Science of the Total Environment 580 (2017) 1185–1196

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microbial community structures in high rate algae ponds for bioconversion of agricultural wastes from livestock industry for feed production

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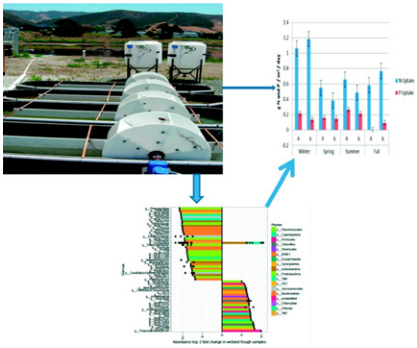
HIGHLIGHTS

- The microbiome profile from the different sources formed distinct clusters.
- All three DNA isolation kits provided differential taxa abundance by the sample types.
- DNA samples isolated from the Zymo kit had a higher proportional abundance of microalgae.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 September 2016
Received in revised form 12 December 2016
Accepted 12 December 2016
Available online 18 December 2016

Editor: D. Barcelo

Keyword:
Microbial community
High rate algae ponds
Dairy lagoon effluent
Algae production
Cyanobacteria
Microalgae

ABSTRACT

Dynamics of seasonal microbial community compositions in algae cultivation ponds are complex. However, there is very limited knowledge on bacterial communities that may play significant roles with algae in the bioconversion of manure nutrients to animal feed. In this study, water samples were collected during winter, spring, summer, and fall from the dairy lagoon effluent (DLE), high rate algae ponds (HRAP) that were fed with diluted DLE, and municipal waste water treatment plant (WWTP) effluent which was included as a comparison system for the analysis of total bacteria, *Cyanobacteria*, and microalgae communities using MiSeq Illumina sequencing targeting the 16S V4 rDNA region. The main objective was to examine dynamics in microbial community composition in the HRAP used for the production of algal biomass. DNA was extracted from the different sample types using three commercially available DNA extraction kits; MoBio Power water extraction kit, Zymo fungi/bacterial extraction kit, and MP Biomedicals FastDNA SPIN Kit. Permutational analysis of variance (PERMANOVA) using distance matrices on each variable showed significant differences ($P = 0.001$) in beta-diversity based on sample source. Environmental variables such as hydraulic retention time (HRT; $P < 0.031$), total N ($P < 0.002$), total inorganic N ($P < 0.002$), total P ($P < 0.002$), alkalinity ($P < 0.002$), pH ($P < 0.022$), total suspended solid (TSS; $P < 0.003$), and volatile suspended solids (VSS; $P < 0.002$) significantly affected microbial communities in DLE, HRAP, and WWTP. Of the operational taxonomic units (OTUs) identified to phyla level, the dominant classes of bacteria

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http://dx.doi.org/10.1016/j.scitotenv.2016.12.076
0048-9697/Published by Elsevier B.V.

identified were: *Cyanobacteria*, *Alpha-*, *Beta-*, *Gamma-*, *Epsilon-*, *and Delta-proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Planctomycetes*. Our data suggest that microbial communities were significantly affected in HRAP by different environmental variables, and care must be taken in extraction procedures when evaluating specific groups of microbial communities for specific functions.

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1. Introduction

The need to control manure-derived nutrient pollution is straining the concentrated animal feeding operations (CAFOs) industry. California is the top milk producing state in the US and has some of the strictest manure-derived nutrient pollution regulations. These operations have little access to affordable land to use manures as a crop fertilizer or access to more land for manure application/disposal, since they produce large numbers of animals in small land areas allowing for competitive animal production. Roughly 20% of the nitrogen and 35% of the phosphorus are recovered from manure in CAFOs using land application as crop fertilizer. Substantial amounts of nutrients in the manure remain unused in the fields, and find their way into ground and surface waters (Kellogg et al., 2000). In 2–5% of all counties in the U.S., including the agricultural regions of California, Virginia, and much of the southeast, the amount of nutrients present in the manure produced by CAFOs is greater than the entire assimilative nutrient capacity of all the cropland and pastureland available in those counties (USEPA, 2003). As a result, large amounts of manure are over-applied, exported to other locations or stored on site. The primary source of agricultural non-point source contamination in California's groundwater is the improper disposal of manure wastes by CAFOs manure (Helperin et al., 2001). Thus, there is a critical and urgent need to control manure-derived greenhouse gases and nutrient pollution while reclaiming water and nutrients.

A highly productive crop is needed that will convert manure N and P into feed but in smaller land areas than traditional crops such as corn. Algae are a candidate feed with annual yields typically 7–13 times greater than soy or corn. With beyond 40–50% protein content, algae contain fatty acids, favorable amino acid contents, pigments, and vitamins that are valuable in animal feeds, especially for adding value to milk. Microalgae have great potential both for sustainable bioremediation of wastewaters and as a feedstock. Microalgae are more efficient for N and P reclamation than higher plants, due in part, to higher rates of biomass production but also because algae lack the large stores of structural carbon (i.e., cellulose) characteristic of land plants (Kumar and Das, 2012). They are photosynthetic organisms that assimilate N and P during growth. The subsequent biomass generated can be converted to high energy and feed stock after further processing. Thus, the C/N ratio of higher plants ranges from 18 to 120 (by atoms) while microalgae ranges from 5 to 20 (Collos and Berges, 2003) indicating that water reclamation and nutrient recovery can be accomplished more rapidly and in a smaller area using algae rather than terrestrial plants.

Mass-culture of algae on manure N and P is an alternative to land spreading of manure effluents, particularly in the case of CAFOs where groundwater contamination is problematic and more land for manure application is restricted. Microalgae are photosynthetic organisms with high productivity (many strains double in less than a day) that removes eutrophying nutrients from water sources. They have been used for over 50 years in municipal wastewater treatment (Oswald, 1988) and more recently for bioremediation of manure effluents (Woertz et al., 2009; Mulbry et al., 2008). Some microalgae produce lipids, rather than carbohydrates, as their primary carbon storage molecules and thus, there has been considerable interest in coupling wastewater treatment to produce feedstock for renewable fuel production. Like higher plants, microalgae produce tricyglycerides that can be readily converted to fatty acid methyl esters, a substitute for fossil-derived diesel fuel. Microalgae also have high protein content and the ability to grow in saline or wastewaters not suitable for agricultural irrigation. Of interest for both algae biofuel and algae feed is the high amounts of oil (lipid) produced, primarily as triglycerides. Algal neutral storage lipids are similar in structure and molecular weight (carbon chains ranging from 12 to 22) to the oils extracted from terrestrial plants. Microalgae can have oil contents that vary from 15 to 77% of the dry weight (Banerjee et al., 2002; Chisti, 2007) compared with 4% in corn. The high oil content of some algal strains is also of interest to the dairy industry as lipids are a key component in feed supplements for lactating dairy cows.

The growth of microalgae may be impeded by many contaminants in the bioreactors or in HRAP. A key hurdle in sustainable large-scale algae production, in common with terrestrial crop production, is species control. Pond performance can be negatively affected by the establishment of zooplankton grazers that can consume much of the algal biomass within a few days (Montemezzani et al., 2015). Populations of zooplankton grazers, parasitic fungi and infective bacteria and viruses are inevitable in outdoor ponds. High ammonium levels (Schluter and Groeneweg, 1985; Lincoln et al., 1983), low nocturnal pO_2 levels, large colonial forms of algae and associated microbial consortiums may effectively control some zooplanktonic herbivores (O'Brien and De Noyelles, 1972; Schluter and Groeneweg, 1981; Montemezzani et al., 2015). There are many methods for the control of zooplanktons in HRAP without detrimental effects on microalgae communities and water quality, and may include physical, chemical, biological, and enzymatic methods (Montemezzani et al., 2015). Microbial community dynamics in pond operations may provide special benefits for the investigation of population and community dynamics to determine early warning signs of a deteriorating pond that may be contaminated by zooplanktons, *Cyanobacteria*, or pathogens. A number of Cyanobacteria species produce potent hepatotoxins or neurotoxins that can be transferred through the food web where they may kill other life forms such as zooplankton, shellfish, fish, birds, marine mammals and humans that feed, either directly or indirectly, on them (Warrington, 2001).

In this study four identical HRAPs were installed adjacent to the dairy waste lagoons at the 300-head dairy at California Polytechnic State University, San Luis Obispo (CPSLO), CA (Fig. 1). The algae ponds are paddle wheel-mixed raceways that simulate standard 30-cm deep algae production ponds. The main objective of our study was to examine dynamics in microbial community composition in the HRAP used for the production of algal biomass. For a successful study of bacterial community dynamics in HRAP, we added a secondary objective to examine which extraction kit will produce the highest diversity of *Cyanobacteria* and other microalgae community in the HRAPs. We hypothesized that changes in solar radiation, temperature, hydraulic and solids residence times, and nutrients will influence bacterial, Cyanobacterial and other microalgae community compositions in the HRAPs. We used MiSeq Illumina V4 16S rDNA sequencing to examine microbial community compositions in HRAPs. MiSeq Illumina sequencing provides sufficient sequencing depth to cover the complex microbial communities (Shendure and Ji, 2008) in the pond to enable studies on the bacterial communities that may provide different functions in the ponds. For these reasons, we believe that the elucidation of bacterial and algal taxa based on deep sequencing may provide some insights into bacterial and algal communities and their interactions in HRAPs, and facilitate monitoring of presence of toxigenic cyanobacteria.

Fig. 1. Four outdoor HRAPs for algae cultivation used to monitor seasonal nutrient uptake rates, determine changes in microbial community, identified dominant algal species present, initiated bioflocculation selection, and for total biomass production. This was set up adjacent to the dairy waste lagoons at the 300-head dairy at California Polytechnic State University, San Luis Obispo (CPSLO), CA.

2. Materials and methods

2.1. Apparatus and operation

Four identical HRAPs were installed adjacent to the DLE at the 300-head CPSLO dairy (Fig. 1). The HRAPS are paddle wheel-mixed raceways that simulate standard 30-cm deep algae production ponds as previously described for municipal wastewater treatment research (Ward, 2011; Weissman et al., 1988). These ponds were operated as two sets of duplicates to conduct controlled experiments to determine parameters to be used in optimization of wastewater treatment and algae feed production. Physical parameters of the units, such as, volume and the relationship between paddle wheel RPM and channel water velocity, as well as cross sectional flow patterns that are critical for developing bio flocculating algal communities were analyzed.

The algae ponds were fed dairy flush water from the stored DLE. Data were collected from the outdoor pilot scale HRAPs under steady-state conditions (Fig. 1) during winter, spring, summer, and fall. Nutrient additions as well as fractions of nutrients from DLE were adjusted to meet the growth needs of the algae enabling development of a productivity model based on multiple variables, including: hydraulic residence time (HRT), solar radiation, water temperature, available nutrient concentration, and primary nutrient source. Nutrient adjustment was achieved by adding fertilizer in the form of water soluble Miracle Grow, 24-8-16 (N-P-K; Home Depot, San Luis Obispo, CA). Fertilizer was added as supplemental nutrients to determine if the units fed DLE were inhibited due to the dark color of the units (which promotes bacterial instead of algal growth). The N consisted of ammonia (3.5%) and urea (20.5%), while the P was in the phosphate form. As a function of routine dairy operation and weather, the nutrient concentrations (total N, total inorganic N, nitrate, total P) in the DLE were changing throughout the trials. Samples of the DLE were characterized to gain an understanding of the types of nutrients present. Analysis of N and P nutrients, solids, chemical oxygen demand (COD) and alkalinity were determined. Water quality data that were collected and sorted by season includes: total suspended solids (TSS), volatile suspended solids (VSS), total nitrogen concentration, total inorganic nitrogen concentration, total phosphorus, alkalinity, Secchi disk visibility, oxygen concentration, pH, and temperature. The data were analyzed to determine algal productivity and nutrient uptake rates for each season at HRTs of 4 vs. 6 days. The above experiments require analyses of samples of influent and effluent at least once per week for the following: total and volatile suspended solids, total ammonia N, nitrate, nitrite, and alkalinity; twice per month for the following, total biochemical oxygen demand, and soluble carbonaceous biochemical oxygen demand. The four HRAPs as well as the DLE were monitored for these compounds. The data were analyzed to determine nutrient uptake rates and algal biomass productivity for each HRT and season.

2.2. Analysis of microbial and cyanobacterial community structure in pond samples

Seasonal samples (fall, winter, spring, and summer) were collected (in duplicate) from the four HRAPs and DLE at the CPSLO dairy. Samples were also collected from the San Luis Obispo city waste water treatment plant (WWTP) for DNA analysis and comparison purposes. Samples from the three sources were collected on the same day in 1 L plastic bottles, maintained at about 4 °C, and transported to the different laboratories for further processing. DNA was extracted from these samples for analysis of total bacteria (targeting 16S rRNA-encoding genes), *Cyanobacteria* and microalgae using Illumina's MiSeq next generation sequencing (NGS) platform (Illumina Inc., San Diego, CA). DNA extraction was undertaken using the Power Water extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA), Fungi/bacterial DNA Mini Prep kit (Zymo Research, Irving CA, USA), and FastDNA SPIN Kit (MP Biomedicals , Solon, OH, USA) for the analysis of total bacteria and *Cyanobacteria*. All samples were stored at −20 °C after further cleanup steps with DNA Clean and Concentrator (Zymo Research Corp- Irvine CA, USA). Extracted DNA (2 μL) was quantified using a Nanodrop ND-2000 C spectrophotometer (Nanodrop Technologies, Wilmington DE), and run on a 1.0% agarose gel before it was used for V4 16S Illumina MiSeq sequencing. DNA was extracted from duplicate samples (collected 30 min apart) and pooled for the sequencing step.

2.3. Illumina MiSeq sequencing of V4 16S rDNA

Microbiota from HRAPs, WWTP, and DLE samples were profiled using Second Genome's Microbiome Signature Discovery service (San Bruno, CA, USA). Illumina sequencing across the four seasons was used to track microbial population dynamics in the three sample types. All samples were quantified via the Qubit Quant-iT dsDNA High Sensitivity Kit (Invitrogen, Life Technologies, Grand Island, NY) to ensure that they met minimum concentration and DNA mass expectations. The V4 region of the 16S rRNA genes was amplified using fusion primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso et al., 2011), and then pooled for sequencing using MiSeq (Illumina, San Diego, CA, USA).

2.4. Statistical analysis of sequence data

Using the software QIIME (Kuczynski et al., 2011), UCLUST (Edgar, 2010) and MOTHUR (Schloss et al., 2009), sequences were clustered into reference OTUs and assigned taxonomic classification from the Greengenes database (McDonald et al., 2012). Sequenced paired-end reads were merged, quality filtered, and dereplicated with USEARCH (Edgar, 2013). The resulting unique sequences were then clustered at 97% similarity by UPARSE (de novo OTU clustering) and a representative consensus sequence per de novo OTU was determined (Edgar, 2013). The clustering algorithm also performs chimera filtering to discard likely chimeric OTUs. Sequences that passed

quality filtering were then mapped to a set of representative consensus sequences to generate an OTU abundance table. Representative OTU sequences were assigned taxonomic classification via MOTHUR's bayesian classifier at 80% confidence; the classifier was trained against the Greengenes reference database of 16S rRNA gene sequences clustered at 99%. After the taxa were identified for inclusion in the analysis, the values used for each taxa-sample intersection were populated with the abundance of reads assigned to each OTU in an OTU table. A corresponding table of OTU Greengenes classification was generated. Alpha-diversity (within sample diversity) metrics were calculated to estimate sample richness and Shannon diversity. Beta-diversity (sample-to-sample dissimilarity) metrics were calculated, for the inter-comparison in a pair-wise fashion, to determine dissimilarity score in a distance dissimilarity matrix. Abundance-weighted sample pair-wise differences were calculated using the Bray-Curtis dissimilarity. All analyses were generated using Second Genome R package (Vegan: R package version 2.2-1). Bray-Curtis dissimilarity was calculated using the ratio of the summed absolute differences in counts to the sum of abundances in the two samples using the Jaccard index. Hierarchical clustering maps of the samples in the form of dendrograms and principal coordinate analysis (PCoA) were used to visualize complex relationships between samples. Permutational analysis of variance (PERMANOVA) was utilized to find significant differences among discrete categorical or continuous variables based on the Monte Carlo permutation test. Univariate differential abundance of OTUs was tested using a negative binomial noise model for the over dispersion and Poisson process intrinsic to these data, as implemented in the DESeq2 package (Love et al., 2014), and described for microbiome applications (McMurdie and Holmes, 2013).

3. Results

3.1. Nutrient removal in the raceway ponds

Water quality data were collected weekly from all four algae growth reactors and sorted by season (winter, spring, summer, and fall)-Fig. 2A, B and C). Total N and P uptakes from the four algae production bioreactors were monitored and the data are summarized based on seasons. The rates were calculated for the collected data points and the weekly data were averaged to determine seasonal uptake rates. N uptake rates were lower than expected, averaging 1.10 g N/m^2/day during winter months. The uptake rates were significantly ($P < 0.002$) lower during spring, summer and fall. P uptake rates averaged 0.16 g P/m^2/day. Generally, spring and summer rates reach about 0.2 g P/m^2/day but were significantly lower in fall ($P < 0.002$) than in spring and summer (Fig. 2A). Dissolved oxygen concentration was also monitored and recorded daily in the early afternoon along with pH and temperature (data not shown). The afternoon dissolved oxygen and pH were higher in bioreactor units 2 and 4 than in units 1 and 3. Units 2 and 4 received a combination of DLE and fertilizer while units 1 and 3 received DLE only. The higher DO and pH indicate greater photosynthesis rates and higher productivity. TSS was lower in winter and fall than summer and spring. During spring and summer TSS was significantly higher ($P < 0.003$) using the 6 day HRT vs. the 4 day HRT (Fig. 2B). VSS was significantly higher in spring and summer ($P < 0.002$) using the 6 day HRT vs. the 4 day HRT (Fig. 2C).

The N and P data were analyzed to determine algal productivity and nutrient uptake rates for each season and HRT. The uptake rates were calculated from the algae productivity numbers as algae contains 10%

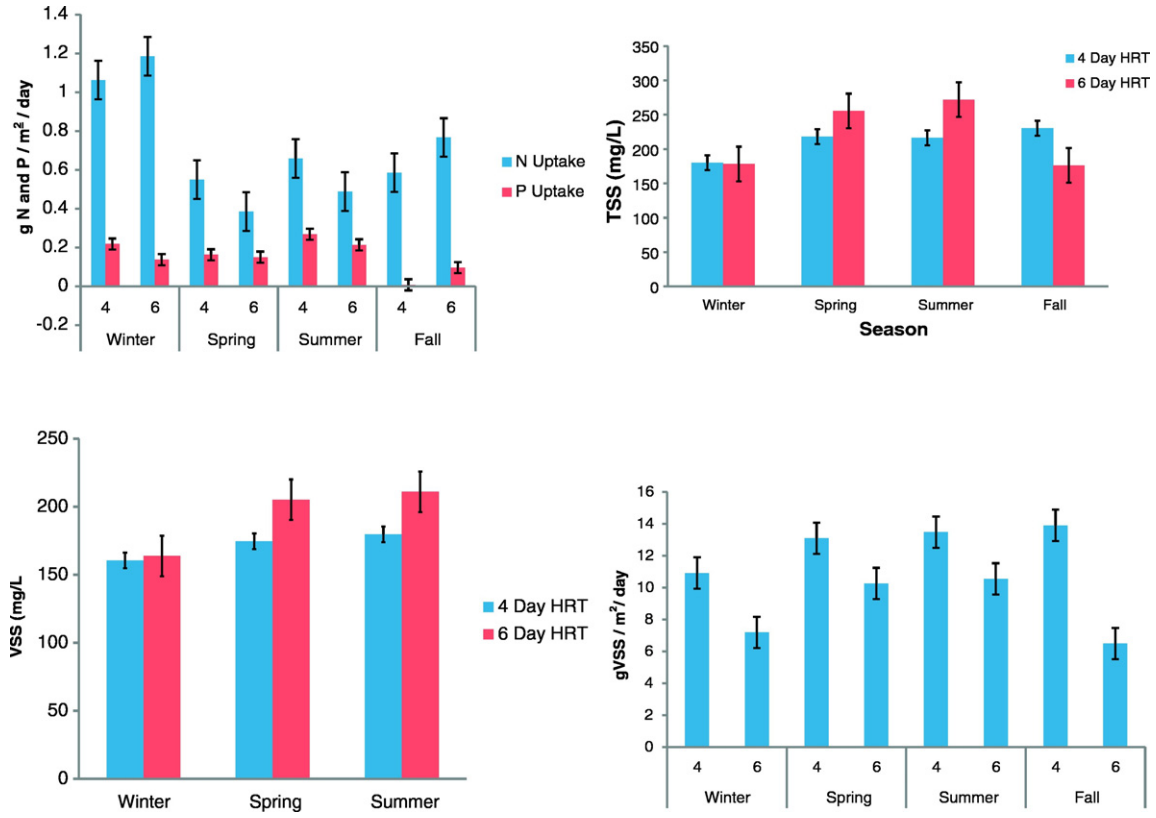


Fig. 2. Water quality data collected weekly from HRAPs growth reactors and sorted by season. (A) Total N and P uptakes from HRAPs production bioreactors monitored weekly. (B) TSS for 6 day vs. 4 day HRT. (C) VSS using the 6 day HRT vs. the 4 day HRT.

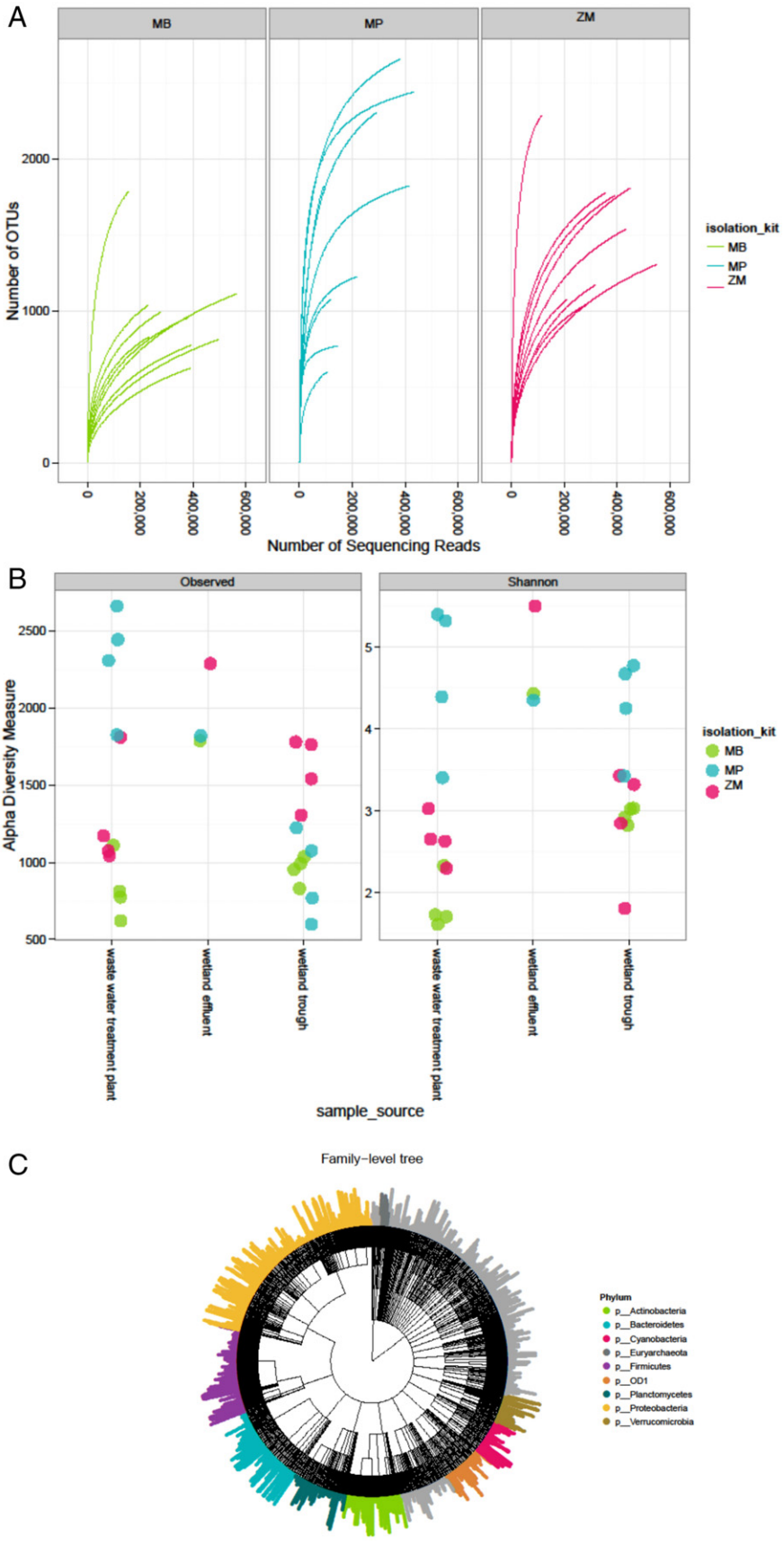


Fig. 3. Numbers of DNA sequences and diversity indices per sample type. (A) Rarefaction curves. Average number of OTUs detected versus sequencing library size. Sample names are annotated at the end (right) of each curve. A curve approaching a horizontal slope is nearly saturated, with few new OTUs undiscovered. A curve with a steep slope remaining in the plot has not been sequenced to saturation, and a substantial quantity of additional OTUs are expected upon further sequencing. (B) Microbiome alpha-diversity estimates for each sample Shannon diversity indices. (C) Phylogenetic tree at the Order level. The height of each bar indicates the number of samples containing that particular Order. The most abundant Phylum-level clades are colored with the remainders in light gray.

Table 1
Shannon diversity indices of samples based on DNA extraction method.

Sample source	MO BIO	ZYMO	MP BIO
Wetland trough-1	2.922931	1.807525	4.250287
Wetland trough-2	3.034928	3.428931	4.673853
Wetland trough-3	3.025278	2.850793	3.424096
Wetland trough-4	2.825826	3.318973	4.773748
Waste water treatment plant	1.612938	2.656298	5.399947
Waste water treatment plant	1.706715	2.631	5.324516
Waste water treatment plant	1.731622	2.30066	3.402613
Waste water treatment plant	2.332175	3.029716	4.392655
DLE effluent	4.430778	5.502392	4.350843

Replace "wetland trough" with, e.g., DLE-fed algae production ponds.

N and 1.25% P. Algal productivity was determined by calculating the daily algae production from the VSS measurements (Fig. 2D). VSS was significantly higher ($P < 0.05$) for the shorter HRT, averaging about 13 g VSS/m^2/day in spring, summer, and fall, and 11 g VSS/m^2/day in winter. These data suggest that about 140 to 210 kg of algae can be produced/4,047 m^2/year in bioreactors operating at 4 and 6 day HRTs, respectively. This indicates a high potential to produce algal biomass for enhanced animal feed supplements.

3.2. Diversity of microbial communities

Bacterial V4 16S rRNA gene analysis was performed to characterize the microbial communities associated with the DLE, HRAPs and WWTP effluent. After processing and chimera removal, numbers of DNA sequences per sample ranged from a minimum of 90,767 to a maximum of 562,789 reads (Fig. 3A). All three DNA isolation kits were able to identify and differentiate taxa abundance by sample type. However, the MP Bio kit yielded higher numbers of OTUs than Mo Bio and ZYMO kits based on rarefaction curves (Fig. 3A), whereas, the Mo Bio kit produced the highest numbers of sequence reads (562,789) while MP Bio produced the lowest (410,000). Except for the DLE, Shannon diversity (H') was significantly higher ($P < 0.001$) with the MP Bio kit than Mo Bio and ZYMO kits for all other test samples (Table 1). Holding the sampling depth constant for the 3 kits, MP Bio samples showed the highest number of unique OTUs in waste water samples, but lowest in HRAPs (Fig. 3B). Of the OTUs identified to taxa level, the dominant classes of bacteria identified in all samples were *Cyanobacteria*, *Alpha-*, *Beta-*, *Gamma-*, *Epsilon-*, *and Delta-proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Planctomycetes*. All percentages from the different OTUs are presented in Table 2.

When these samples were reanalyzed based on sources instead of extraction method *Proteobacteria* had the highest percent concentration followed by *Firmicutes*, *Bacteroidetes*, and *Cyanobacteria* (Fig. 3C). The clades that were more abundant in DLE were also abundant in HRAP. However, higher abundance was found in DLE than in HRAP and WWTP. Furthermore, we compared HRAPs, DLE, and WWTP microbial composition at the genus level. The HRAP samples had higher heterogeneity within the group than DLE samples, as the former samples occupied a bigger area in ordination space. Community composition among the three sample sources depended on differences in environmental parameters (i.e., nutrients, pH, hydraulic retention times, etc.). Environmental variables such as hydraulic retention time (HRT; $P < 0.031$), total N ($P < 0.002$), total inorganic N ($P < 0.002$), total P ($P < 0.002$), alkalinity ($P < 0.002$), pH ($P < 0.022$), total suspended solid (TSS; $P < 0.003$), and volatile suspended solids (VSS; $P < 0.002$) significantly affected microbial communities in DLE, HRAP, and WWTP. The 100 most significant OTUs were examined in HRAP and each OTU was considered significant if their corrected *P*-value was ≤0.05, and the absolute value of their log2-fold change was ≥1 (Tables S1). Similar to HRAPs comparison, the dairy pond samples had higher heterogeneity within the group, as they occupied a bigger area in ordination space. The 100 most significant different OTUs detected in DLE (Table S2) and WWTP (Table S3) are presented for comparisons. Due to the toxicity nature of *Cyanobacteria* to algae, a total of 418 Cyanobacteria were identified (Table S4). Further analysis of the 418 species showed that 7.65% came from DLE and these species were significantly higher in DLE than in HRAP and WWTP. On the contrary, only 4.31% and 2.63% were significantly higher in WWTP and HRAP, respectively than in DLE (Table 3). This analysis further showed that only OUT 3, 39, 2402, and 2823 were present at significant relative percent concentrations in the three sources. Five OTUs (19, 73, 185, 906, and 10,098) were present in HRAP but not in the DLE, suggesting that these OTUs may have been enriched within the HRAP unit. Seasonally dominant algal species present in HRAPS were identified using microscopy and a digital camera. This predominantly identified *Scenedesmus* species. Other isolates identified were *Scenedesmus*, *Chlorella*, *Desmodesmus* and a variety of small *Chlorophyta*. These may likely be some of the OTUs identified by sequencing.

The relative abundances of nine major phyla isolated with the three kits are shown in Fig. 4A. Relative abundance data showed that extraction technique did not influence the percent relative abundance of most of the nine major phyla presented in Fig. 4A except for the Zymo extraction kit which produced the highest relative abundance of Cyanobacteria. At the family level, nine bacterial taxa were relatively dominant in the sample sources, with Mo Bio kit producing significant relative abundances of Oxalobacteraceae and Spingomonadaceae, while samples extracted with Zymo kit showed a higher proportional abundance of Chlamydomonadaceae, a family of microalgae (Fig. 4B),

Table 2
Detection of significantly different OTUs from each DNA extraction kit.

Phylum	Extraction kit (%)		
	MP	MoBio	Zymo
Cyanobacteria	3.78	2.5	2.5
Proteobacteria	38.2	40.9	28.7
Beta	6.4	9.5	4.6
Alpha	12.6	16.3	11.7
Gamma	7.6	9.3	5.8
Epsilon	1.01	2.7	1.5
Delta	8.4	2.5	4.4
Actinobacteria	3.7	4.9	4.9
Bacteroidetes	15.83	20.1	18.9
Verrucomicrobia	7.8	8.8	8.3
Chloroflexi	1.32	1.6	2.5
Firmicutes	7.76	4.5	9.3
Tenericutes	0.3	0.6	1.1
SR1	0.6	0	0
Acidobacteria	1.7	0.2	0
Chlorobi	0.7	0.6	0.4
Planctomycetes	5.82	1.8	3.7
OD1	2.65	2.2	5.2
Fibrobacteres	0.03	0	0
Gemmatimonadetes	0.1	0.2	0.4
Chlamydiae	0.1	0.2	0.1
Spirochaetes	0.8	0.4	1.1
TM7	0.7	0.2	0.2
GN02	0.1	0.6	0.7
Caldithrix	0.1	0.2	0.1
Thermi	0.1	0	0
NKB19	0.3	0	0.2
Armatimonadetes	0.1	0	0
TM6	0.1	0	0.1
Fusobacteria	0.4	6	0.5
BRC1	1.2	0	0
OP3	0.3	2	1.6
WPS-2	0.3	0	0
LD1	0.1	0.2	0.1
Elusimicrobia	0.1	0.2	0
WWE1	0	1.1	0.8
Lentisphaerae	0	0.6	1.5

Table 3
Cyanobacteria with percent significant differences between sample sources based on greengenes analysis.

OTU	Order	Family	Genus	Species	DLE-*P* value	HRAP-*P* value	WWTP-*P* value
3	*Chlorophyta*	*Chlamydomonadaceae*	Unclassified	Unclassified	0.000629	1.26E-15	5.12E-14
19	*Chlorophyta*	*Chlamydomonadaceae*	94otu30054	97otu5870		1.12E-07	2.85E-06
23	*Chlorophyta*	91otu763	94otu34503	97otu72584	0.004926		
39	*Chlorophyta*	91otu4387	Unclassified	Unclassified	1.36E-06	2.92E-07	2.85E-06
73	*Stramenopiles*	91otu4345	94otu10147	97otu13102		3.50E-07	
185	*Chlorophyta*	*Chlamydomonadaceae*	94otu41005	97otu92736		0.0023327	3.13E-06
292	*Stramenopiles*	91otu4445	94otu10273	Unclassified	0.0003755		
369	*Chroococcales*	*Cyanobacteriaceae*	*Cyanobacterium*	97otu65507	5.36E-06		
490	*Stramenopiles*	91otu672	94otu29984	97otu33082	6.87E-07		
906	*Stramenopiles*	91otu5457	94otu17156	Unclassified		0.0114097	
946	85otu1728	91otu8766	94otu4415	97otu5105	4.52E-06		
1312	*Stramenopiles*	91otu4345	Unclassified	Unclassified	1.38E-05		
1346	85otu754	91otu6314	Unclassified	Unclassified	0.000277		
1372	*Streptophyta*	91otu8468	94otu16468	97otu36443			0.0002054
1631	*Chlorophyta*	91otu4387	Unclassified	Unclassified	0.006901		
1725	*Chlorophyta*	*Chlamydomonadaceae*	94otu32417	97otu83273	0.0008228		
1726	85otu1728	91otu12004	94otu23100	Unclassified	0.0001622		
2402	*Chlorophyta*	*Chlamydomonadaceae*	Unclassified	Unclassified	0.0193302	0.0116155	0.0006197
2504	*Pseudanabaenales*	*Pseudanabaenaceae*	*Leptolyngbya*	97otu90470	0.0030998		
2518	YS2	91otu5915	94otu16622	97otu5543			0.0086599
2662	*Stramenopiles*	Unclassified	Unclassified	Unclassified	0.0002578		
2692	*Chlorophyta*	*Chlamydomonadaceae*	Unclassified	Unclassified	3.16E-05		
2823	*Stramenopiles*	91otu4345	94otu12325	Unclassified	5.16E-06	0.0004138	6.48E-11
2839	unclassified	Unclassified	Unclassified	Unclassified	0.0002829		
2908	*Chlorophyta*	*Trebouxiophyceae*	94otu2543	97otu29136			0.0013869
3047	YS2	unclassified	Unclassified	Unclassified	0.0052925		
3265	*Chlorophyta*	*Chlamydomonadaceae*	Unclassified	Unclassified			0.0021237
3584	Unclassified	Unclassified	Unclassified	Unclassified			0.0129087
3869	*Synechococcales*	*Acaryochloridaceae*	94otu14616	97otu53199	0.0110053		
3923	*Chroococcales*	*Xenococcaceae*	Unclassified	Unclassified	0.0014368		0.0069497
4489	*Oscillatoriales*	*Phormidiaceae*	Unclassified	Unclassified	0.0051075		
5032	*Chlorophyta*	91otu763	94otu28051	97otu78782	0.0120289		
5654	*Stramenopiles*	Unclassified	Unclassified	Unclassified			0.0110093
8609	*Chlorophyta*	*Chlamydomonadaceae*	Unclassified	Unclassified			0.0186376
10,098	*Stramenopiles*	91otu4345	Unclassified	Unclassified		3.18E-07	
10,922	*Chlorophyta*	*Chlamydomonadaceae*	94otu30054	97otu5870	0.0025111		
11,356	*Chlorophyta*	91otu4387	Unclassified	Unclassified			0.0026257
11,670	*Chlorophyta*	*Chlamydomonadaceae*	94otu41005	97otu92736	0.0067226		
13,148	*Chlorophyta*	*Chlamydomonadaceae*	94otu30054	97otu5870			0.0018061
13,175	*Stramenopiles*	91otu4345	Unclassified	Unclassified	0.0005337		
13,399	YS2	91otu5915	94otu16622	97otu5543			0.0084372
14,374	85otu1728	91otu8766	Unclassified	Unclassified	0.0011139		
14,858	*Chlorophyta*	*Chlamydomonadaceae*	Unclassified	Unclassified			3.50E-06
15,732	*Stramenopiles*	91otu4345	94otu10147	Unclassified	0.0160404		
19,265	SM1D11	91otu7744	94otu9796	97otu76577	7.30E-06		
Percent					7.65	2.63	4.31

with Chlamydomonadaceae, being the most abundant taxa at the family rank, followed by Oxalobacteraceae.

3.3. Drivers of microbial community composition in HRAPs, DLE, and WWTP effluent

Dimensional reduction of the Bray-Curtis distance between microbiome samples using PCoA analysis showed that sample type was the main driver for the microbiome difference in the sample set. HRAPs and WWTP effluent were separated along Axis 1 in the abundance ordination analysis, which accounted for 35.4% of the variance. Furthermore, the DLE samples were separated from the rest of the samples along Axis 2, which accounted for 15.9% of the variance (Fig. 5A). PCoA analysis by taxa membership metrics only explains 10% of variance in the two highest axes combined, suggesting that taxa presence/absence is not as strong a driving force in sample differentiation as abundance metrics (data note shown). Hierarchical clustering based on Bray-Curtis distance separated the sample types into three distinctive clades (Fig. 5B). PERMANOVA with Monte Carlo permutation test showed that the type of DNA isolation kit had no significant effects ($P < 0.268$) on beta diversity, but samples types were major significant factors in microbial beta diversity ($P < 0.001$).

4. Discussion

4.1. Pilot scale HRAPs

Data were collected from the outdoor pilot scale HRAPs for one year to develop a predictive growth model for algal based treatment systems. Data were collected to help develop a productivity model based on multiple variables which included: hydraulic residence time (HRT), solar radiation, water temperature, available nutrient concentration, and primary nutrient source. A pH control system was set up during this period to introduce carbon dioxide into the system as needed, based on unit pH. This pH control system ensured carbon limitation would not retard biomass production. As a function of dairy operation and weather, the nutrient concentrations (total nitrogen, total inorganic nitrogen, nitrate, total phosphate) in the DLE were changing throughout the trials, therefore samples of the DLE were characterized continuously to gain an understanding of the types of nutrients present in the HRAPs. As a result DLE effluent was diluted to 3–7% of the daily refill volume depending on the season to promote green water or algae-based treatment system as opposed to brown water or bacteria-based treatment system. Water quality data are presented in Fig. 2 and these include TSS, VSS, total nitrogen concentration, total phosphorus, alkalinity, oxygen

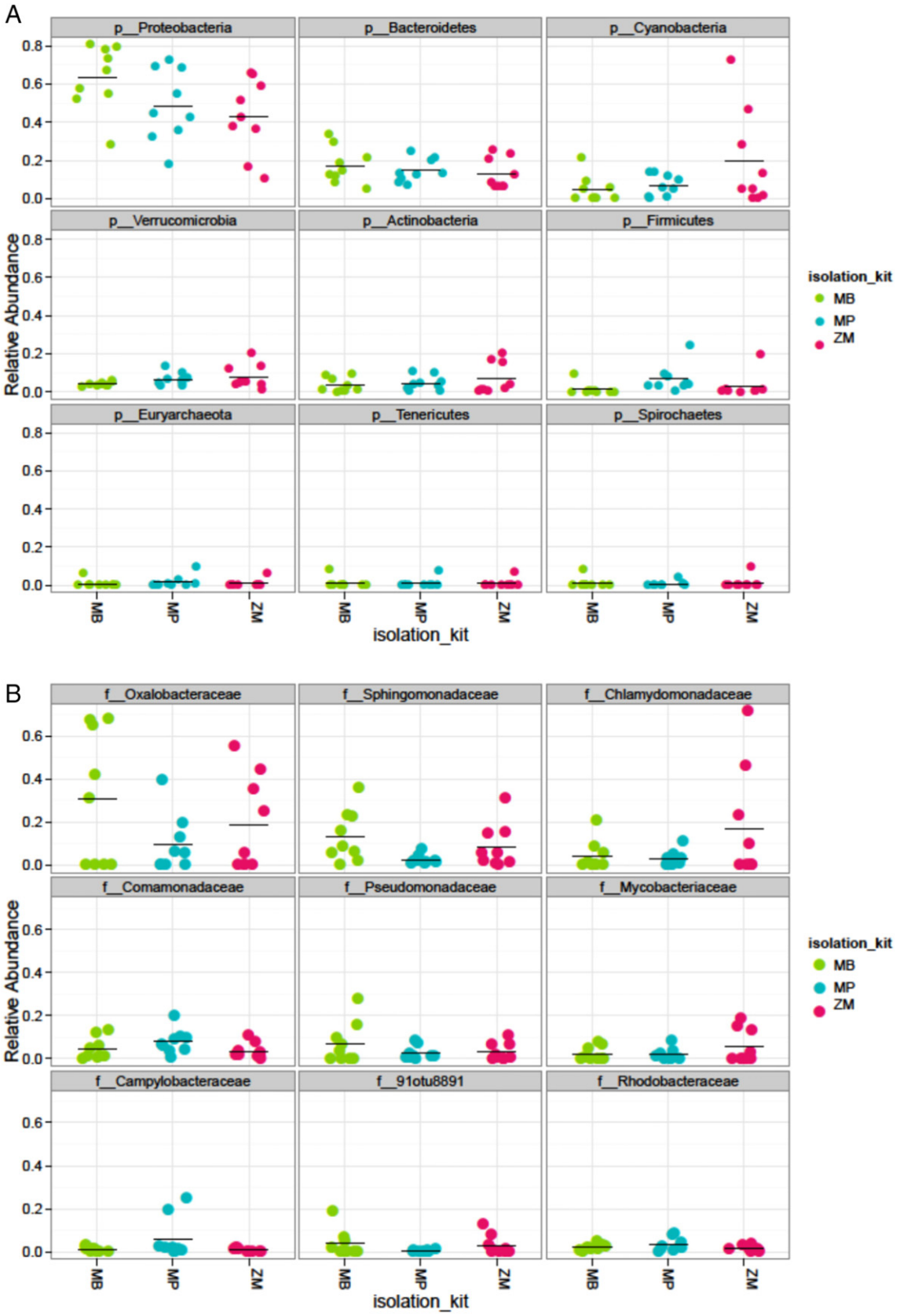


Fig. 4. Composition of bacteria at the phylum and family levels. (A) Relative abundance of each phylum per sample. (B) Most abundant nine groups at the taxonomic level of family.

concentration, pH, and temperature. The data were analyzed to determine algal productivity and nutrient uptake rates for each season at HRTs of 4 and 6 days (Fig. 2A–D). Algal productivity values ranged 6.5–13.9 g/m^2/day, whereas, nutrient uptake values ranged 0.8–1.4 g N/m^2/day and 0.08–0.17 g P/m^2/day and were all higher at HRTs of 4 days in all seasons.

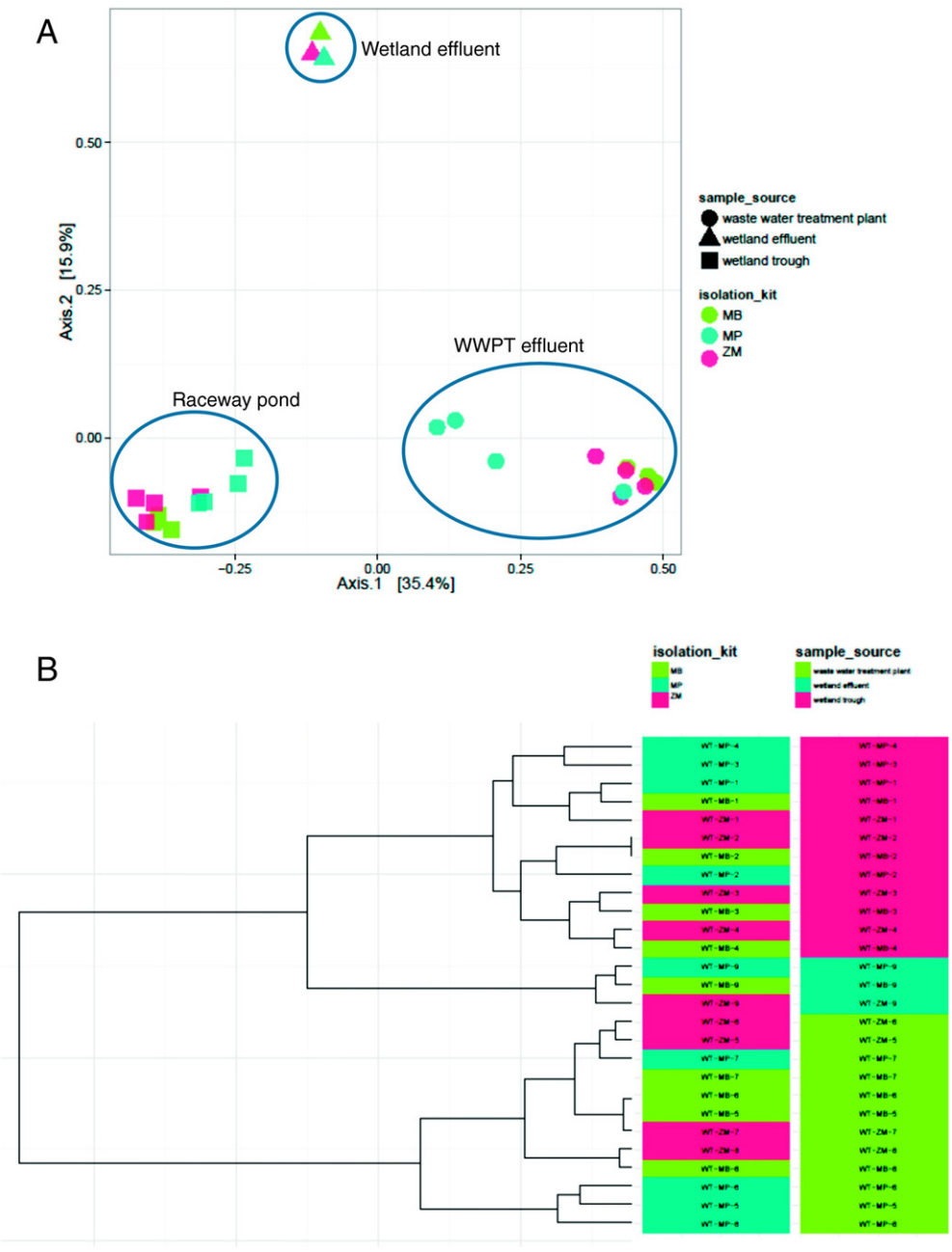
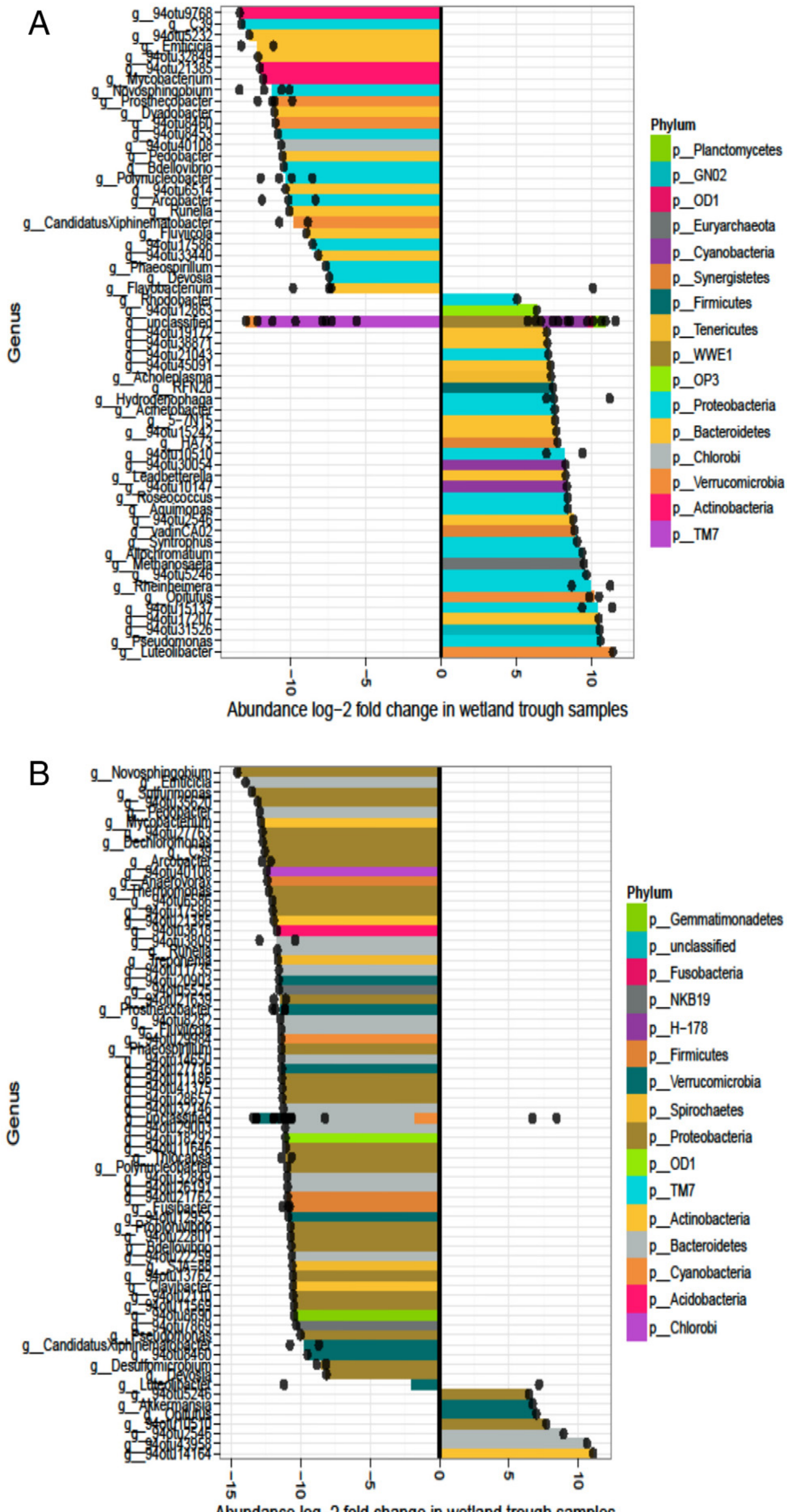


Fig. 5. Ordination using abundance. (A) Dimensional reduction of the Bray-Curtis distance between microbiome samples, using the PCoA ordination method. (B) Hierarchical clustering; ward clustering, Bray-Curtis distance.

Raceway ponds are probably the most common technology used for treatment of municipal and agricultural wastewaters in the US, with over 7000 publicly-owned treatment pond and lagoon systems (USEPA, 2008). However, nutrient removal is an increasingly common regulatory requirement, and conventional ponds are not well suited for nitrogen and phosphorus removal. Newer pond technologies (e.g., paddle wheel-mixed raceway ponds (HRAPs), Fig. 1, and newer variants of aerated lagoons) have advanced the reliability and effectiveness. In this study the removal rate of N was between 75 and 85% from DLE total N load to the HRAPs. The removal rate for N was better than the removal rate for P, but similar to many studies in the literature (Taziki et al., 2015). At dairies and swine farms, for example, algae production/wastewater treatment systems can be used to remove N, P, and dissolved organic matter from barn flush water, while producing high protein biomass (Whitten et al., 2015). Such treatment is already commercially available for municipal wastewater treatment in the US and for dairy in New Zealand, albeit with only a few installations and without algae feed production.

4.2. Microbial communities in the HRAPs

The dominant phyla from our study showed *Proteobacteria* with the highest relative abundance (0.1–0.8) for all the phyla identified. This was followed by *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Verrucomicrobia*, and *Cyanobacteria*. All classifications were based on the Greengenes reference database of 16S rRNA gene sequences clustered at 99. DNA samples isolated from the ZYMO kit had a higher proportional abundance of *Chlamydomonadaceae*, a family of green microalgae. The presence of *Chlamydomonadaceae*, which belongs to the Chlorophyta phylum, may support H_2 production by dark fermentation under anaerobic conditions (Yang et al., 2013). *Cyanobacteria* are important components of the HRAPs (Tables S1–S3) providing photosynthetic oxygen production


A
Genus
Abundance log−2 fold change in wetland trough samples
−10
−5
0
5
10
Phylum
p__Planctomycetes
p__GN02
p__OD1
p__Euryarchaeota
p__Cyanobacteria
p__Synergistetes
p__Firmicutes
p__Tenericutes
p__WWE1
p__OP3
p__Proteobacteria
p__Bacteroidetes
p__Chlorobi
p__Verrucomicrobia
p__Actinobacteria
p__TM7
B
Genus
Abundance log−2 fold change in wetland trough samples
−15
−10
−5
0
5
10
Phylum
p__Gemmatimonadetes
p__unclassified
p__Fusobacteria
p__NKB19
p__H−178
p__Firmicutes
p__Verrucomicrobia
p__Spirochaetes
p__Proteobacteria
p__OD1
p__TM7
p__Actinobacteria
p__Bacteroidetes
p__Cyanobacteria
p__Acidobacteria
p__Chlorobi

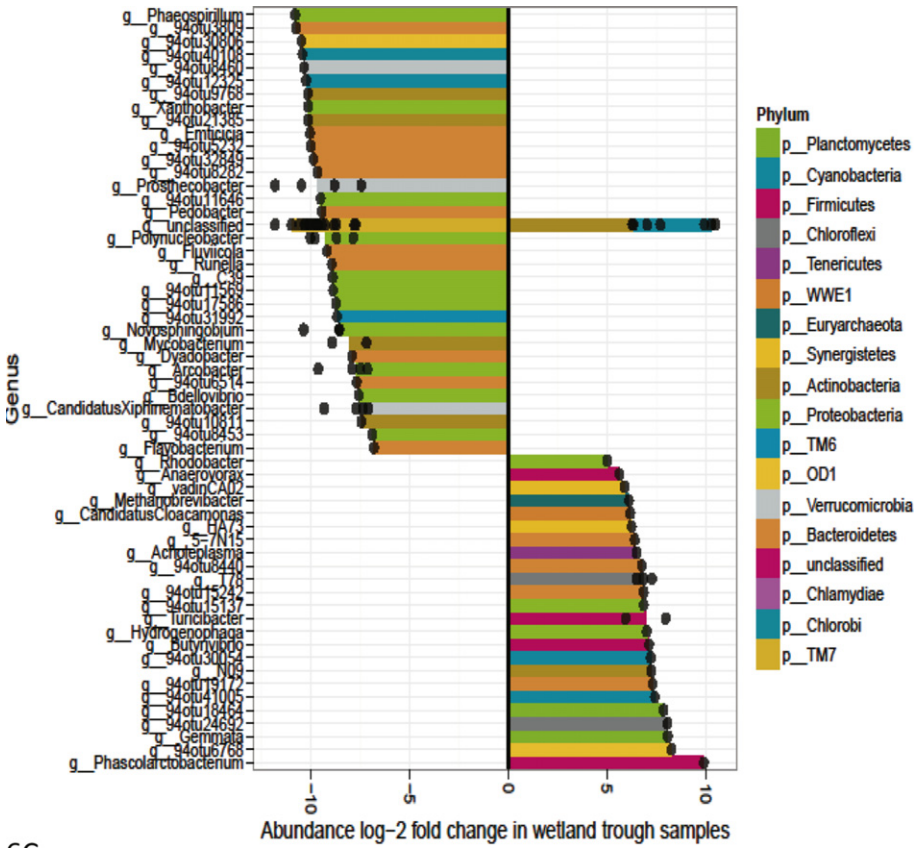


Fig. 6. Graphic summary of feature (OTU) selection. (A) Mo Bio kit: graphic summary of feature (OTU) selection. B) MP BIO: graphic summary of feature (OTU) selection. (C) ZYMO: graphic summary of feature (OTU) selection.

indicating that *Cyanobacteria* and other microalgae can contribute to wastewater nutrient remediation. The other dominant photoautotrophs identified to genus level included the *Leptolyngbya*. This photoautotroph was significantly more abundant ($P < 0.0031$) in HRAPs than in DLE. This genus was only identified using the MP Bio extraction kit, suggesting the possible role of extraction techniques in bacterial community composition analysis. It should be noted that algal reference sequences are not typically as well represented as bacteria in 16S rRNA sequence databases, and that even more careful targeting (e.g., 18S rRNA), could lead to large proportions of unidentified sequences (Xiao et al., 2015).

Bacterial family sequence numbers were adjusted to the median sequence number for the three sample sources and differences at the family levels are shown in Fig. 5B. One of the species identified was *Polynucleobacter cosmopolitanus* of the *Oxalobacteraceae* family which possess a large photosynthetic gene cluster containing all key genes of anoxygenic photosynthesis (Hahn et al., 2010, 2012). Among the *Rhodobacteraceae* (*Alpha-proteobacteria*) and the genus *Rhodobacter* was detected in all the sample sources and these genera include species which possess an extensive range of metabolic capabilities. *Rhodobacter sphaeroides* is the most-studied photosynthetic organism in terms of the structural and functional light reactions, and the metabolic capabilities of each species generate great interest within the research community, especially with regards to renewable energy sources. *Rhodobacter* are found in freshwater or marine environments (Xiao et al., 2015). Bacterial taxa with potential negative effects on algal growth, e.g. *Pseudomonas* and *Bdellovibrionaceae* (Carney et al., 2014), were identified in all of our samples. Notable *Xanthomonadaceae* species identified in ponds included the common aquatic bacterium *Aquimonas* genus with some species like *Aquimonas voraii*, which has extracellular phosphatase activity (von Tigerstrom and Stelmaschuk, 1987) that could improve phosphorus availability for algae (Fig. 6).

As shown in Table 2 and in Tables S1–S3, the relative abundances of the species detected was more dependent on the samples sources rather than on the choice of DNA extraction procedure (Fouhy et al., 2016). Notable differences occurred based on phyla, namely the *Cyanobacteria* cells had a higher relative abundance using the ZYMO procedure compared to the Mo Bio or MP Bio procedures. These results suggest subtle differences occur in sequencing data as a result of the DNA extraction protocol used. Extraction methods had a lesser effect on overall composition, since no significant differences ($P < 0.249$) were identified based on extraction procedure. DNA extraction procedure may have significant impact on sequencing results (McOrist et al., 2002). Several studies have previously shown the effects of using different commercial kits for DNA extraction from fecal samples on sequencing outcomes (Nechvatal et al., 2008; Kennedy et al., 2014; Walker et al., 2015). Our approach was to focus specifically on three extraction methods commonly used in microbial ecology studies to establish if they produce the same level of relative abundances of major bacterial families that may coexist with algae in an algal production pond. The three extraction procedures yielded DNA that gave comparable results with respect to phylogeny, but with some minor differences in taxa diversity at the genus level Tables S1–S3).

5. Conclusion

The use of next generation sequencing has significantly enhanced our understanding of microbial community dynamics in HRAPs used for the bioconversion of animal waste to nutrients. However, for success in this kind of study, the best DNA extraction procedure must be followed for good quality DNA. As a result many OTUs were identified using the fungi/bacterial DNA Mini Prep kit from Zymo Research which were similar to *Scenedesmus*, *Chlorella*, *Desmodesmus* and a variety of small *Chlorophyta* which were also identified by microscopy. These

OTUs or species identified microscopically may facilitated the conversion of DLE N and P to algal biomass thus facilitating the removal of N and P from animal waste and improving the quality of the resultant effluent water. The technology provides many advantages to society as a whole by providing better quality water with less residual N and P that may contaminate ground and surface water, building algal biomass that may be used as an animal feed, alternative energy source, or for other products.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.12.076.

Acknowledgements

This research was supported by the USDA-AFRI-NIFA Award # 2013-67019-21374. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. The U.S. Department of Agriculture (USDA) prohibits discrimination in all its programs and activities on the basis of race, color, national origin, age, disability, and where applicable, sex, marital status, familial status, parental status, religion, sexual orientation, genetic information, political beliefs, reprisal, or because all or part of an individual's income is derived from any public assistance program.

References

Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U.C., 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit. Rev. Biotechnol. 22, 245–279.

Caporaso, J.G., Lauber, C., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., et al., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. U. S. A. 15, 4516–4522.

Carney, L.T., Reinsch, S.S., Lane, P.D., Solberg, O.D., Jansen, L.S., Williams, K.P., Trent, J.D., Lane, T.W., 2014. Microbiome analysis of a microalgal mass culture growing in municipal wastewater in a prototype OMEGA photobioreactor. Algal Res. 4, 52–61.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Collos, Y., Berges, J.A., 2003. Nitrogen metabolism in phytoplankton. In: Duarte, C.M. (Ed.), Encyclopedia of Life Support Systems. EOLSS Publishers. (UNESCO). http://www.EOLSS.net/PDF.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.

Fouhy, F., Clooney, A.G., Stanton, C., Claesson, M.J., Cotter, P.D., 2016. 16S rRNA gene sequencing of mock microbial populations- impact of DNA extraction method, primer choice and sequencing platform. BMC Microbiol. 16 (123). http://dx.doi.org/10.1186/s12866-016-0738-z.

Hahn, M.W., Lang, E., Brandt, U., Lu, H., Wu, Q.L., Stackebrandt, E., 2010. Polynucleobacter cosmopolitanus sp. nov., free-living planktonic bacteria inhabiting freshwater lakes and rivers. Int. J. Syst. Evol. Microbiol. 60 (2010), 166–173.

Hahn, M.W., Scheuerl, T., Jezberova, J., Koll, U., Jezbera, J., et al., 2012. The passive yet successful way of planktonic life: genomic and experimental analysis of the ecology of a free-living polynucleobacter population. PLoS One 7 (3), e32772. http://dx.doi.org/10.1371/journal.pone.0032772.

Helperin, A.N., Beckman, D.S., Inwood, D., 2001. California's Contaminated Groundwater - Is the State Minding the Store? National Resources Defense Council.

Kellogg, R.L., Lander, C.H., Moffitt, D.C., Gollehon, N., 2000. Manure nutrients relative to the capacity of cropland and pastureland to assimilate nutrients-spatial and temporal trends for the United States. GSA Publ. nsp00-0579. USDA-NRCS-ERS, Riverside, CA.

Kennedy, N.A., Walker, A.W., Berry, S.H., Duncan, S.H., Farquarson, F.M., Louis, P., Thomson, J.M., Ahmad, T., Satsangi, J., Flint, H.J., 2014. The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16s rRNA gene sequencing. PLoS One 9, e88982.

Kuczynski, J., Stombaugh, J., Walters, W.A., González, A., Caporaso, J.G., Knight, R., 2011. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Curr. Protoc. Microbiol. http://dx.doi.org/10.1002/0471250953.bi1007s36 (Chapter 10:Unit 10.7).

Kumar, K., Das, D., 2012. Growth characteristics of *Chlorella sorokiniana* in airlift and bubble column photobioreactors. Bioresour. Technol. 116, 307–313.

Lincoln, E.P., Hall, T.W., Koopman, B., 1983. Zooplankton control in mass algal cultures. Aquaculture 32, 331–337.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. bioRxiv http://dx.doi.org/10.1101/002832.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610–618.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217.

McOrist, A.L., Jackson, M., Bird, A.R., 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. J. Microbiol. Methods 50, 131–139.

Montemezzani, V.I., Duggan, C., Hogg, I.D., Craggs, R.J., 2015. A review of potential methods for zooplankton control in wastewater treatment high rate algal ponds and algal production raceways. Algal Res. 11, 211–226.

Mulbry, W., Kondrad, S., Pizarro, C., Kebede-Westhead, E., 2008. Treatment of dairy manure effluent using freshwater algae: algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. Bioresour. Technol. 99, 8137–8142.

Nechvatal, J.M., Ram, J.L., Basson, M.D., Namprachan, P., Niec, S.R., Badsha, K.Z., Matherly, L.H., Majumdar, A.P., Kato, I., 2008. Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. J. Microbiol. Methods 72, 124–132.

O'Brien, W.J., De Noyelles, F., 1972. Photosynthetically elevated pH as a factor in zooplankton mortality in nutrient enriched ponds. Ecology 53, 605–614.

Oswald, W.J., 1988. Terrestrial approaches to integration of waste treatment. Waste Manag. Res. 9, 477–484.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al., 2009. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.

Schluter, M., Groeneweg, J., 1981. Mass production of freshwater rotifers on liquid wastes. I. The influence of some environmental factors on population growth of *Brachionus rubens* Ehrenberg 1838. Aquaculture 25, 17–24.

Schluter, M., Groeneweg, J., 1985. The inhibition by ammonia of population growth of the rotifer, *Brancionus rebens*, in continuous culture. Aquaculture 46, 215–220.

Shendure, J., Ji, H., 2008. Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135–1145.

Taziki, M., Ahmadzadeh, H., Murry, M.A., Lyon, S.R., 2015. Nitrate and nitrite removal from wastewater using algae. Curr. Biotech. 4, 426–440.

USEPA, 2003. National pollutant discharge elimination system permit regulation and effluent limitation guidelines and standards for concentrated animal feeding operations (CAFOs). Fed. Regist. 69 (29), 7176–7274.

US-EPA, 2008. An Evaluation of a Covered Anaerobic Lagoon for Flushed Dairy Cattle Manure Stabilization and Biogas Production. United States Environmental Protection Agency, Washington, D.C.

von Tigerstrom, R.G., Stelmaschuk, S., 1987. Comparison of the phosphatases of *Lysobacter enzymogenes* with those of related bacteria. J. Gen. Microbiol. 133, 3121–3127.

Walker, A.W., Martin, J.C., Scott, P., Parkhill, J., Flint, H.J., Scott, K.P., 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome. 3, 1–11.

Ward, P.E., 2011. Bioflocculation and CO_2 Supplementation in Wastewater Treatment by Algal High-rate Ponds. (MS thesis). Civil and Environmental Engineering Department, California Polytechnic State University, San Luis Obispo.

Warrington, P., 2001. Aquatic pathogens – cyanophytes – the blue-green algae. Available Online: http://www.elp.gov.bc.ca/wat/wq/reference/toxicalgae.html.

Weissman, J.C., Goebel, R.P., Benemann, J.R., 1988. Photobiorector design: comparison of open ponds and tubular reactors. Bioeng. Biotech 31, 336–344.

Whitten, R., Mevel, A.L., Pidou, M., Ometto, F., Villa, R., Jefferson, B., 2015. Influence of microalgal N and P composition on wastewater nutrient remediation. Water Res. 91, 371–378.

Woertz, I.C., Lundquist, T.J., Feffer, A.S., Nelson, Y.M., 2009. Lipid productivity of algae grown on dairy and municipal wastewaters for biofuel feedstock. J. Environ. Eng. ASCE 135 (11), 1115–1122.

Xiao, L., Young, E.B., Grothjan, J.J., Lyon, S., Zhang, Z., He, Z., 2015. Wastewater treatment and microbial communities in an integrated photo-bioelectrochemical system affected by different wastewater algal inocula. Algal Res. 12, 446–454.

Yang, S., Guarnieri, M.T., Smolinski, S., Ghirardi, M., Pienkos, P.T., 2013. De novo transcriptomic analysis of hydrogen production in the green alga *Chlamydomonas moewusi*i through RNA-Seq. Biotechnol. Biofuels. 6, 1–17.