the sun and the sides of the aquaria tanks, the effective LE-SA:V ratio is probably between these two values.

Table 4. Light-Exposed Surface Area to Volume Ratio (LE-SA:V) of All Vessels Used in This Study

Scale	Vessel	Light-Exposed Surface Area (LE-SA) (m^2)	LE-SA:V (m^{-1})
Large	1000 L Raceway Pond	3.47	5
Medium	100 L Aquaria Tank	0.22−1.16	3−12
Small	0.25 L Flask	0.00112	112

The surface areas of the large-scale RWPs were 3.47m^2. The sides of the RWPs were made of an opaque plastic which did not allow additional light to enter the system. The LE-SA:V of the large-scale RWP is 5m^{-1}, which is similar to that of the AT. Further increasing the scale of the large-scale RWPs to a commercial-scale vessel of the same design will allow the LE-SA:V ratio to stay the same as the depth of the raceway ponds and remain constant, but the overall surface area will increase proportionally with volume.

The small-scale glass flasks had significantly more light exposure when compared to the RWPs or ATs. The small-scale LE-SA:V is 10 to 20 times larger than those of the medium- and large-scale vessels and clearly had an effect on the algae growth rates (Table 1). Similarly, the medium- and large-scale systems, which were exposed to the same biological, nutrient, light, and environmental conditions, also exhibited statistically significantly different biomass growth rates.

Mixing Dynamics. The large-scale vessels were mixed using a paddle wheel, medium-scale vessels were mixed using overhead stirrers, and small-scale vessels were mixed on a shaking platform, as shown in Figure 1. All vessels showed complete mixing. The tracer test used in the raceway pond resulted in complete mixing within 30 s (data not shown). Despite these tests, it was seen that due to the adherent properties of algae and bacterial cells walls some microorganisms were able to attach to the sidewalls or corners and create algae-bacterial flocs.[53] These biofilms were seen on the sidewalls of the medium- and large-scale vessels but were removed at the beginning of every week. This attachment to surfaces creates biofilm and microenvironments where conditions can be significantly different from the bulk system.[23,54] In this case, it is possible that while the overall systems were well mixed some microenvironments were limited in nutrients or anoxic. These types of microenvironments can allow microbes, unsuited for life in the bulk of the vessel, to flourish. This contrasts with the small-scale studies, performed in flasks with smooth glass walls and no corners, which make the development of these microenvironments unlikely over the seven-day experimental period. The effect of these microenvironments that will develop at a large scale cannot be accounted for in these small-scale studies where they do not develop.

Environmental Conditions. During the months with warmer water temperatures, lower dissolved oxygen concentrations were seen, as presented in Figure 2. Daily fluctuations in dissolved oxygen follow this same trend, where the warmest parts of the day showed the lowest dissolved oxygen concentrations, as shown in Figure 3. These annual and daily trends are expected as gas solubility decreases with increasing temperature. Daily fluctuations were also seen in pH which can be explained by the occurrence of photosynthesis. During times where photosynthesis is occurring, CO_2 is consumed, and pH increases. During times where respiration is occurring, pH decreases. These pH increases and decreases are shown in Figure 3 and coincide with the light and dark times of the day where photosynthesis is or is not occurring.

Influence of Competing Microorganisms. While axenic cultures may be used in photobioreactors fed with sterile nutrient media, they are not appropriate for open pond systems

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

or those fed with unsterilized wastewater. All experiments in this study, even the small-scale, used a mixed algae−bacteria culture, containing approximately 376 species (as determined from the metagenomic analysis), and were fed with raw, unsterilized leachate. However, many studies, including those looking to use wastewater at a large scale, use axenic algae cultures and sterilized media.[55] Axenic cultures and sterilized wastewater is not an accurate representation of algae growth at large-scale conditions.

All experiments in this study were inoculated with a culture containing many types of algae and bacteria. Additionally, the untreated leachate used as the nutrient source contained more bacteria, serving as a source for additional microbial diversity during the experiment. An important difference between the small-, medium-, and large-scales is that the medium- and large-scale cultures were exposed to the open air, which could carry in algae and bacteria by wind through the open windows of the greenhouse, whereas the small-scale cultures were in an enclosed environmental chamber. Medium- and large-scale cultures were exposed to additional sources of algae and bacteria, which have the potential to out-compete inoculated cultures; this is an important aspect to consider in commercial algae growth.

In this study, ammonia oxidizing bacteria (AOB) were present in all cultures. During the first half of this study, no significant amount of nitrite or nitrate was measured. During this time period, leachate influent volumes were kept low and slowly increased from 5 to 25 L of leachate per week, as shown in Figure S1 of the Supporting Information. However, after 20 weeks, in August 2016, near complete conversion of ammonia to nitrite was regularly seen. This occurred during a month where low dissolved oxygen concentrations and high water temperatures were observed. This continued for approximately 10 weeks, until the middle of October 2016. After 2 weeks of nitrite concentrations above 50 mgN/L, leachate influent was reduced to zero, but weekly removal of biomass and liquid with water replacement continued. High nitrite concentrations is a somewhat regular challenge in open biological wastewater treatment systems.[56,57] However, since nitrite is toxic to aquatic and land species, this biological activity is not encouraged.[58] In this study, excessive nitrite concentrations were managed by removing a larger portion of the liquid and biomass than the usual weekly amount and reducing the leachate influent concentration. As nitrite concentrations lowered, small amounts of nitrate were detected. Once nitrite concentrations were measured below 50 mgN/L, leachate additions continued. After nitrite concentrations dropped below 10 mgN/L, larger leachate volumes were added, approximately 25−40 L weekly, from November 2016 through the end of the study in February 2017. The change in the active microbial community as well as the change in leachate influent volumes may have caused the greater variation in ammonia and nitrogen removal rates during the second half of this study. For example, the average ± standard deviation of the ammonia removal rates from the medium- and large-scale tanks for the first 26 weeks was 1.65 ± 0.949 mgN/L/day, while the last 26 weeks was 3.41 ± 4.69 mgN/L/day.

The detection of nitrite and nitrate in the vessels is indicative of ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively). Metagenomic analysis confirmed that AOB and NOB comprise a large portion of the microbial community, with between 12.89% (using M5nr database) and 13.28% (using RefSeq database) of the reads being attributed to these taxa (Table S9). A recent investigation of 10 full-scale nutrient removal treatment plants by Yao and Peng[58] revealed that nitrifying bacteria composed between 1% and 10% of the microbial population. As the plants studied by Yao and Peng were operated, in part, for nitrification purposes, the high abundance of nitrifying bacteria reads in the metagenomic results indicates that the microbial community in the current study possesses the potential to carry out nitrification to a large degree.

From this study, small-scale growth rates and total nitrogen removal rates by an algal−bacterial consortium would not be appropriate input values for large-scale predictive studies. If biomass growth or total nitrogen or ammonia removal rates are important to the applied study, then using a raceway pond (or other vessel similar to the proposed commercial vessel) is likely necessary to get an accurate prediction of productivity that will occur at a commercial scale. For example, if average rates from the small-scale vessels of this study were used to estimate a 10-acre-ft commercial facility, the annual biomass production and nitrogen removal capabilities would be approximately 81,000 and 14,200 kg/year, respectively. If the same calculations were done using the average rates from the large-scale vessels of this study, the annual biomass production and nitrogen removal capabilities would be estimated to only be 54,000 and 7,300 kg/year, respectively. Ideally, future studies used as predictive input data for commercial-scale growth will be carried out with cultures exposed to the same outdoor air/bacterial influences, light, temperature, nutrient sources, and algae cultures and use vessels as close to the intended full scale as possible.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b03975.

Figure S1: Weekly leachate additions, liters of leachate added each week. Figure S2: Biomass growth rates from medium- and large-scale vessels over the year-long study. Table S1: Chemical components of leachate at each collection time point. Table S2: MG-RAST identification information and quality control statistics. Table S3: Ranges of initial conditions at each scale Table S4: Domain-level relative abundance. Table S5: Phylum-level relative abundance (top 10 most abundant). Table S6: Relative abundance of taxa falling within Chlorophyta using RefSeq annotations. Table S7: Relative abundance of taxa falling within Cyanobacteria using RefSeq annotations. Table S8: Relative abundance of nitrifying and anammox bacteria (Genus-level)*. Table S9: Nonstandardized β values of regression. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mso28@drexel.edu.

ORCID

Kaitlyn D. Sniffen: 0000-0002-1684-2099

Mira S. Olson: 0000-0001-7780-9711

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Abdel-Raouf, N.; Al-Homaidan, A. A.; Ibraheem, I. B. Microalgae and wastewater treatment. *Saudi J. Biol. Sci.* **2012**, *19* (3), 257−75.

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(2) *A Compilation of Cost Data Associated with the Impacts and Control of Nutrient Pollution*, U.S. Environmental Protection Agency, 2015.
(3) Lin, L.; Chan, G. Y. S.; Jiang, B. L.; Lan, C. Y. Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. *Waste Manage.* **2007**, *27* (10), 1376−1382.
(4) Kumari, M.; Ghosh, P.; Thakur, I. S. Landfill leachate treatment using bacto-algal co-culture: An integrated approach using chemical analyses and toxicological assessment. *Ecotoxicol. Environ. Saf.* **2016**, *128*, 44−51.
(5) Sniffen, K. D.; Sales, C. M.; Olson, M. S. Nitrogen removal from raw landfill leachate by an algae-bacteria consortium. *Water Sci. Technol.* **2016**, *73* (3), 479−485.
(6) Zhao, X.; Zhou, Y.; Huang, S.; Qiu, D.; Schideman, L.; Chai, X.; Zhao, Y. Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production. *Bioresour. Technol.* **2014**, *156*, 322−328.
(7) Mustafa, E. M.; Phang, S. M.; Chu, W. L. Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system. *J. Appl. Phycol.* **2012**, *24* (4), 953−963.
(8) Edmundson, S. J.; Wilkie, A. C. Landfill leachate–a water and nutrient resource for algae-based biofuels. *Environ. Technol.* **2013**, *34* (13−16), 1849−57.
(9) Martins, C. L.; Fernandes, H.; Costa, R. H. Landfill leachate treatment as measured by nitrogen transformations in stabilization ponds. *Bioresour. Technol.* **2013**, *147*, 562−8.
(10) Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **2007**, *25* (3), 294−306.
(11) Rawat, I.; Ranjith Kumar, R.; Mutanda, T.; Bux, F. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Appl. Energy* **2011**, *88* (10), 3411−3424.
(12) Li, Q.; Du, W.; Liu, D. Perspectives of microbial oils for biodiesel production. *Appl. Microbiol. Biotechnol.* **2008**, *80* (5), 749−56.
(13) Lardon, L.; Helias, A.; Sialve, B.; Steyer, J.; Bernard, O. Life-Cycle Assessment of Biodiesel Production from Microalgae. *Environ. Sci. Technol.* **2009**, *43* (17), 6475−6481.
(14) Amer, L.; Adhikari, B.; Pellegrino, J. Technoeconomic analysis of five microalgae-to-biofuels processes of varying complexity. *Bioresour. Technol.* **2011**, *102* (20), 9350−9359.
(15) Moody, J. W.; McGinty, C. M.; Quinn, J. C. Global evaluation of biofuel potential from microalgae. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (23), 8691−8696.
(16) Quinn, J. C.; Davis, R. The potentials and challenges of algae based biofuels: A review of the techno-economic, life cycle, and resource assessment modeling. *Bioresour. Technol.* **2015**, *184*, 444−452.
(17) Schreiber, C.; Behrendt, D.; Huber, G.; Pfaff, C.; Widzgowski, J.; Ackermann, B.; Müller, A.; Zachleder, V.; Moudříková, S.; Mojzeš, P.; et al. Growth of algal biomass in laboratory and in large-scale algal photobioreactors in the temperate climate of western Germany. *Bioresour. Technol.* **2017**, *234*, 140−149.
(18) Enfors, S. O.; Jahic, M.; Rozkov, A.; Xu, B.; Hecker, M.; Jürgen, B.; Krüger, E.; Schweder, T.; Hamer, G.; O'Beirne, D.; et al. Physiological responses to mixing in large scale bioreactors. *J. Biotechnol.* **2001**, *85* (2), 175−185.
(19) Bylund, F.; Collet, E.; Enfors, S. O.; Larsson, G. Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation. *Bioprocess Eng.* **1998**, *18* (3), 171−180.
(20) Junker, B. H. Scale-up methodologies for Escherichia coli and yeast fermentation processes. *J. Biosci. Bioeng.* **2004**, *97* (6), 347−364.
(21) Hewitt, C. J.; Nienow, A. W. The Scale-Up of Microbial Batch and Fed-Batch Fermentation Processes. *Adv. Appl. Microbiol.* **2007**, *62*, 105−135.
(22) George, S.; Larsson, G.; Enfors, S. O. A scale-down two-compartment reactor with controlled substrate oscillations: Metabolic response of Saccharomyces cerevisiae. *Bioprocess Eng.* **1993**, *9* (6), 249−257.
(23) Kesaano, M.; Sims, R. C. Algal biofilm based technology for wastewater treatment. *Algal Res.* **2014**, *5*, 231−240.
(24) Einsele, A. Scaling up bioreactors. *Process Biochemistry* **1978**, *13* (7), 13−14.
(25) Xiao, Y.; Li, Z.; Li, C.; Zhang, Z.; Guo, J. Effect of Small-Scale Turbulence on the Physiology and Morphology of Two Bloom-Forming Cyanobacteria. *PLoS One* **2016**, *11* (12), e0168925.
(26) Sforza, E.; Simionato, D.; Giacometti, G. M.; Bertucco, A.; Morosinotto, T. Adjusted light and dark cycles can optimize photosynthetic efficiency in algae growing in photobioreactors. *PLoS One* **2012**, *7* (6), e38975.
(27) Scoma, A.; Giannelli, L.; Faraloni, C.; Torzillo, G. Outdoor H(2) production in a 50-L tubular photobioreactor by means of a sulfur-deprived culture of the microalga Chlamydomonas reinhardtii. *J. Biotechnol.* **2012**, *157* (4), 620−7.
(28) Sorokin, C.; Krauss, R. W. The Effects of Light Intensity on the Growth Rates of Green Algae. *Plant Physiol.* **1958**, *33* (2), 109.
(29) Davis, R. E.; Fishman, D. B.; Frank, E. D.; Johnson, M. C.; Jones, S. B.; Kinchin, C. M.; Skaggs, R. L.; Venteris, E. R.; Wigmosta, M. S. Integrated Evaluation of Cost, Emissions, and Resource Potential for Algal Biofuels at the National Scale. *Environ. Sci. Technol.* **2014**, *48* (10), 6035−6042.
(30) Converti, A.; Casazza, A. A.; Ortiz, E. Y.; Perego, P.; Del Borghi, M. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. *Chem. Eng. Process.* **2009**, *48* (6), 1146−1151.
(31) Chen, G. Q.; Jiang, Y.; Chen, F. Variation of lipid class composition in Nitzschia laevis as a response to growth temperature change. *Food Chem.* **2008**, *109* (1), 88−94.
(32) Lin, L.; Chan, G. Y. S.; Jiang, B. L.; Lan, C. Y. Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. *Waste Manage.* **2007**, *27* (10), 1376−1382.
(33) Price, J. R.; Keshani Langroodi, S.; Lan, Y.; Becker, J.; Shieh, W. K.; Rosen, G. L.; Sales, C. M. Emerging investigators series: untangling the microbial ecosystem and kinetics in a nitrogen removing photosynthetic high density bioreactor. *Environmental Science: Water Research & Technology* **2016**, *2* (4), 705−716.
(34) Meyer, F.; Paarmann, D.; D'Souza, M.; Olson, R.; Glass, E. M.; Kubal, M.; Paczian, T.; Rodriguez, A.; Stevens, R.; Wilke, A.; et al. The metagenomics RAST server − a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinf.* **2008**, *9* (1), 386.
(35) Gomez-Alvarez, V.; Teal, T. K.; Schmidt, T. M. Systematic artifacts in metagenomes from complex microbial communities. *ISME J.* **2009**, *3* (11), 1314−1317.
(36) Langmead, B.; Trapnell, C.; Pop, M.; Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **2009**, *10* (3), R25.
(37) Cox, M. P.; Peterson, D. A.; Biggs, P. J. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinf.* **2010**, *11* (1), 485.
(38) Sniffen, K. D.; Sales, C. M.; Olson, M. S. Comparison of Scale in a Photosynthetic Reactor System for Algal Remediation of Wastewater. *J. Visualized Exp.* **2017**, *121*, e55256.
(39) Lee, C.-G.; Palsson, B.Ø. High-density algal photobioreactors using light-emitting diodes. *Biotechnol. Bioeng.* **1994**, *44* (10), 1161−1167.
(40) Miller, S.; Abeliovich, A.; Belfort, G. Effects of High Organic Loading on Mixed Photosynthetic Wastewater Treatment. *Journal (Water Pollution Control Federation)* **1977**, *49* (3), 436−440.
(41) Meals, D. W. D.; Steven, A. *Surface Water Flow Measurement for Water Quality Monitoring Projects*; Tech notes 3; U.S. Environmental Protection Agency, Tetra Tech Inc., 2008.
(42) Eaton, A.; Clesceri, L. S.; Greenberg, A. E.; Franson, M. A. H. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association, 1998.
(43) *IBM SPSS Statistics for Macintosh*; IBM Corp.: Armonk, NY, 2016.

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(44) Wang, L.; Min, M.; Li, Y.; Chen, P.; Chen, Y.; Liu, Y.; Ruan, R.; Wang, Y. Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. *Appl. Biochem. Biotechnol.* **2010**, *162* (4), 1174−1186.
(45) Woertz, I.; Feffer, A.; Lundquist, T.; Nelson, Y. Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *J. Environ. Eng.* **2009**, *135* (11), 1115−1122.
(46) Craggs, R.; Sutherland, D.; Campbell, H. Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. *J. Appl. Phycol.* **2012**, *24* (3), 329−337.
(47) Sturm, B. S. M.; Peltier, E.; Smith, V.; deNoyelles, F. Controls of microalgal biomass and lipid production in municipal wastewater-fed bioreactors. *Environ. Prog. Sustainable Energy* **2012**, *31* (1), 10−16.
(48) Brister, J. R.; Ako-Adjei, D.; Bao, Y.; Blinkova, O. NCBI Viral Genomes Resource. *Nucleic Acids Res.* **2015**, *43* (D1), D571−D577.
(49) O'Leary, N. A.; Wright, M. W.; Brister, J. R.; Ciufo, S.; Haddad, D.; McVeigh, R.; Rajput, B.; Robbertse, B.; Smith-White, B.; Ako-Adjei, D.; et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res.* **2016**, *44*, D733−D745.
(50) Tatusova, T.; DiCuccio, M.; Badretdin, A.; Chetvernin, V.; Nawrocki, E. P.; Zaslavsky, L.; Lomsadze, A.; Pruitt, K. D.; Borodovsky, M.; Ostell, J. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* **2016**, *44* (14), 6614−6624.
(51) Wilke, A.; Harrison, T.; Wilkening, J.; Field, D.; Glass, E. M.; Kyrpides, N.; Mavrommatis, K.; Meyer, F. The M5nr: a novel non-redundant database containing protein sequences and annotations from multiple sources and associated tools. *BMC Bioinf.* **2012**, *13* (1), 141.
(52) Lindgreen, S.; Adair, K. L.; Gardner, P. P. An evaluation of the accuracy and speed of metagenome analysis tools. *Sci. Rep.* **2016**, *6*, 19233.
(53) Sutherland, I. W. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **2001**, *147* (1), 3−9.
(54) Donlan, R. M. Biofilms: Microbial Life on Surfaces. *Emerging Infect. Dis.* **2002**, *8* (9), 881−890.
(55) Zhu, L. D.; Wang, Z.; Shu, Q.; Takala, J.; Hiltunen, E.; Feng, P.; Yuan, Z. Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. *Water Res.* **2013**, *47* (13), 4294−4302.
(56) Wang, L.; Min, M.; Chen, P.; Liu, Y.; Wang, Y.; Ruan, R.; Li, Y.; Chen, Y. Cultivation of Green Algae Chlorella sp in Different Wastewaters from Municipal Wastewater Treatment Plant. *Appl. Biochem. Biotechnol.* **2010**, *162* (4), 1174−1186.
(57) Kim, D. J.; Lee, D. I.; Keller, J. Effect of temperature and free ammonia on nitrification and nitrite accumulation in landfill leachate and analysis of its nitrifying bacterial community by FISH. *Bioresour. Technol.* **2006**, *97* (3), 459−468.
(58) Yao, Q.; Peng, D. C. Nitrite oxidizing bacteria (NOB) dominating in nitrifying community in full-scale biological nutrient removal wastewater treatment plants. *AMB Express* **2017**, *7* (1), 25.

DOI: 10.1021/acs.est.7b03975
Environ. Sci. Technol. XXXX, XXX, XXX−XXX