

Microbial Ecology

Longitudinal analysis of microbiota in microalga *Nannochloropsis salina* cultures --Manuscript Draft--

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Corresponding Author:	Todd William Lane, Ph.D. UNITED STATES					
Corresponding Author Secondary Information:						
Corresponding Author's Institution:						
Corresponding Author's Secondary Institution:						
First Author:	Haifeng Geng, Ph.D.					
First Author Secondary Information:						
Order of Authors:	Haifeng Geng, Ph.D. Kenneth L. Sale, Ph.D. Mary Bao Tran-Gyamfi, B.S. Todd William Lane, Ph.D. Eizadora Yu, Ph.D.					
Order of Authors Secondary Information:						
Funding Information:	<table border="1"> <tr> <td>Sandia National Laboratories, National Nuclear Security Administration (US)</td><td>Dr. Eizadora Yu</td></tr> <tr> <td>U.S. Department of Energy, Genomic Science Program (SCW1039)</td><td>Dr Todd William Lane</td></tr> </table>		Sandia National Laboratories, National Nuclear Security Administration (US)	Dr. Eizadora Yu	U.S. Department of Energy, Genomic Science Program (SCW1039)	Dr Todd William Lane
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Abstract:	Large-scale open microalgae cultivation has tremendous potential to make a significant contribution to replacing petroleum-based fuels with biofuels. Open algal cultures are unavoidably inhabited with a diversity of microbes that live on, influence, and shape the fate of these ecosystems. However, there is little understanding of the resilience and stability of the microbial communities in engineered semi-continuous algal systems. To evaluate the dynamics and resilience of the microbial communities in microalgae biofuel cultures, we conducted a longitudinal study on open systems to compare the temporal profiles of the microbiota from two multi-generational algal cohorts, which include one seeded with the microbiota from an in-house culture and the other exogenously-seeded with a natural occurring consortia of bacterial species harvested from the Pacific Ocean. From these month-long, semi-continuous open microalga <i>Nannochloropsis salina</i> cultures, we sequenced a time-series of 46 samples, yielding 8804 operational taxonomic units derived from 9,160,076 high-quality partial 16S rRNA sequences. We provide quantitative evidence that clearly illustrates the development of microbial community is associated with microbiota ancestry. In addition, <i>N. salina</i> growth phases were linked with distinct changes in microbial phylotypes. Alteromonadeles dominated the community in the <i>N. salina</i> exponential phase whereas Alphaproteobacteria and Flavobacteriia were more prevalent in the stationary phase. We also demonstrate that the <i>N. salina</i>-associated microbial community in open cultures is diverse, resilient, and dynamic in response to environmental perturbations. This knowledge has general implications for developing and testing design principles of cultivated algal systems.					
Response to Reviewers:	We have responded to all of the reviewers' comments. Please see attached file.					

We have made all of the requested revisions to the manuscript as detailed below. In each case the reviewers comments are in *italics* and our responses are in **bold**.

Reviewer 1

MECO review MECO-D-15-00435

Recommendation: minor revisions

Comments to authors:

The reviewed manuscript tracks bacterial community progression and algae growth rates in outdoor open ponds that are started from different founder communities. On appears to be a lab generated community while the other is a mix of the lab community and a natural open coast community collected some distance away from where the study was performed. Succession of the bacterial community and its response to an ammonium-induced environmental shift were analyzed in both types of founder communities using NGS. Surprisingly, this study observed both community types to be very stable and revert back to very close to the original community after one perturbation. This was observed in duplicates of each community. This is a nice piece of work using sophisticated methods of analysis. However some of the conclusions appear to overreach the evidence provided. The authors need to be more cautious about conclusions made from one study in one place at one point in time. There is no evidence that results shown here will apply to additional runs in the same location, let alone runs in other locations. They do a nice job of tying in results from other studies but there have been too few to support the observed patterns. There is also no discussion here on how seasonality may affect the succession of these communities. Again, a nice piece of work and definitely a step in the right direction, but until results are confirmed as repeatable and stronger effects on algae productivity are observed, conclusions should be limited.

Title reads strangely: "biofuel microalga"

Response: removed biofuel in title

P 4, L 31: Remove "microbial and"

Response: removed "microbial and"

P6, L66: Clarify what is meant by "passaged six times". It is unclear from this description where the bacteria originated from for the domestic sample. Were these from the ambient laboratory environment or were these exposed to local outdoor conditions.

For each "passage" the cultures were diluted ten fold with fresh medium (essentially a 90% harvest) and regrew them. The bacteria originated from the from the ambient laboratory environment prior to exposure to local outdoor conditions. We have updated and incorporate these changes in manuscript on P6L66.

P6, L70. Were any steps taken to make sure that the bacterial load was the same for starting D and S cultures? If not, how could different starting bacterial concentrations have affected the results.

We did not fix the bacterial loading because, according to our experimental data as well as that from the literature, total bacterial numbers vary with the algal growth phase. Differential starting bacterial concentrations may indeed have some effects but our investigation focused on the microbiota structure and behaviors, which arise during and after outdoor incubation rather than that from the laboratory culture. Outdoor incubation should apply selective pressure on both the species present and their abundance

P6, L68-9: add “Was derived from a combination”

: added “Was derived from a combination”.

P6, last paragraph: Were there 4 or 6 ponds? Six starter cultures are mentioned but only two treatment in duplicate are described. Explain proximity and layout of outdoor ponds and replicates. Are they laid out in a way that would prevent cross contamination?

: It was 6 ponds as mentioned at the beginning of this paragraph. D-treatment and S-treatment (3 ponds each) were alternately laid out (DSDSDS) to minimize variations of local environmental conditions across different cultures. To prevent cross contamination, we reserved headspace approximately 10 cm in depth of each culture so that culture will not overflow. Accordingly we added the following text in P6L73-74. “Triplicated D- and S- treated algal open cultures (16L ESAW medium each) were alternately laid out in D-S-D-S-D-S format to minimize variations of local environmental conditions.”

P7, L80: cells should be singular

: updated and changed to singular.

P14, last paragraph: With such a weak R^2 value, it is difficult to end on such a conclusion.

: Various uncontrolled environmental factors like temperature and light density would have effects on algal growth rates. When we regressed growth rate against single microbiota richness index, the linear model did not incorporate these other latent factors for growth rates. A Low R^2 indicates that our regression model is missing some other things that account for the other 76% of the variation in growth rate. The P value just indicates the likelihood of the variation in growth rate not being affected by changes in richness is very low. Low P values would suggest that growth rate and richness were significantly correlated.

There was no variation in other abiotic parameters such as T, salinity, light, N, O, across the different experimental setups so the inclusion of those in a model would not be useful. Since there is no variability in these parameters they are unlikely to contribute to variation in growth rates.

P15, L 235: change to “N. salina cultures in an open system”

: changed to “N. salina cultures in an open system”

P15, L236: What constitutes “multi-generational”? A 3 month study may indeed be the longest published but papers cited here track community changes long enough to account for multiple generations.

N. salina divides approximately once per day thus a 1.5 month-long study consisted of 45 generations over 5 culture passages: defining a multi-generation time course.

P17, L286-8: The final sentence needs more explanations and perhaps more caveats. The data from this study represents one culture run in one time and one place. Additionally, there is a lack of metagenomics studies out there concerning algae cultivation systems. We are just starting to touch the surface on what types of communities are going to be ubiquitous and stable in these systems.

: updated this sentence as requested and added “Nevertheless, there is a lack of whole genome shotgun sequencing data at resolution down to bacterial species or gene levels concerning *N. salina* cultivation systems. This investigation represents only one of arising case studies that would touch on microalgae microbiota composition, structure and social traits.” P17 L294-P18 L297

P18, L304: keys should be singular

: We made this correction on P18L313

P18, L309-10: This statement seems mostly conjecture. There is no clear link sown here between richness and culture health. Please clarify this sentence.

: The reviewer is correct, there is no clear mechanistic data linking richness to culture health. Species richness and culture health were correlated, but lacked a causal relationship. We discussed bacterial roles and effects on microalgae cultures in P18 L310-312.

P18, 317-8: This is a very interesting statement and should be a more dominant argument in this ms, esp. compared to some other lines of suggestion. This is essentially what was done here, although specific community make-up was not intentional, and it appears to have been fairly successful in aiding community stability. Would like to see follow-up studies where more intentional communities are forced and how this affects algae productivity.

: We are working on WGS-metagenomics studies on this to further define microbial composition, functionality and biological state as occurred in algae cultivation system.

Reviewer #3: The manuscript describes the microbial communities in microalgae open cultures and their dynamics and resilience. Although there are many similar papers published in the literature several years ago, the authors' approaches are original and the results of interest for the people working with microalgae cultures. The problem with this manuscript is that includes (almost exclusively) observations without entering in the relationships developed among the various organisms. Discussion should be extended to the physiology of the organisms and suggestions concerning synergistic/antagonistic relationships should be provided.

:

: We have extended the discussion of the physiology of species observed on in two location in the revised manuscript: from P16L263 to P17L278 and P17L286 to P18L29 where we added the following text: “It has been shown that algal culture growth phase and physiological state could serve as selective niches filtering bacterial compositions and governing bacterial community structure [47]. These selective activities could involve both positive and negative effects from both partners in many cases. It have been reported negative interactions of algicidal bacteria that kill phytoplankton as well as antibacterial compounds secreted from algae that kill or inhibit bacterial species [48-50]. Meanwhile, positive interactions from acquisition of vitamins or iron by algae with the aid of heterotrophic bacteria possibly in exchange of carbon source have been documented [51, 52]. Nevertheless, there is a lack of whole genome shotgun sequencing data at resolution down to bacterial species or gene levels

concerning *N. salina* cultivation systems. This investigation represents only one of arising case studies that would touch on microalgae microbiota composition, structure and social traits.”

These changes required the Deletion of the last sentence in the discussion section: “Thus, the observed consistent major taxa might have important implications for future diagnostic and prognostic target on specific biomarkers for maintaining algal culture homeostasis.”

[Click here to view linked References](#)

Longitudinal analysis of microbiota in microalga *Nannochloropsis salina* cultures

Haifeng Geng¹, Kenneth L. Sale², Mary Bao Tran-Gyamfi², Todd W. Lane*¹ and Eizadora T. Yu^{1,3}

¹ Department of Systems Biology, Sandia National Laboratories, 7011 East Ave, Livermore, CA 94550 Livermore

² Department of Biomass Science and Conversion Technology, Sandia National Laboratories, 7011 East Ave Livermore, CA 94550 Livermore

³ Present address Institute of Chemistry, National Science Complex, University of the Philippines, Diliman Quezon City, Philippines 1101

* Corresponding author

Telephone: 925-294-2057

Fax: 925-294-3020

Email: twlane@sandia.gov

Keywords: biosystem, microbiota, algae, stability

Running Title: Algal culture-associated microbiota succession and dynamics

Abstract

Large-scale open microalgae cultivation has tremendous potential to make a significant contribution to replacing petroleum-based fuels with biofuels. Open algal cultures are unavoidably inhabited with a diversity of microbes that live on, influence, and shape the fate of these ecosystems. However, there is little understanding of the resilience and stability of the microbial communities in engineered semi-continuous algal systems. To evaluate the dynamics and resilience of the microbial communities in microalgae biofuel cultures, we conducted a longitudinal study on open systems to compare the temporal profiles of the microbiota from two multi-generational algal cohorts, which include one seeded with the microbiota from an in-house culture and the other exogenously-seeded with a natural occurring consortia of bacterial species harvested from the Pacific Ocean. From these month-long, semi-continuous open microalga *Nannochloropsis salina* cultures, we sequenced a time-series of 46 samples, yielding 8804 operational taxonomic units derived from 9,160,076 high-quality partial 16S rRNA sequences. We provide quantitative evidence that clearly illustrates the development of microbial community is associated with microbiota ancestry. In addition, *N. salina* growth phases were linked with distinct changes in microbial phylotypes. *Alteromonadeles* dominated the community in the *N. salina* exponential phase whereas *Alphaproteobacteria* and *Flavobacteriia* were more prevalent in the stationary phase. We also demonstrate that the *N. salina*-associated microbial community in open cultures is diverse, resilient, and dynamic in response to environmental perturbations. This knowledge has general implications for developing and testing design principles of cultivated algal systems.

Introduction

Aquatic microalgae offer unique features that make them attractive alternatives to land plants for use as feedstocks for production of advanced biofuels [1, 2]. Microalgae grow rapidly in open systems (ponds and raceways), proliferate in a wide variety of marginal water sources, and do not displace arable land for food production [1, 2]. However, established algae facilities are challenged to economically generate the biomass production yields required to make microalgae biofuels a viable renewable source [3]. Reasons for this challenge include both loss of production due to culture instabilities and suboptimal yields due to unfavorable environmental conditions [3, 4]. Attaining and maintaining culture stability, in part by either limiting the impact of deleterious species or by creating a culture that is more robust to environmental fluctuations, is critical to the sustainable production of algae biomass. Developing strategies for monitoring the health of outdoor algal culture systems and the future diagnosis, treatment, and prevention of pond crashes is dependent on understanding the diverse, complex microbial communities associated with algal production ponds whose component species are intricately interconnected and dynamic in their response to changing environments [5-7].

Several factors, including the diversity of the ecosystem and the composition of the community, are associated with the function, stability and dynamics of ecosystems. Changes in biodiversity pertaining to species evenness (the relative abundance of species) and richness (the number of species) have been associated with ecosystem function, stability and dynamics [8, 9]. Greater species evenness has been shown to confer ecosystems with robust functions under varying environmental stresses [10] and to be an important element in controlling invasion of managed ecosystems [11]. In microcosms, microbiota diversity has been shown to have strong effect on the functional stability of ecosystems [10]. For example, initial bacterial community diversity

determines the ecosystem productivity as evidenced by significant relationships between microbiota overall resilience and the rate of biomass turnover in structured microbial communities[12]. On the other hand, methanogenic bioreactor microbial communities are resilient against differential sludge loading rates, suggesting the importance of microbial community stability in maintaining robust methanogenic activity and substrate removal efficiency [13].

Evaluating the resilience and long-term health of algal biofuel ponds requires a clear understanding of the make-up and dynamics of the entire ecological community, including the the associated microbial community. A number of studies have identified the phylotypes of microbial communities associated with microalgae in experimental algae production cultures [3, 14, 15]; however, less is known about either the dynamics of the microbiota or the resilience of the community in manipulated algal cultivated system as a function of changing environmental conditions. To this end, we conducted a longitudinal study to evaluate the dynamics and resilience of the microalgae microbiota in response to environmental perturbations. The algal microbiota cohorts consisted of duplicated domestic microbiota that resided with *N. salina* for over 3 months in laboratory cultures and, separately, duplicated conditioned-microbiota prepared by a distinct collection of bacterial species separately harvested from coastal areas of the Pacific Ocean. Changes to the composition of the microbiota over the time course of multi-generations in open algae cultured systems were mapped using 16S profiles of the microbiota generated using next-generation sequencing. From these month-long, semi-continuous *N. salina* cultures in uncontrolled, open environments, we found that variation (β -diversity) between domestic and conditioned microbiota was larger than the variation (β -diversity) between replicates of either the domestic or the conditioned microbiota during cultivation trajectories, suggesting relative

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46 stability of ancestry microbiota in open algae cultures and thereby suggesting their persistent
47 influences on the outcome of the descendant microbial community. In addition, it showed the
48 microbial communities were resilient to both periodic dilution and an ammonia-induced
49 disturbance. This stability and resilience of microbiota confirms the potential of microbiota as a
50 target to increase the tractability of stable microalgae cultures.

Materials and Methods

Strains and Culture Growth Conditions. Axenic cultures of *Nannochloropsis salina* (CCMP 1776) were obtained from the Culture of Marine Phytoplankton at Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, MA). The cultures were maintained in ESAW medium at 21°C under constant light 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and aeration [16].

Sampling of coastal marine microbiota. Two water samples each 500 ml were collected from the Pacific Ocean off the coast of Santa Cruz, CA, in May 2012 and returned to the laboratory in the dark at ambient temperature. Aliquots (500 mL) were filtered sequentially through 2 μm pore size polycarbonate GTTP membranes (Millipore, Bedford, MA) to remove larger eukaryotes including phytoplankton, followed by 0.2 μm pore size polycarbonate GTTP membranes to capture bacteria. Lastly, membranes were then washed twice with 500 mL sterile ESAW. The biomass retained by the 0.2 μm pore size membranes was then suspended in 500 mL mid log phase xenic culture of *N. salina*. The endogenous microbiota associated with xenic laboratory *N. salina* cultures was treated as the domestic microbiota (referred to as D-treatment), which were developed during acclimation of *N. salina* under aeration and passaged (splitted and regrew) six times in the indoor laboratory conditions for 3 months. In comparison, the resulting Santa Cruz microbiota-treated *N. salina* culture and associated microbial consortium (referred to as S-treatment) was derived from combination of microbiota from domestic and that from the marine sample microbial species.

Outdoor cultivation. Six one-liter starter cultures of D- and S- *N. salina* were cultivated to mid-log phase under laboratory conditions (20 °C under light intensity 100 $\mu\text{M photons m}^{-2} \text{ s}^{-1}$ with a 14 h light/ 10h dark cycle, constant aeration) and used to seed outdoor open cultures. Triplicated D- and S- treated algal open cultures (16L ESAW medium each) were alternately laid out in D-

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4 74 S-D-S-D-S format to minimize variations of local environmental conditions. These cultures
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6 75 were inoculated at an initial cell density of ~200,000 cells/ml. These cultures were grown with
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9 76 continuous aeration in semi-continuous mode. Ninety percent of each culture was harvested
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11 77 every 4~6 days and replaced with fresh sterile ESAW medium for a total of five cycles. Sterile
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14 78 distilled water was added daily to the cultures to compensate evaporative loss. Culture
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16 79 temperature, pH, NH₃/NH₄⁺ levels, dissolved oxygen, and light levels were continuously
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19 80 monitored using autonomous Seneye REEF monitors (Seneye Ltd, Norwich, UK). Phosphate and
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21 81 nitrate concentrations were measured using standard colorimetric methods [17, 18]. *N. salina* cell
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23 82 counts were measured using an Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA).
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26 83 Daily biomass samples from 500 mL of culture were obtained by filtration (0.2 µm pore size
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29 84 filters) and stored at – 80 °C prior to total DNA extraction.

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32 85 *DNA extraction and 16S rRNA library preparation.* Genomic DNA was extracted from samples
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34 86 using a ZR Fungal/Bacterial DNA MiniPrep (ZYMO Research, Irvine, CA) following the
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37 87 manufacturer's protocol. Each sample was PCR amplified, targeting the 177 bp V3 region of 16S
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39 88 rRNA gene using the 341F forward and 518R reverse barcoded primer set as previously
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42 89 described [19]. PCR runs for each sample were performed in triplicate under the following
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44 90 conditions: 100 µM dNTPs, 0.2 µM primers, and 1U of Phusion Taq DNA polymerase in 1X
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47 91 reaction buffer (New England Biolabs, Beverly, MA) with an initial denaturing step at 94 °C for
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49 92 5 min, followed by 25 cycles at 94 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72
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51 93 °C for 1 min each, and ended with 72 °C for 7 min extension. Triplicate PCR products for each
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54 94 sample were pooled, purified using QIAquick PCR purification kit (Qiagen, Valencia, CA), and
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56 95 quantified using a Nanodrop ND spectrophotometer (Thermo Science, Wilmington, DE).
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59 96 Libraries were constructed by mixing equal amounts of 24 samples (each with unique index
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sequence), gel-purified using QIAquick gel extraction kit (Qiagen), and quantified on a Bioanalyser DNA 7500 chip (Agilent Technologies, Santa Clara, CA). Thirty percent (v/v) PhiX control (Illumina, Hayward, CA) was added to 16S rRNA library prior to sequencing of the 151-nucleotide paired-end multiplex sequences on MiSeq (Illumina). Image analysis, base calling, error estimation, and demultiplexing according to index sequence were carried using the Illumina Analysis Pipeline (Illumina).

Sequence quality filtering. Paired-end reads were assembled by aligning the 3' ends of forward and reverse reads using SHE-RA software [20]. Sequence reads were filtered to remove sequences of poor quality using an algorithm modified from QIIME [21]. Sequence reads were truncated 5 bases off the individual read. Sequences were trimmed off when two or more continuous base calls below 30. Reads with less than 75 bases or ambiguous bases were excluded from the downstream analysis.

Data analysis. Filtered sequences were aligned, clustered and assigned to operational taxonomic units (OTUs) using the UCLUST algorithm and Greengenes database in QIIME [21, 22]. Sequences were clustered using 97% sequence similarity threshold and phylotypes were assigned according to Greengenes taxonomy, using a 0.80 confidence threshold. Relative abundance of OTUs per sample was normalized by dividing the OTU reads by the sum of usable reads. Alpha rarefaction was performed using the Shannon index, Chao1, and observed species metrics [21, 23]. Ten sampling repetitions were performed, without replacement, at each sampling depth, starting from 10 sequences/sample up to 30,000 sequences/sample (in 100 sequences/sample increments) to obtain α -diversity rarefaction curves. Beta diversity was calculated using unweighted UniFrac distances between samples [24]. Principal component analysis (PCA) was applied to cluster samples based on their between-samples unweighted UniFrac distances

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120 metrics. Jackknifing was performed by resampling 100 times with replacement at a depth of
121 100,000 sequences per sample, and used to build a rooted pairwise similarity tree based on
122 hierarchical clustering using the Unweighted Pair Group Method with Arithmetic Mean
123 (UPGMA) in QIIME [21].

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Results

Overview of algal growth and microbial community 16S library

To investigate the temporal dynamics of the microbiota-algae community structure to changing environmental conditions, abiotic data that included pH, light, temperature, dissolved oxygen, total and free ammonia were archived every 1 hr (Table S1), and *N. salina* densities were counted by flow cytometer for each round starting with similar cell density in the range of 2.6×10^5 cells/ml (Fig. 1). Overall, the growth rates of *N. salina* were not significantly different between the Domestic (D-treated) and Santa Cruz conditioned-microbiota (S-treated) cultures within each dilution passage round. However, significant differences in growth rate were observed between different passage rounds (Fig. 1, Table 1) (One-way ANOVA, $p < 0.01$), round two being the slowest growing cycle (0.58 ± 0.08), round one (0.83 ± 0.04) and three being the fastest growing cycle (0.83 ± 0.04), round four (0.69 ± 0.02) and round five having intermediate growth rates (0.73 ± 0.01) (Tukey's HSD test, $p < 0.05$). From day three to day four (R2, Day3-Day4) in the second passage round, we observed an approximately fourteen fold increase in total ammonia over basal in all ponds (Fig. 4A) due to unidentified sources. There were no other observed abnormalities in other abiotic factors (Table 1).

To characterize microbiota in *N. salina* cultures, we ribotyped a total of 46 samples collected by filtering 200 ml cultures from 4 microcosms throughout the algae cultivation. The samples include 2 initial starting samples (Dday0 and Sday0), and 11 time points per alga *N. salina* culture (Day 2, Day 4 from Rounds 1-5 and Day 14 from Round 5 only). We obtained a combined 21,066,422 reads that were successfully assigned to individual samples in two Miseq runs (24 multiplexed samples/run), and a combined 9,160,076 high-quality 16S rRNA gene sequences from the 5' and 3' ends after quality filtration (average per sample: $195,022 \pm 46,515$), which removed 11,900,346 low-quality reads (56%). We subsequently excluded 4,985,354 reads

corresponding to chloroplast. The final 4,180,722 reads (45% of post quality-filtered sequences) were clustered into 97%-identical groups and were assigned to 8804 unique OTUs.

Three percent of the OTUs were not assignable to the bacterial domain, perhaps due to PCR errors or non-specific pick up from eukaryotes during PCR amplification (Fig. 2). In order to compare the taxonomic distribution across all 46 samples, we used the normalized relative abundances of OTUs (family level) to generate a distribution map, classifying the OTUs as high (> 10 %), medium (>1 % and < 10 %), and low (< 1 %) abundance (Fig. 3). The data showed that 14 bacterial groups of OTUs at the family level mainly contributed to the microbial community. In addition, the same group of OTUs tend to co-occur either high/medium (> 1%) or low abundant (< 1%) across all microbiota, suggesting that open algal cultures select for a limited number of specialized microbial groups.

Changes in microbiota diversity corresponded to environmental disturbance

We tracked the microbial community structure during the course of algal outdoor cultivation. Rarefaction showed that both species richness and within-sample species diversity (α -diversity) reach an asymptote in all tested samples, indicating adequate coverage of the microbial community (Fig. S1). We applied the Chao1 metric included in the QIIME pipeline [21, 23] to assess the species richness of all collected samples. As shown in Fig. 4A, the species richness remained relatively constant across all samples and no general discernible differences were observed between the D- and S-treated samples. However, the Shannon index (a measure of α -diversity) of both D and S- communities were significantly reduced in the samples obtained from Round 2 (respectively, 0.79 ± 0.16 and 1.29 ± 0.12 for R2Dday4 and R2Sday4) as compared to the remaining 42 samples (paired t-test, $P < 0.05$, Fig. 4B). We related the α -diversity in these

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4 171 samples to the metadata of culture conditions, and found the reduction in α -diversity of these 4
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6 172 samples occurred concurrently with a spike in total ammonia (Fig. 5A).
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10 173 To compare the variation of the community structure between samples, β -diversity was measured
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12 174 using the unweighted UniFrac distance metric [25]. Principal components analysis (PCA)
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14 175 revealed that the UniFrac distances separate the four ammonia-perturbed samples into a distinct
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17 176 group that is well separated from the cluster of unperturbed samples (Fig. 5). The outgroup of β -
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19 177 diversity in these four samples (R2Dday4 and R2Sday4) indicated that the unperturbed
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21 178 community formed a single cluster, changed its structure in response to environmental
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24 179 perturbation, and reverted to the original cluster when environmental conditions returned to
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27 180 regular conditions. These data indicated that the microbial communities are resilient in that they
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29 181 harbor ecological traits, which not only allow them to react and respond to changing
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31 182 environmental conditions (such as changes in ammonia concentration) but also provides them
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34 183 with certain resilient properties.
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38 184 **Succession of microbiota is confined by the initial founder community**

39 185 To better understand the stability of the founder microbiota the phylogenetic trajectories among
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41 186 the S-treated and D-treated microbiota were compared by performing clustering analysis of the
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43 187 microbiota based on the unweighted Unifrac distance matrix. As shown in Figure 6A,
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46 188 community assembly was nonrandom: samples from D-microbiota cluster together (black), and
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48 189 are distinct from the samples from the S-treated microbiota clusters (yellow), indicating samples
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50 190 from the same initial starting microbiota were, through time, more similar to each other than
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52 191 those from different founder microbiota. In parallel, PCA analysis of samples showed microbiota
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54 192 from the same founder microbiota were much more similar to one another than samples from
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57 193 different founder microbiota (D vs. S) (Fig. S2). Taken together, these results reflect that
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variations of within-founder community were minor compared with those of inter-founder differences. Clustering of the β -diversity of microbiota was performed by jackknifing the diversity distance matrix 1000 times and using the unweighted pair group method with arithmetic mean (UPGMA) tree (Fig. 6B) [21]. Consistent with the PCA results (Fig. 5), environment-perturbed samples (R2day4) demonstrated a well-separated outgroup among all samples. Meanwhile, the D-seeded microbiota samples, from the first four rounds, formed distinct clusters from their counterparts of S-seeded microbiota, and the two cohorts do not appear to mix until round 5 (Fig. 6B). These results indicate that tested microbiota, if developed from the same founder, tend to cluster together over 17-day time frame, providing quantitative evidences for the stability of microbiota in tested open *N. salina* cultures.

Correlations between microbiota and microalga *N. salina*

The bacterial communities associated with algae play key roles in development, growth and metabolic activities of algae [7, 26]. We assessed taxonomic distributions at the family-level across D-treated and S-treated samples, grouped by culture passage rounds (Fig. 2). The largest taxa in D-treated and S-treated microbiota were distinct populations of *Alteromonadales* in *Gammaproteobacteria*, which constituted a greater proportion (53 % on average) of microbiota in early algal exponential growth phase (Day 2 and Day 4, Fig. 2) but diminished in late stationary phase (Day 14, Fig. 2) in algal cultures. These data suggest they may fill a specialized niche where they thrive well in the early algal exponential growth phase. In contrast, the microbiota in late algal stationary phase (Day 14, Fig. 2) was dominated by *Rhodobacteraceae* and *Flavobacteriia*, respectively 20.43 % and 43.56 % on average, indicating changed niches in different algal growth phases, presumably endowed by different nutrient sources or associated environments, specifically selected for specialized bacterial population. In addition, while

Bacteriovoracaceae successfully increased in abundance in algal culture for a brief period in round 3 day 2 (R3day2), but they failed to sustain in the ensuing cultures, indicating that this population lacks necessary fitness to grow sustainably in open algal cultures.

Since algal growth rates varied across the 5 passage rounds (Table 1), we explored the association between microbial communities and algae culture by regressing growth rates of each round with either bacterial species richness or diversity (Fig. S3). Algal growth rates were not significantly associated with bacterial richness ($P=0.64$), but they were significantly ($P<0.05$), albeit weakly ($R^2=0.24$), associated as a function of bacterial population diversity, providing empirical evidence for the relationship between the diversity of the community and *N. salina* growth.

Discussion

Elucidating the traits and constituents of microalgae-microbiota biosystems that promote stability and productivity is essential for developing a deeper understanding of the ecological functioning of the microbiota associated with microalgae production in open ponds. We took a systems biology approach to investigate the microbiota traits associated with microalgae and characterized the microbial community structure and dynamics, as a response to perturbations encountered in typical outdoor algal cultivation practices. This report detailed microbial community taxonomical composition derived from high-quality 16S sequences along 5 rounds of experimental *N. salina* cultures in an open system. To the best of our knowledge, this is the first quantitative temporal study to characterize the microbial community during multigenerational outdoor algal cultures. We found the microbial communities associated with *N.salina* were diverse, displayed resilience to environmental perturbations, and had compositions that were specific to algal growth phases. In addition, while the microbiota was subjected to unpredictable environmental conditions and exposures, the development of the descending microbiota were nevertheless inherently attributed by the initially seeded microbiota (i.e., the founder microbiota).

The results of this longitudinal study focused on characterizing a critical component for the wellbeing of algal cultures, the dynamics of the microbial community in artificial microalgae biofuel cultures. Hierarchical clustering of the samples shows that the trajectories of microbiota succession are constrained and conditioned by the initially seeded microbial community, arguing against the hypothesis that the microbiota in open microalgae cultures is solely determined by environmental culture conditions. There are several possible causes underlying this persistent, nonrandom pattern of microbial assemblage attributed to the founder microbial community. One

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4 251 explanation is that the microbiota maintains stability at the population level rather than
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6 252 individual species level. This collective stability of microbial consortia may stem from the fact
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9 253 that ecosystems maintain requisite biological functions in modules or networks composed of
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11 254 multiple, defined interacting bacterial species that collectively deliver the ecological services,
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14 255 such as biofilm formations or intercellular communications [27, 28]. Ecological processes, like
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16 256 nutrient cycling and metabolism complementation and cross feeding [29] can also potentially
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19 257 contribute to the formation of these ecosystem modules. A second possible explanation for the
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21 258 lasting effects of founder microbiota is that the seeding of the initial microbiota provide the
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24 259 diverse genetic foundational basis of core taxa as well as many occasional rare taxa in the form
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26 260 of ‘seed bank’ (long range of rare diverse taxa) in the organized community, which allows
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29 261 microbial community to survive and persist for long periods of time [30].
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32 262 Taxonomic analysis showed that the microbiota associated with manipulated *N. salina* open
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34 263 culture was mainly comprised of a limited number of phylotypes in an algal growth phase-
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37 264 dependent manner. *Alteromonadeles* were dominant in early algal exponential growth phase
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39 265 whereas *Alphaproteobacteria* and *Flavobacteriia* were prevalent in late stationary phase
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41 266 cultures. *Alteromonadaceae* are marine bacterial specialists and multiply in response to the
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44 267 presence of organic matter from actively growing phytoplankton [31, 32] and dominate
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46 268 microbiome in large-scale cultivation of microalgae cultures [3]. *Flavobacteriia* are well known
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49 269 as fast-growing specialists during algae blooms and inhabit complex organic matter and produce
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51 270 abundant enzymes for catalyzing deconstruction of algal, such as carbohydrate-active enzymes
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54 271 and TonB-dependent transporters [33-35]. The *Roseobacter* clade (*Alphaproteobacteria*) is well
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56 272 known for the production of secondary metabolites, and many *Roseobacter* manifest symbiotic
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59 273 traits when physically attached to algae [19, 36-38]. Members of *Roseobacter* clade also possess
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diverse metabolic capabilities and are capable of degrading algal-derived compounds such as dimethylsulfoniopropionate (DMSP) and producing bioactive molecules that act as antibiotics [19, 36, 39-41]. Indeed, the *Roseobacter*-derived antibiotic tropodithietic acid inhibits growth of various taxa of bacteria [36, 42]; therefore, *Roseobacter* might play a key role in bacteria-microalgae symbiosis and may also drive and shape the algal-associated microbial community structure [43]. These specialized phylotypes were also identified as the key members of microbiota of natural diatom-dominated blooms at German Bight in spring 2009 [35] and were the major phylotypes represented in annually recurring algal blooms off the southern California coast from 2000 to 2004 [44]. A small number of microbiome studies on algal biofuel pond systems have identified similar phylotypes as being affiliated with *Alphaproteobacteria*, *Alteromonadeles*, and *Flavobacteriia* [3, 45, 46]. The reproducible occurrence of the same set of dominant phylotypes from independent studies, in both natural blooms and artificial algal cultures, emphasizes the specialized fitness of these phylotypes for thriving in algal blooms, and suggests that only a limited number of well-defined niches are present in this community. It has been shown that algal culture growth phase and physiological state could serve as selective niches filtering bacterial compositions and governing bacterial community structure [47]. These selective activities could involve both positive and negative effects from both partners in many cases. It have been reported negative interactions of algicidal bacteria that kill phytoplankton as well as antibacterial compounds secreted from algae that kill or inhibit bacterial species [48-50]. Meanwhile, positive interactions from acquisition of vitamins or iron by algae with the aid of heterotrophic bacteria possibly in exchange of carbon source have been documented [51, 52]. Nevertheless, there is a lack of whole genome shotgun sequencing data at resolution down to bacterial species or gene levels concerning *N. salina* cultivation systems. This investigation

represents only one of arising case studies that would touch on microalgae microbiota composition, structure and social traits.

The results of our *N. salina* microbiota study also highlights resilience of the *N. salina* associated microbiota to exogenous perturbations, which suggests that microalgae microbiota likely maintain ecological service rendered on algal culture and hence might play an important role in ensuring the continuous functioning of the algal culture ecosystem. While community structure is perturbed in response to abnormal ammonium levels during one of the culture rounds, the community rebounded back to previous microbial community in phylogenetic distances, rather than being displaced by different organisms with redundant functions. This observed resilience trait in microbiota is not isolated, as it was also observed in other model systems [13, 53]. For instance, microbiota in methanogenic bioreactors grown under similar conditions does not change significantly as a function of culture time [13].

Sluggish algal growth was weakly associated with a decrease in microbial diversity. Our data showed that the *N. salina* growth rates between passage rounds ranged from 0.58 to 0.83 day⁻¹ on average, which were within the reported 0.18~1.3 day⁻¹ growth rate of *N. salina* performance in outdoor conditions [54]. The correlation between microbial community diversity and *N. salina* growth observed here, albeit lacking causal relationship, substantiates other studies showing that microalgae microbiota play key roles in affecting algal metabolisms, where the microbial community provides nutrients, vitamins and cofactors to the host, as well as participate in metabolic cooperation [5-7]. The weak relationship in algal growth and microbial diversity observed in this study might be confounded by other factors known to influence algal growth, such as light, temperature and aeration [55], which are not controlled in our open culture experiments. Given the similarity of richness in microalga microbiota, it appears that evenness of

microbial community is closely correlated with algal health. Community evenness is predicted to benefit microbial functional stability because higher evenness provides more redundant functional pathways [10, 13] and hence higher buffering capacities in dealing with environmental perturbations [56].

Thus, open cultivated microalga *N. salina* microbiota demonstrated several traits that maintain stable ecological functions, such as their community stability, resilience, and dynamics specific to environmental perturbations, providing a lens through which to view the algae-microbiota ecosystem. The stability of microbiota highlights that early seeding microbiota, if exogenously inoculated in early algal cultures, has long-term implications in culture interventions. A holistic network investigation on algae-microbiota interactions that examines the collective species in defined modules of microalgae-microbiota ecosystem may provide an avenue to feasibly engineer microbial consortia with optimized functions. In the future, these principles can be used to build models to ultimately inform and predict the symbiosis of algae and microbial communities in response to a wide range of variable environments, ultimately informing biomass production processes engaged by these two partners.

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Table 1. Outdoor algal growth and culture conditions.

Cycle	D1		D2		S3		S4		Temperature	
	μ^a	NH ₃	μ	NH ₃	μ	NH ₃	μ	NH ₃	low	high
R1	0.78	0.001 ^b (0.007 ^c)	0.82	0.001 (0.008)	0.83	0.002 (0.011)	0.87	0.001 (0.007)	22.0 ^d (21.0 ^f)	30.0 ^e (30.2 ^g)
R2	0.58	0.008 (0.036)	0.49	0.008 (0.055)	0.56	0.006 (0.024)	0.68	0.004 (0.018)	19.8 (19.0)	29.0 (29.8)
R3	0.88	0.002 (0.012)	0.84	0.003 (0.014)	0.82	0.003 (0.008)	0.78	0.001 (0.006)	18.7 (18.2)	27.1 (28.5)
R4	0.68	0.001 (0.014)	0.71	0.001 (0.005)	0.68	0.001 (0.007)	0.70	0.002 (0.009)	18.3 (16.0)	26.6 (28.5)
R5	0.74	0.008 (0.014)	0.72	0.002 (0.017)	0.73	0.002 (0.013)	0.72	0.001 (0.001)	17.9 (15.2)	25.8 (28.8)

^a μ is growth rate calculated accordingly [55].

^b NH₃ is free ammonia mean value in ppm (recorded by Seneye monitor).

^c highest free ammonia concentration reported in ppm (recorded by Seneye monitor).

^{d, e} average daily high and low temperatures, respectively (°C)

^{f, g} lowest and highest recorded temperatures, respectively (°C)

Figures

Fig1.

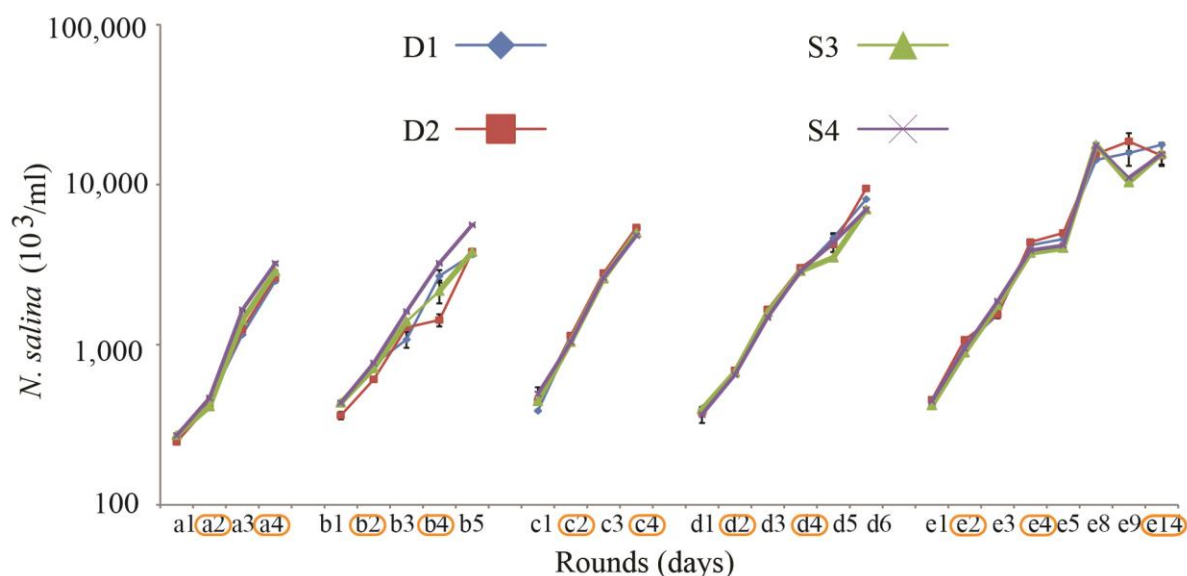


Fig1. Time series of algal culture growth determined by cell densities with respect to passage rounds. Growth curves of duplicated *N. salina* cultures, separately seeded with D- or with S-treatment microbiota, were displayed along each passage rounds (denoted by a~e) and days (1~14). Total DNAs were extracted at day 2, 4, or day 14 marked with yellow oval boxes.

Fig2.

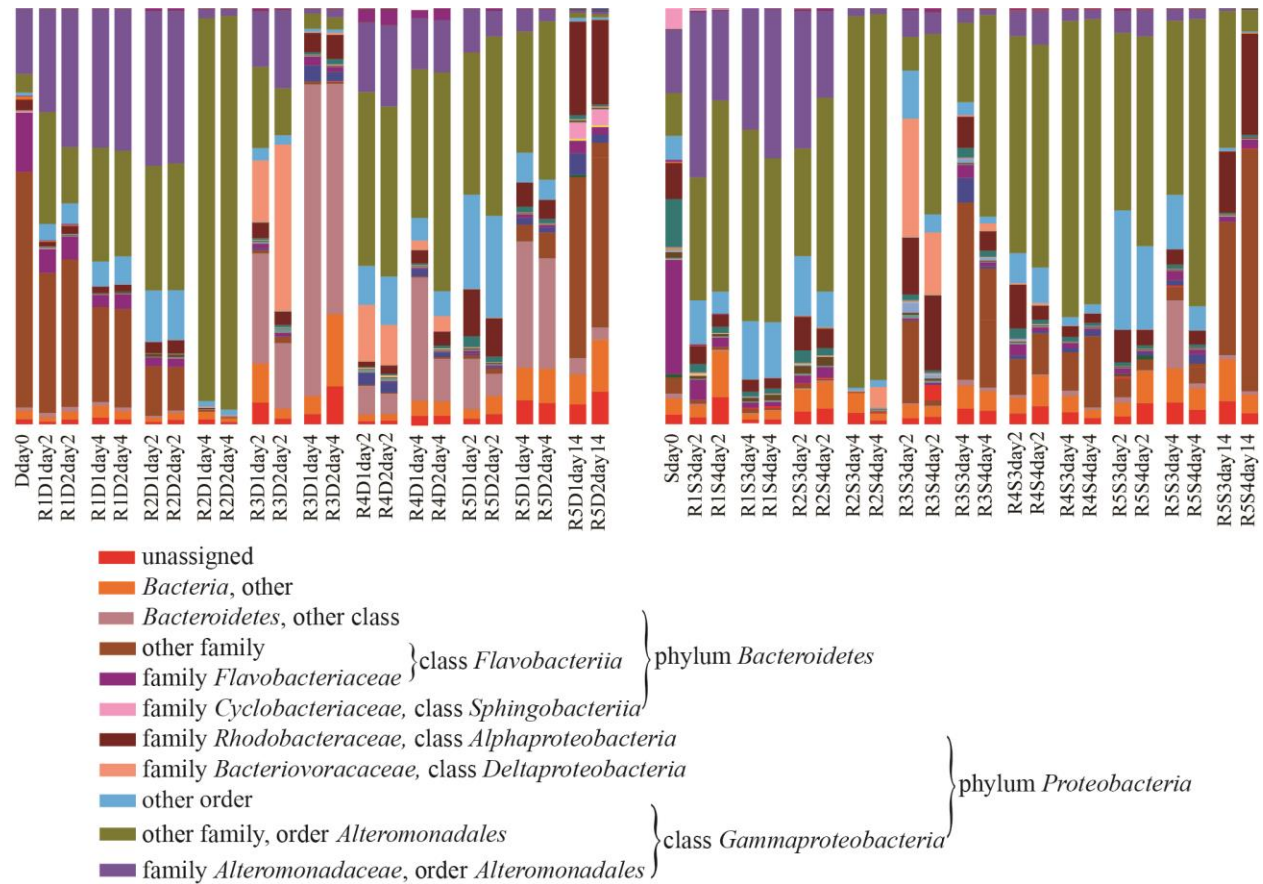


Fig2. Family-level taxonomic compositions for all algal microbiota in the study. Samples are grouped per founder community. Direct environmental sample microbiota (ES), starting domestic (Dday0) and S-treated (Sday0) microbiota, and individual microbiota in respective passage rounds (R) and culture days are labeled (i.e., R1D1day2 is Round 1 Domestic1 day 2 cohort). Replicate samples are grouped together. Taxonomic affiliations of OTUs were determined at the family level (see legend colors) using the Greengenes database [22].

Fig3.

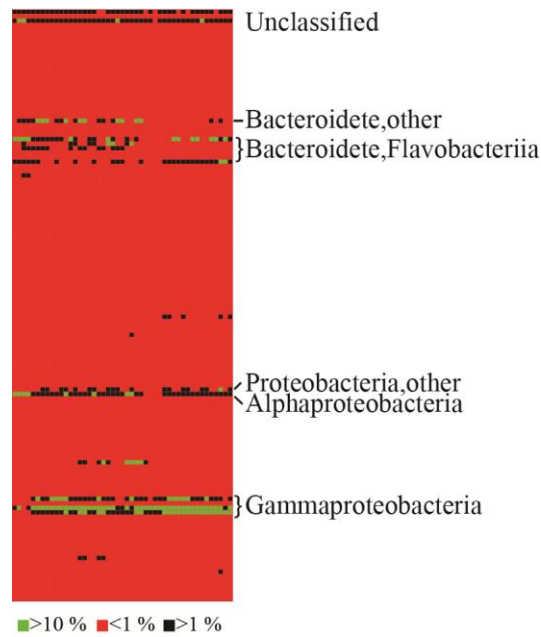


Fig3. Heat map diagram of the relative abundance of bacterial families from the time-course study of algal culture-associated microbial communities. Each column represents unique OTU and each row represents a microbiota sample. Taxonomic affiliations of OTUs were determined at the family level and the relative abundances normalized to generate the distribution map, classifying OTUs as high abundance (> 10%; green), medium abundance (> 1%, <10 %; black), and low abundance (< 1%, red).

Fig4.

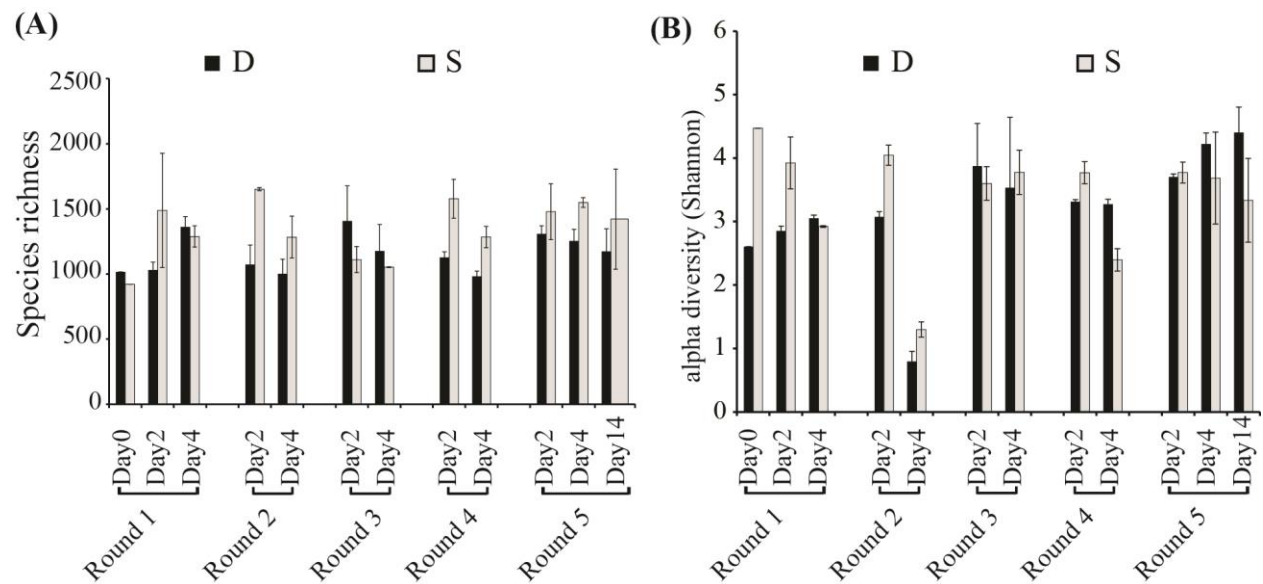


Fig4. Phylogenetic diversity decrease sharply in cultures that experienced environmental disturbance. (A) Average of estimated species richness within domestic (D, black bars) and Santa Cruz (S)-treated microbiota (gray bars) pairs over time. Error bars indicate standard deviations of each replicate. (B) Average of species diversity within Domestic and S-treated microbiota.

Fig5.

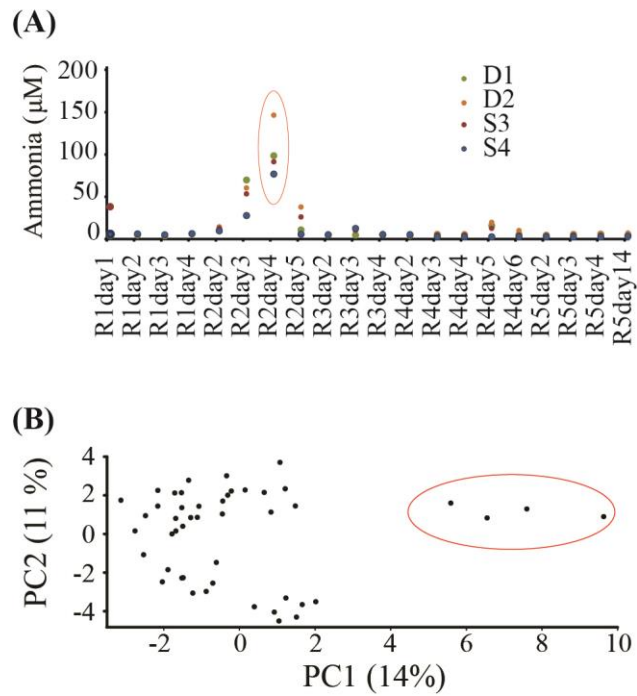
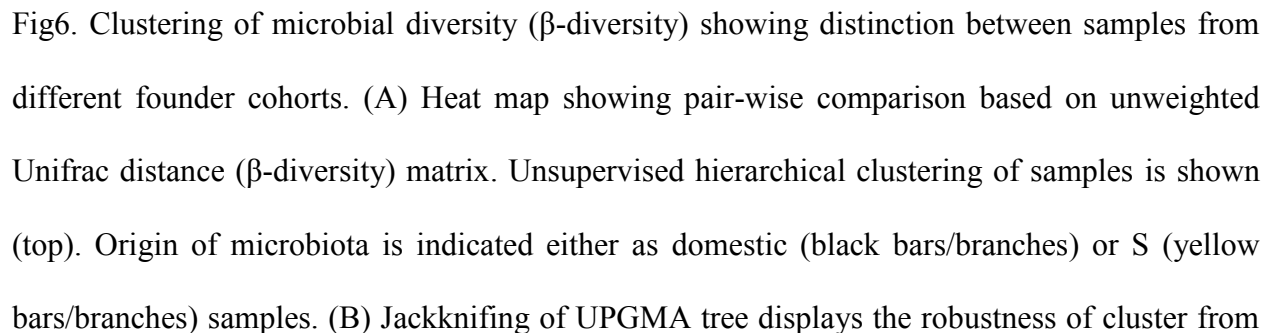


Fig5. Segregated cluster of microbiota is correlated with ammonia-perturbed samples. (A) Profiles of total ammonia during the course of open algal cultures. (B) The first two axes of the PCA ordination of the unweighted UniFrac matrix derived from microbial community. Data points from perturbed samples are encircled (red).

Fig6.



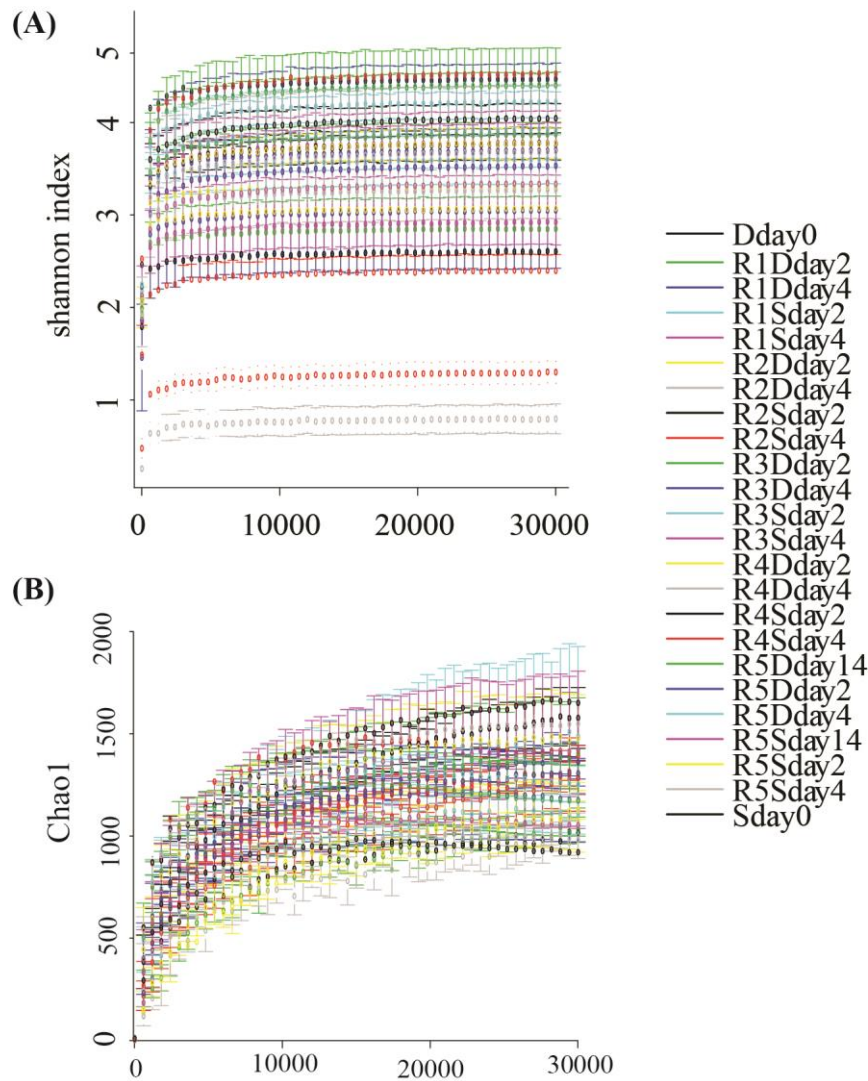
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597 domestic versus S samples. Bootstrap values higher than 0.50 for a total of 22,500 sequences per
598 sample iterated 1,000 times are shown at the nodes of the tree, indicating percentage of
599 jackknifed data supports the node. The outgroup samples in the tree are the ammonia-perturbed
600 samples from round 2.

601

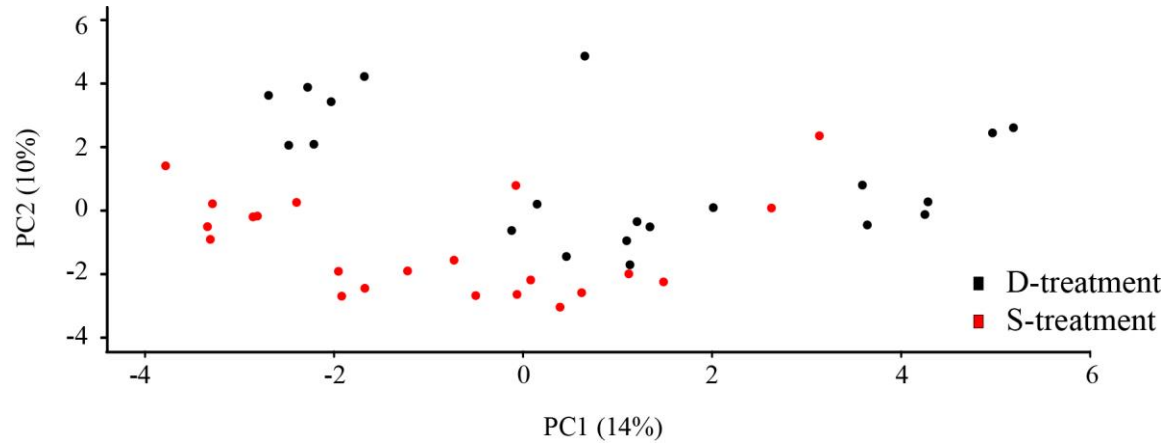
Supplementary Figures:

FigS1.



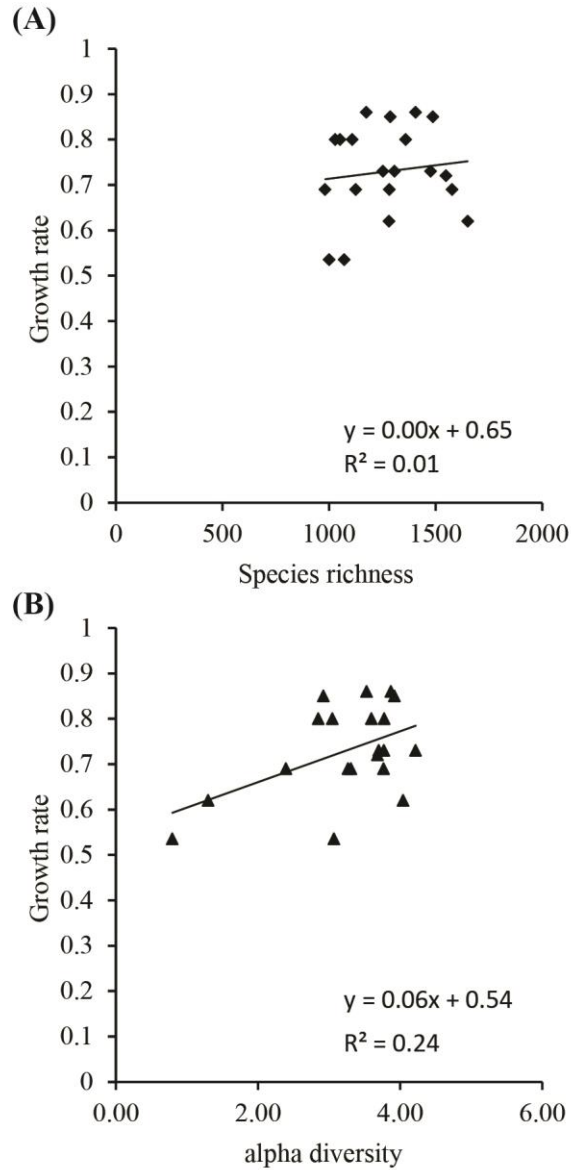
FigS1. Rarefaction analysis comparing microbial community of species richness (Chao1) and diversity (Shannon index) in 16S libraries from algal microbiota. Error bars depicted standard deviations during iterative resamplings from each duplicated samples.

FigS2.



FigS2. A PCA plot based on unweighted Unifrac distance generally, with few exceptions, separates two groups of microbial community. Total ammonia-disturbed samples in passage 2 day4 were excluded in this case to allow environmental conditions in those samples to be comparable. The origins of samples were indicated by colors (see legend).

FigS3.



FigS3. The comparison of algal growth rate as a function of species richness (A) and as a function of population diversity (B). Algal growth rate was significantly associated with microbial community diversity ($P < 0.05$).

Figure 1

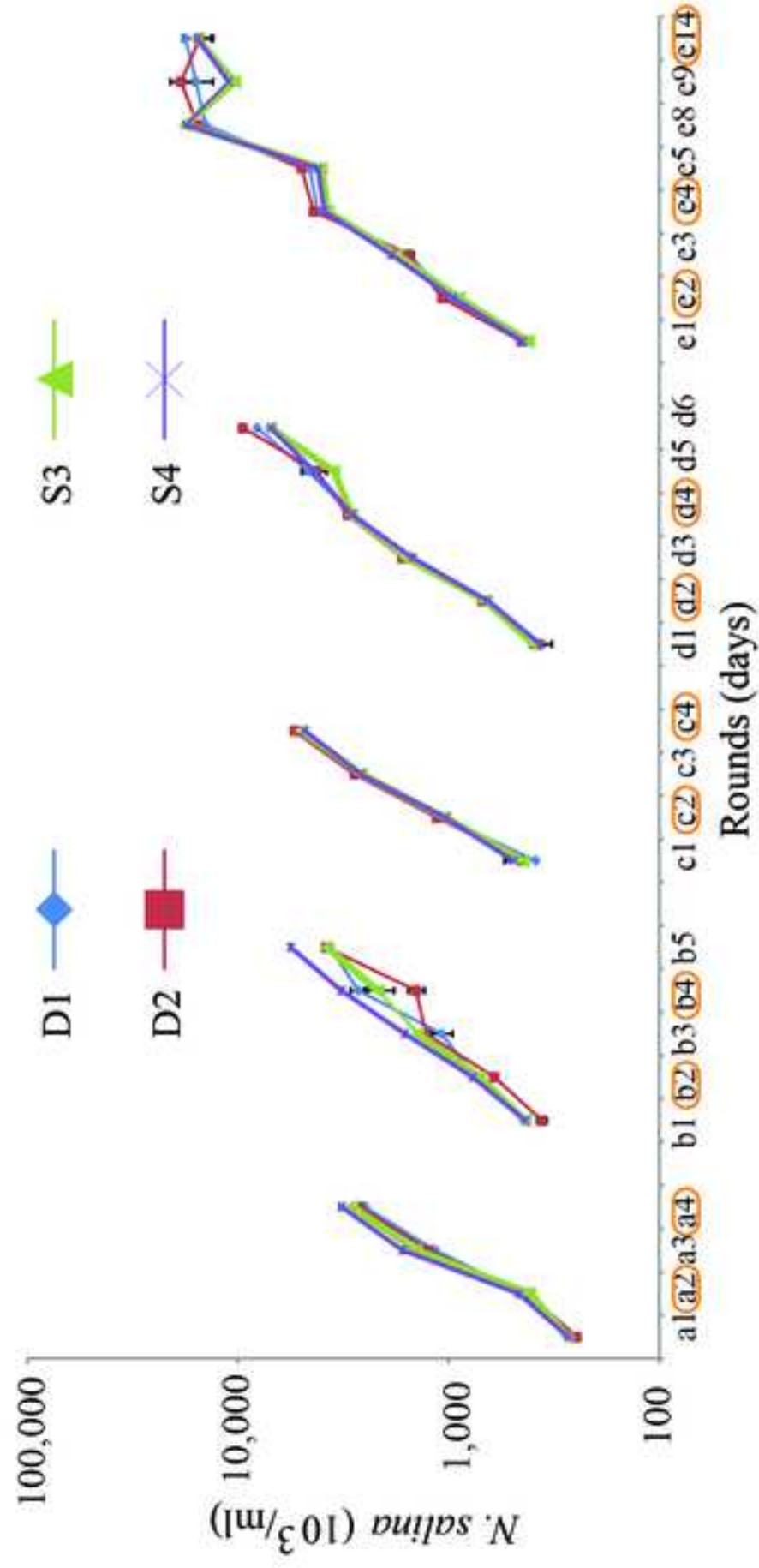
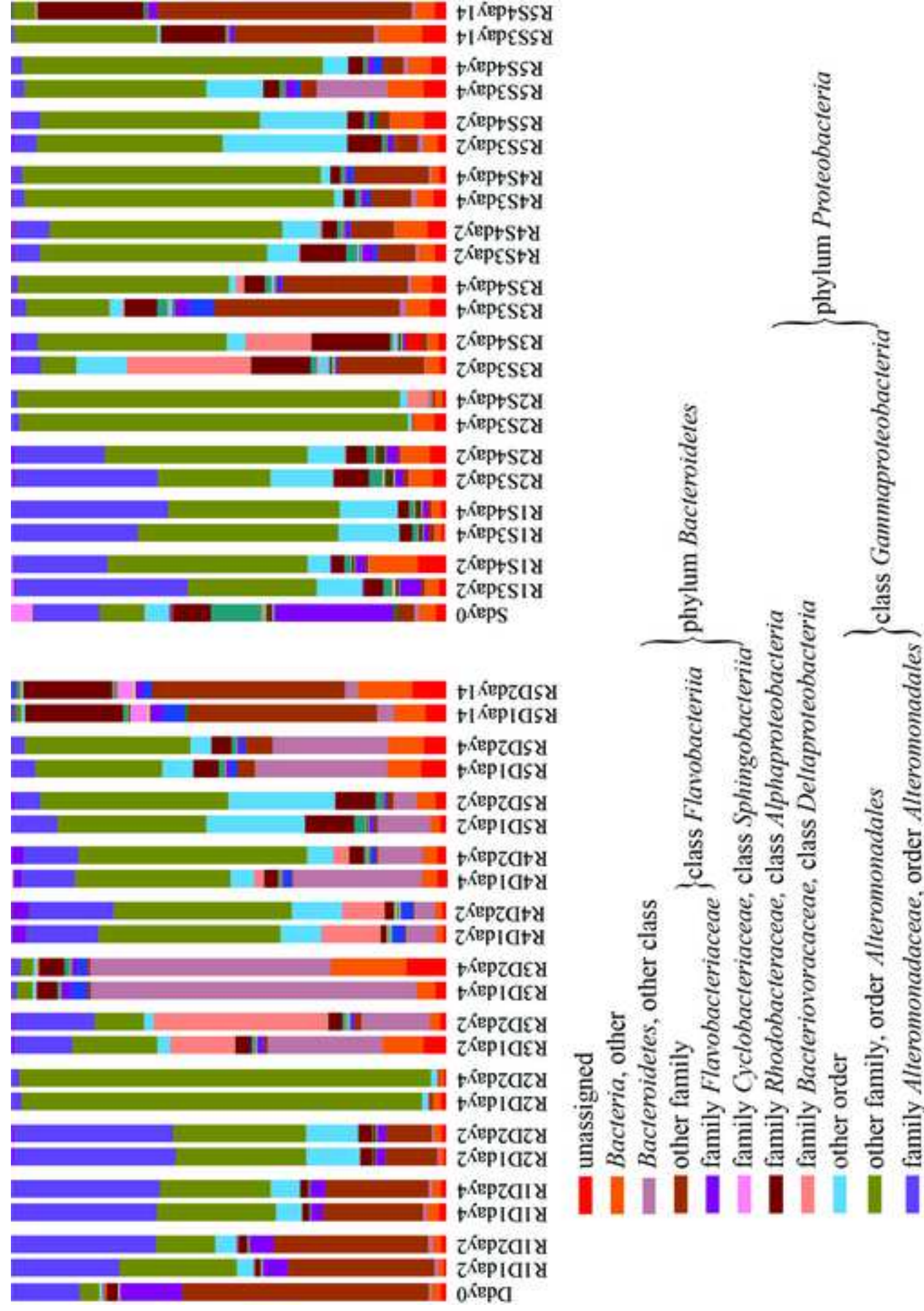
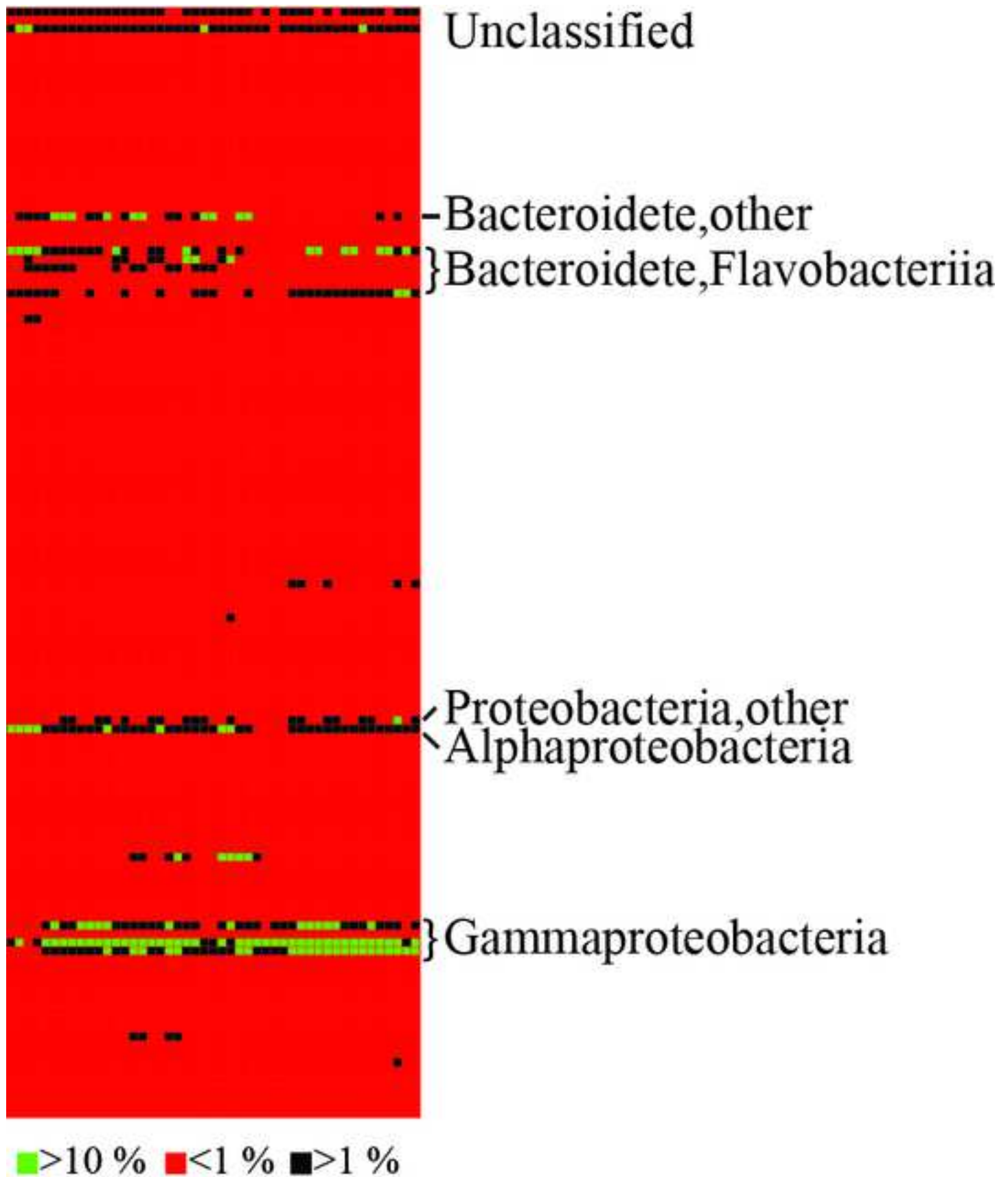


Figure 2





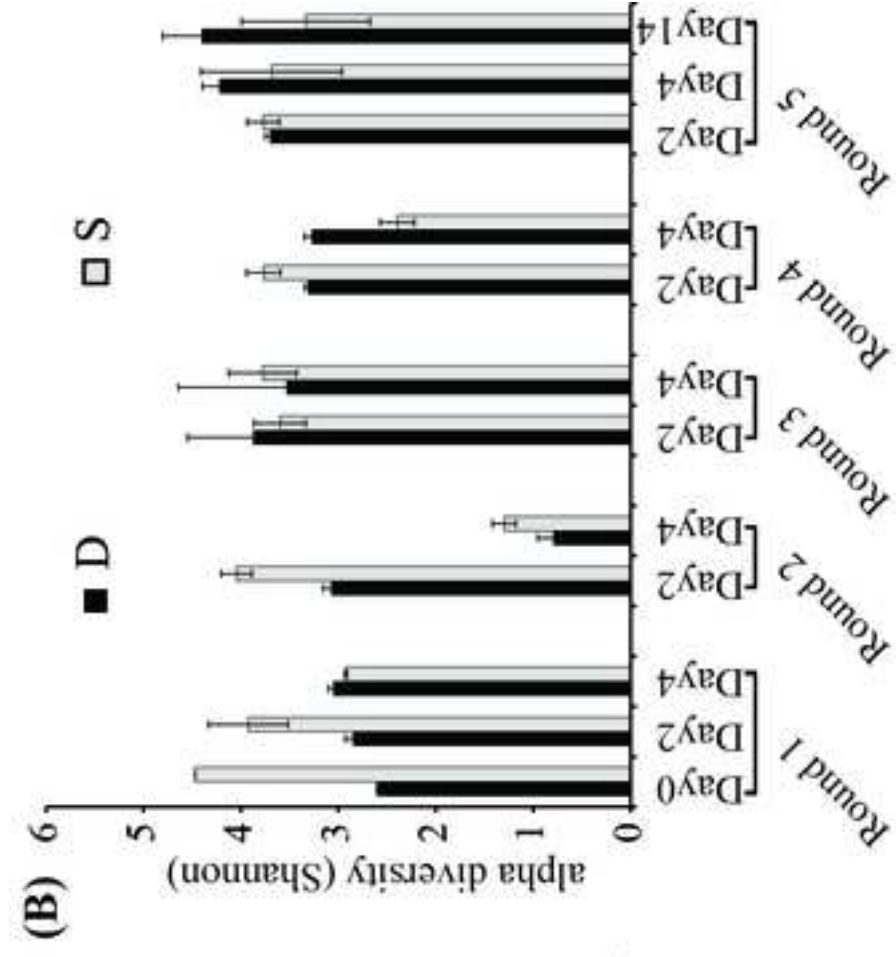
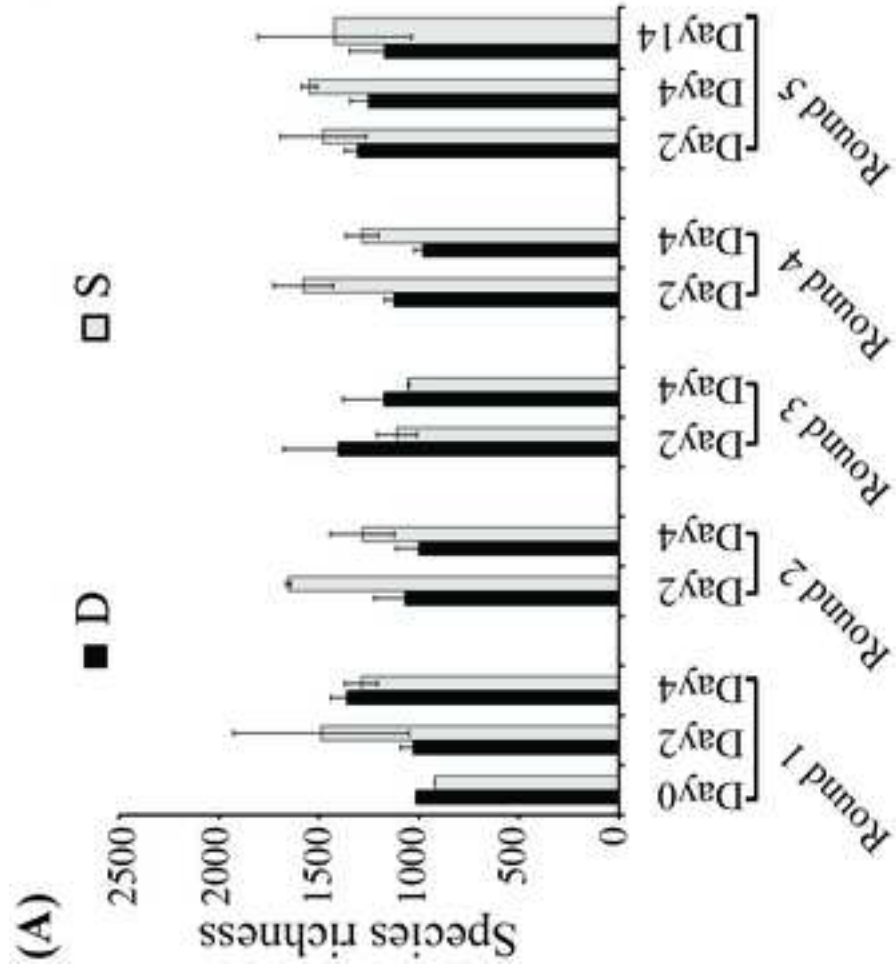


Figure 5

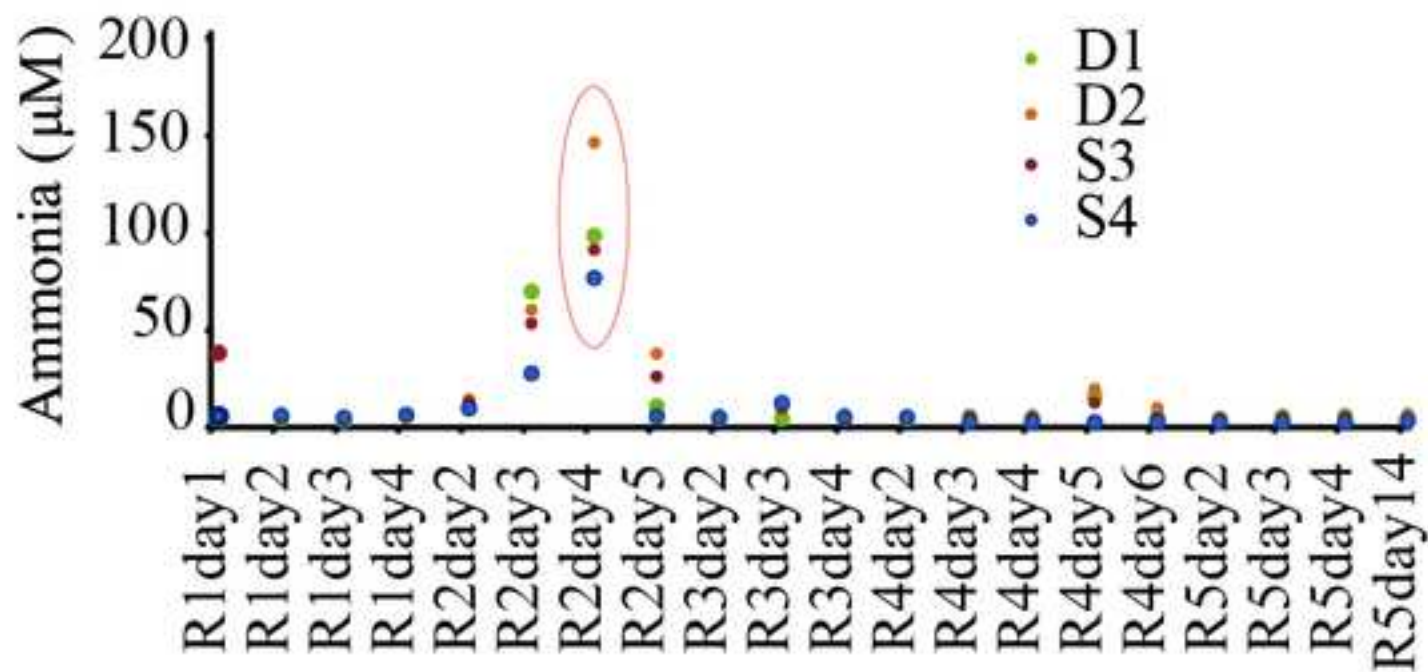
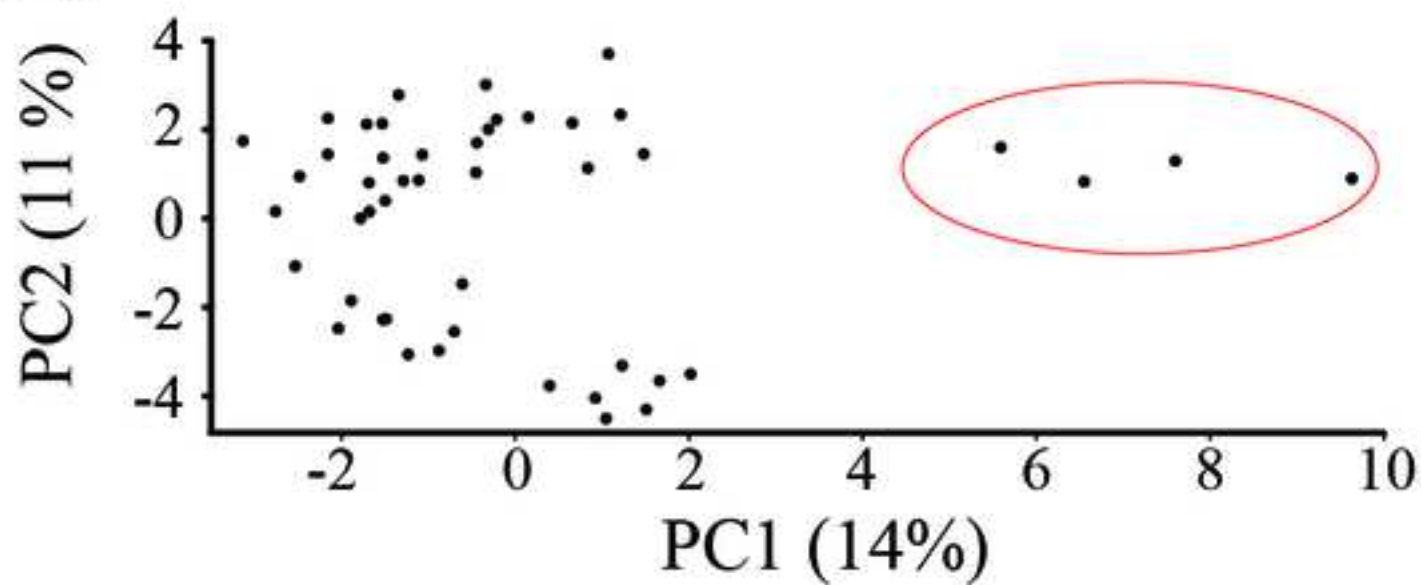
[Click here to download Figure Fig. 5.jpg](#)**(A)****(B)**

Table 1. Outdoor algal growth and culture conditions.

Cycle	D1		D2		S3		S4		Temperature	
	μ^a	NH ₃	μ	NH ₃	μ	NH ₃	μ	NH ₃	low	high
R1	0.78	0.001 ^b (0.007 ^c)	0.82	0.001 (0.008)	0.83	0.002 (0.011)	0.87	0.001 (0.007)	22.0 ^d (21.0 ^f)	30.0 ^e (30.2 ^g)
R2	0.58	0.008 (0.036)	0.49	0.008 (0.055)	0.56	0.006 (0.024)	0.68	0.004 (0.018)	19.8 (19.0)	29.0 (29.8)
R3	0.88	0.002 (0.012)	0.84	0.003 (0.014)	0.82	0.003 (0.008)	0.78	0.001 (0.006)	18.7 (18.2)	27.1 (28.5)
R4	0.68	0.001 (0.014)	0.71	0.001 (0.005)	0.68	0.001 (0.007)	0.70	0.002 (0.009)	18.3 (16.0)	26.6 (28.5)
R5	0.74	0.008 (0.014)	0.72	0.002 (0.017)	0.73	0.002 (0.013)	0.72	0.001 (0.001)	17.9 (15.2)	25.8 (28.8)

^a μ is growth rate calculated accordingly [1].

^b NH₃ is free ammonia mean value in ppm (recorded by Seneye monitor).

^c highest free ammonia concentration reported in ppm (recorded by Seneye monitor).

^{d, e} average daily high and low temperatures, respectively (°C)

^{f, g} lowest and highest recorded temperatures, respectively (°C)

1. Wood AM, Everroad RC, Wingard LM (2005) Measuring growth rates in microalgal cultures. In: Anderson, RA (ed.) Algal culturing techniques. Elsevier Academic Press, Burlington, Mass., pp. 269-286