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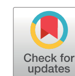
Host selection and stochastic effects influence bacterial community assembly on the microalgal phycosphere



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ABSTRACT

Microalgae have major functions in global biogeochemical cycles and are promising sources of renewable energy, yet the relationships between algal hosts and their associated microbiomes remain relatively underexplored. Understanding community organization of microalgal microbiomes, such as how algal species identity influences bacterial community structure, will aid in efforts to engineer more efficient phototrophic ecosystems. Here, we examined the community assembly of phycosphere-associated (attached) and free-living bacterial taxa associated with two marine microalgae: the diatom *Phaeodactylum tricornutum* and eustigmatophyte *Microchloropsis salina*. Samples were collected from outdoor mesocosms, raceway ponds, and laboratory enrichments, and bacterial taxa identified by 16S rRNA gene sequences. In outdoor mesocosms, we found distinct bacterial taxa associated with each algal species, including the Cytophagaceae and Rhodobacteraceae families with *P. tricornutum*, and Rhodobacteraceae, Hyphomonadaceae, and Saprospiraceae with *M. salina*. Additionally, there were host-specific differences in the bacterial genera associated with the phycosphere, including *Novosphingobium* and *Rhodopirellula* with *P. tricornutum*, and *Methylophaga* and *Dyadobacter* with *M. salina*. Bacterial communities from outdoor monoalgal *P. tricornutum* and polyalgal *P. tricornutum/M. salina* samples were used as inocula for laboratory enrichments with axenic *P. tricornutum*. Here, similar bacterial communities emerged, suggesting that the algal host exerts substantial influence over bacterial community assembly. Further enrichments for phycosphere-association revealed differing outcomes of community assembly processes contingent on the initial community composition. Phycosphere-associated communities from monoalgal *P. tricornutum* mesocosms were highly similar to one another, suggesting deterministic processes, whereas cultures from mixed *M. salina/P. tricornutum* raceways followed two apparent paths differentiated by the stochastic loss of specific community members and convergence towards or further deviation from the monoalgal samples. These results demonstrate that algal-associated bacterial communities are controlled by algal host, culture conditions, and the initial inoculum composition of the algal microbiome, and this knowledge can inform the engineering of more productive algal systems.

1. Introduction

Microalgae and their microbial partnerships are critical to global productivity in the biosphere, responsible for half of global O_2 production and sustaining the energy requirements of aquatic ecosystems from the tropics to the poles [1,2]. For humans, microalgae can be deleterious through the formation of toxic blooms that result in negative health and economic consequences [3], or beneficial through the production of sustainable biofuels and bioproducts [4] and renewable sources of food through aquaculture. In both natural (lakes, rivers, and oceans) and engineered (algal ponds, photobioreactors) systems, microalgae interact with one another and with surrounding organisms, such as heterotrophic bacteria, through a multitude of mechanisms and interfaces. These can include physical contact between bacteria and algae or through the exchange of dissolved compounds, both of which mediate the bilateral transfer of organic and inorganic chemicals that function in signaling and nutrient acquisition [5,6]. The relationship between algae and their surrounding environment, including other cells of the same species, is regulated to a large extent by the extracellular space surrounding each algal cell, a barrier region known as the phycosphere that limits the diffusion of algal-derived organic matter and inorganic nutrients [7]. Through the phycosphere, algae are chemically connected with their external environment for uptake of nutrients, release of waste, and secretion and sensing of signal compounds to and from symbiotic partners as well as competing organisms. Similarly, the chemical gradient of algal-derived organic matter that is more concentrated at the algal surface attracts chemotactic bacteria towards productive microalgal cells, leading to potentially favorable physical attachment of bacteria to the algal surface [8].

Molecular analysis, especially rRNA gene sequencing, is beginning

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to address some long-standing questions of how microalgal species influence their surrounding bacterial communities [9]. In general, aquatic microalgae produce organic matter that appears to shape the associated microbial communities [10–13], and algal blooms dominated by high biomass of one or a few algal species usually have less diverse bacterial communities than those with multiple algal species [14–16]. A limited number of studies have contrasted the diversity of free-living bacteria versus those specifically associated with the algal phycosphere [17–19] or more generally found attached to particles during algal blooms [20,21]. On limnic and marine particles, some studies have found a large overlap of bacterial taxa in the attached and free-living fractions [22], while other studies have shown that surface water particles promote a strong partitioning of attached and free-living bacterial taxa [23]. In a freshwater lake, a clear difference between free and algal-attached bacteria was found, suggesting that algal species in part determine the composition of attached bacteria [24]. Once cultivated, however, the algae exhibited similar free and attached bacterial communities, suggesting that laboratory conditions favoring high algal biomass promote the attached bacteria to also dominate the free-living community [24]. This trend is not widespread; some species, including the marine diatom *Pseudo-nitzschia*, associate with distinctly different bacterial community members depending on the growth conditions [25], while the picophytoplankton *Ostreococcus* appears to interact specifically with bacteria of the *Marinobacter* genus regardless of conditions [26]. Thus, alterations in bacterial community composition appear to be predominantly influenced by both *in situ* environmental settings and the identity of microalgal species, but assessing their relative contributions has remained elusive.

These variable and sometimes circumstantial results led us to study how heterotrophic bacterial community structure was influenced by two algal species cultured under a variety of conditions. First, we examined the bacterial communities associated with outdoor mono- and polyalgal mesocosms and raceway ponds to determine the taxonomic composition of algal-attached and free-living bacteria incubated under natural sunlight and outdoor mass culture conditions. Second, we inoculated bacterial communities from a subset of the outdoor ponds into previously axenic *P. tricornutum* laboratory cultures to enrich for algal-exudate-utilizing bacteria and assess the similarities and differences in the emerging communities from differing source inocula. Third, we performed repeated passaging of algae and attached bacteria to exert a selective bottleneck for physical attachment. Through this process of enriching bacteria from different outdoor source ponds for phycosphere attachment, we predicted that we would obtain simplified yet convergent bacterial communities primarily selected for by the host alga. Our experiments were stimulated by the need to understand the factors that control bacterial community assembly in the presence of specific microalgal species, as one of our long-term goals is to modify bacterial communities to obtain more robust microalgal growth for biofuels and bioproducts. Although this study did not aim to understand the ecological roles of specific bacterial taxa associated with microalgae, once specific mutualistic bacteria are identified, the factors that lead to their establishment in long-term cultures can be better understood or at least partially constrained.

As the phycosphere is defined by the diffusion gradient of solutes, its bounds are variable for different chemical compounds [27] and it is therefore difficult to constrain the phycosphere to an exact region or distance from an algal cell. In this study we classified phycosphere-associated bacteria as those that were algal-attached, as by definition these bacteria were living in the phycosphere. Some bacterial taxa that do not attach to the algal cells likely have the capability to live close to the algal cells and would be technically living in the phycosphere, thus our analyses are conservative. Furthermore, algal attachment may be a transient process (attachment followed by detachment, or loose attachment) and taxa exhibiting attachment likely have an unattached contingent at any given time.

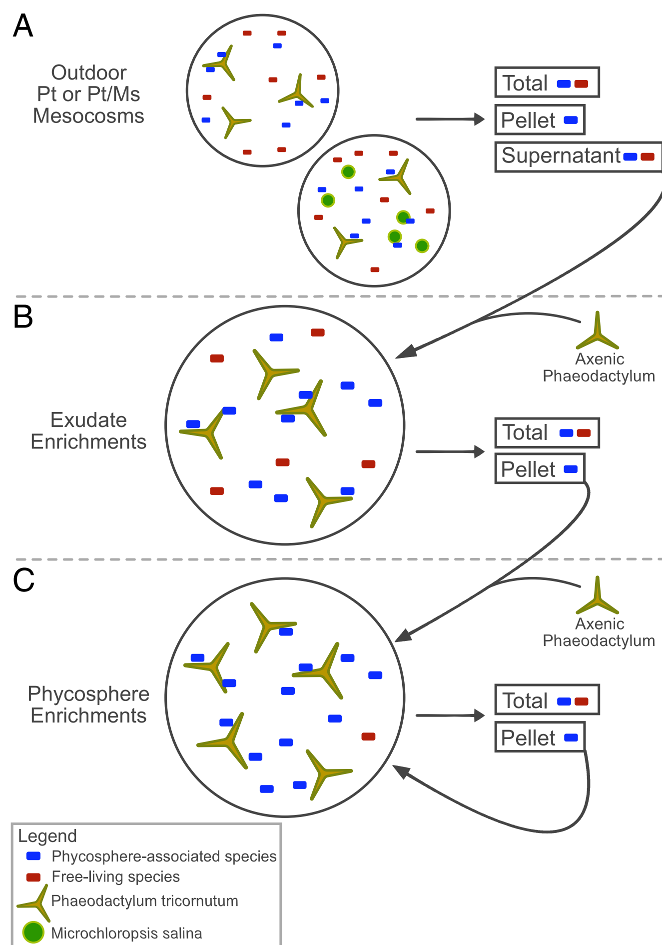

Fig. 1. Sampling workflow for the outdoor mesocosm and indoor enrichment cultures. Biomass was harvested by different methods to isolate different fractions; filtration onto a 0.2 μm filter to isolate the microalgae and prokaryotes (the “Total” fraction), and centrifugation to isolate the microalgae and attached bacteria (the “Pellet” fraction) and the free-living community (the “Supernatant” fraction). Note that individuals from species that are generally phycosphere-associated (blue squares) may also be found unattached in the supernatant fraction as well. A) Outdoor monoalgal mesocosms with *P. tricornutum* (Pt) or polyalgal mesocosms with Pt and *Microchloropsis salina* (Ms). All three fractions were obtained and sequenced. B) The Exudate Enrichments began with the outdoor mesocosm Supernatant fractions (consisting of both free-living and phycosphere-associated species) that were then added to axenic Pt cells to enrich for community members able to subsist solely on Pt-derived exudates. Samples were fractionated into both a Total (used for sequencing) and Pellet (used for the Phycosphere Enrichments) fractions. C) The Phycosphere Enrichments began with the Exudate Enrichment Pellet fractions added to axenic Pt cells. These enrichments underwent several iterations to further select for phycosphere-association by collecting and washing the Pellet fraction to remove unattached community members followed by culturing with fresh media. After several rounds, the Total fraction was obtained for sequencing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Algal strains, source communities, and laboratory growth conditions

Fourteen outdoor samples were obtained from one of two different sources: (1) 100-L outdoor *M. salina* (CCMP 1776, formerly *Nannochloropsis salina* [28]) or *P. tricornutum* (“Flour Bluff” isolate) monoalgal mesocosms cultivated for 7 days in Corpus Christi, TX using natural seawater, and (2) 557-L outdoor *M. salina* and *P. tricornutum* polyalgal raceway ponds cultivated and diluted for 21 days using

natural, diatomaceous earth filtered seawater from Laguna Madre, Corpus Christi, TX. Both sets of outdoor cultures were amended with 2.0 mM NH_4Cl, 2.0 mM pH balanced H_3PO_4, and 0.07 mM FeSO4. We collected 3 samples each from single *P. tricornutum* and *M. salina* monoalgal mesocosms (for each species, two replicates from December 13, 2014 and one replicate from February 11, 2015), and 2 duplicate samples from 4 polyalgal raceways collected on January 28, 2015, resulting in 8 (Fig. 1A). Monoalgal and polyalgal outdoor samples were collected on a 0.2 μm filter to obtain the total bacterial communities, which includes both free-living and algal-attached cells. Additional monoalgal outdoor samples were size fractionated: the samples were centrifuged (3000 $\times g$ for *P. tricornutum* and 5500 $\times g$ for *M. salina*) and the pellet and supernatant were split, the former including algal-attached bacteria and the latter (after filtration on 0.2 μm polycarbonate filters) mostly free-living bacteria, though some algal cells remained in the supernatant.

For the laboratory incubations, the axenic marine diatom *Phaeodactylum tricornutum* CCMP 2561 was acquired from the National Center for Marine Algae and Microbiota (NCMA; ncma.bigelow.org). Cultures were maintained in *f*/2 media using seawater prepared from commercially available sea salts (Instant Ocean, Blacksburg, VA) at 12 h light/dark cycles (PAR irradiance 4395–5860 $W\,m^{-2}$, cool white fluorescent bulbs; temperature 20–22 °C). Throughout the experiments, control axenic cultures were maintained for identification of algal-derived sequences (mitochondria and chloroplasts).

Bacterial filtrates (0.6–1 μm pore size, removing the larger algal cells) were collected from two sources, the outdoor *P. tricornutum* monoalgal mesocosms and the polyalgal raceways, and were used as inoculants into axenic *P. tricornutum* cultures (Fig. 1B). These two “exudate” enrichments consisting of *P. tricornutum* with one of two different source microbial communities were incubated in 13 × 100 mm glass vials and transferred 5 times every 2 weeks before sample collection for sequencing. This approach ensured that enough time passed to enable the bacterial communities in the enrichments to adjust and reflect members that were subsisting on algal-derived organic matter and not on organic matter transferred from the original sources [29].

Next, “phycosphere” enrichments were initiated to obtain phycosphere-associated bacteria by centrifuging the “exudate” samples at 3000 $\times g$, removing the supernatant and resuspending in sterile medium a total of 3 times, in order to remove free-living bacteria and enrich for algal-attached bacteria. These samples, which included algal cells and any attached bacteria, were diluted to transfer from 200 to 2000 algal cells into 200 μl wells containing ~500 axenic *P. tricornutum* cells, creating a total of eleven enrichments (5 from the monoalgal “exudate” community, and 6 from the polyalgal “exudate” community) (Fig. 1C). Due to the large number of smaller scale lab enrichments, both the “exudate” and “phycosphere” enriched samples were fractionated as pellets comprised of algal-attached bacteria, and whole samples filtered on 0.2 μm filters to obtain total cells consisting of algae and both attached and free-living bacteria.

2.2. Sequencing and analysis

DNA was extracted from all frozen samples (filters or pellets) with the DNeasy kit (Qiagen) with 2 added steps: a 10 minute initial lysozyme incubation at RT and a 15 second bead beading step in lysing matrix tubes after the Qiagen AL buffer incubation. Samples were amplified with the V4 16S rRNA gene primer set (515F-806R; [30]) and sequenced on an Illumina MiSeq with barcoding (NCBI BioProject PRJNA390149). Ribosomal amplicon sequence variants (ASVs) were determined using DADA2 version 0.9.5 [31] as follows: 16S rRNA reads were quality-filtered (parameters maxN = 0, maxEE = 2, truncQ = 2) and trimmed from both the 5′ and 3′ to nucleotide positions 10–230 (Forward) and 10–150 (Reverse). Read pairs were then denoised based on a DADA2 error model, merged, and *de novo* chimeras were removed. The output of the DADA2 algorithm is an ASV table (functionally analogous to an “OTU table”) and final ASV sequences. Importantly, ASVs are not the result of a strict clustering cut-off as are OTUs, and therefore have a higher resolution to distinguish between closely related strains [32].

The ASV sequences were then aligned with Muscle version 3.8.31 [33], and a maximum-likelihood phylogenetic tree was generated using FastTree version 2.1.9 [34]. Taxonomy of ASVs was assigned using RDPtools and RDP Release 11.4 [35]. Sequence analysis was done in R [36] primarily using Phyloseq 1.19.1 [37]. Samples with < 500 total reads were removed. ASVs assigned either to the “Chloroplast” taxonomic class or identified as algal mitochondrial sequences were removed, as well as ASVs not counted at least 5 times in at least one sample. A mean of 189,615 (range of 53,487–400,432) read pairs were sequenced per sample, with a mean of 155,959 (range of 23,548–363,657) per sample retained after quality-filtering, and a mean of 65,592 (range of 3448–310,503) per sample retained after chloroplast removal. Phyloseq 1.19.1 was used to calculate Shannon's Evenness, Jensen-Shannon Distances (JSD), and to visualize distances using Non-metric Multidimensional Scaling (NMDS) or Principal Coordinate Analysis (PCoA). PERMANOVA was calculated using the Vegan 2.5.1 R package [38].

3. Results

3.1. Bacterial communities in outdoor ponds

We analyzed the structure of *P. tricornutum* and *M. salina* microbial communities in outdoor monoalgal mesocosms, and found the microbiomes of both algal species were primarily comprised of Proteobacteria followed by Bacteroidetes (Fig. 2A). At the family level, however, the Rhodobacteraceae were dominant in the *P. tricornutum* mesocosms, while there was a more even distribution of families in the *M. salina* mesocosms. Despite the proximity of these two mesocosms and identical initial culture media and environmental conditions, the differing distribution of species suggests these algae created distinct conditions that selected for different microbial communities.

Microalgae, particularly those that are from different lineages, are often grown together in polyalgal cultures in order to increase pond stability and biomass production [39]. We obtained duplicate samples of the total microbial communities associated with mixed *P. tricornutum* and *M. salina* in four parallel outdoor raceway ponds. Similar to the monoalgal mesocosms, the phylum distribution of the bacterial communities in the mixed-species raceways were primarily Proteobacteria and Bacteroidetes, with $< 1\%$ of the reads from other phyla (replicates from a representative raceway are shown in Fig. 2A). Despite the coarse taxonomic similarities, the polyalgal ponds were distinct from the monoalgal mesocosms with several unique taxa present rather than simply a mixture of the two monoalgal communities. One family present only in the polyalgal ponds was the Flavobacteriaceae at an average relative abundance of $> 40\%$ in all pond samples. The sequences were primarily from the *Maribacter*, *Polaribacter*, *Persicivirga*, *Vitellibacter* and *Arenibacter* genera, many of which are members of the Flavobacteriaceae marine clade responsible for a considerable portion of organic matter utilization and remineralization in the world's oceans [40]. Although the four ponds had similar families present, the relative distribution of the Colwelliaceae family was markedly different. This variation was mostly due to a single *Colwellia* 16S rRNA gene amplicon sequence variant (ASV, see Materials and Methods; seq4555) with pond abundances ranging from just 0.3% to 15%. Large abundance ranges of *Colwellia* species have been seen in microbial communities associated with the macroalga *Delisea pulchra*, with *Colwellia* being abundant in diseased tissue, yet absent in healthy tissue [41]. This ASV and other *Colwelliaceae* family members were largely absent from the monoalgal mesocosms. Overall, the bacterial communities from both monoalgal mesocosms were distinct from the polyalgal ponds (p-value = 0.001).

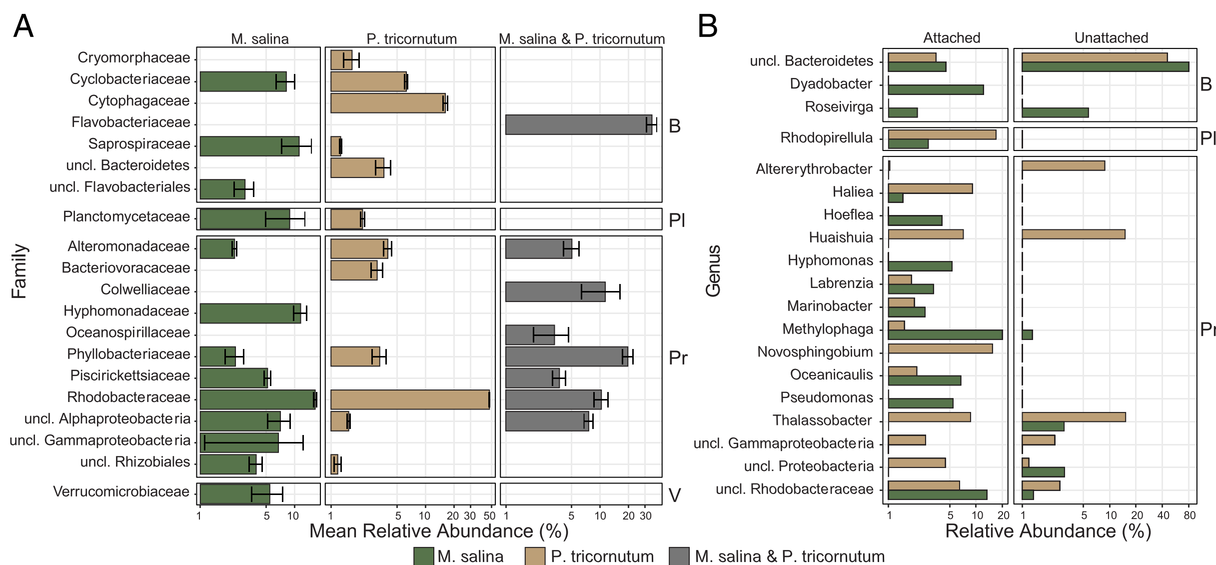

Fig. 2. A) Abundances (relative to the total) of bacterial families in outdoor monoalgal *M. salina* (green), *P. tricornutum* (brown) mesocosms or polyalgal (gray) raceways. Data shown are the mean relative percent abundances of two replicates with standard deviation error bars. B) Relative abundances of genera (found at least > 1% abundance in at least one sample) of the attached and free-living fractions of bacterial communities in *P. tricornutum* (brown) or *M. salina* (green) algal mesocosms. Families and genera are grouped on the right by phylum (B, Bacteroidetes; Pl, Planctomycetes; Pr, Proteobacteria; V, Verrucomicrobia). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This could have important consequences as monoalgal ponds frequently become contaminated with other algal species, resulting in microbial communities whose structure and function may not be readily predictable.

We hypothesized that phycosphere-associated bacteria would be phylogenetically distinct from free-living bacteria. To examine the phycosphere-associated bacteria (here operationally defined as those attached to the algal surface), we physically separated the bacterial communities by centrifugation into algal-attached and unattached fractions from both the *P. tricornutum* and *M. salina* monoalgal mesocosm samples. The sequences in the algal-attached fractions were mostly chloroplasts (mean 63% for *M. salina* and 86% for *P. tricornutum*, respectively) compared to the unattached (mean 0.1% for *M. salina* and 1.4% for *P. tricornutum*, respectively), and accordingly the read depth of the chloroplast-removed samples was lower than the free-living fraction. For both algal hosts, the unattached fractions had a higher number of observed ASVs (means of 299.5 and 274.0 for *P. tricornutum* and *M. salina*, respectively) than the algal-associated communities (means of 142.5 and 128.5 for *P. tricornutum* and *M. salina*, respectively). The higher evenness, however, revealed that the ASVs were more evenly distributed in the attached communities (means of 0.684 and 0.663 for *P. tricornutum* and *M. salina*, respectively) than the unattached communities (means of 0.399 and 0.227 for *P. tricornutum* and *M. salina*, respectively). The low evenness of the unattached communities was due to a single highly-abundant ASV best classified as an uncultivated Bacteroidetes (seq79; Fig. 2B) which appeared to be ecologically successful in the free-living phase of the mesocosms, but its role remains uncharacterized. BLAST analysis revealed low sequence homology to cultivated organisms (best match at 89% identity to *Chryseobacterium*), however, seq79 had 99% identity to an uncultured bacterium found in sponges (NCBI accession KT880429 [42]). Overall, in the outdoor mesocosms the unattached communities from the two algal hosts had higher similarities to each other than did the two algal surface-associated communities (JSD of 0.216 and 0.540, respectively), suggesting a role in host-specific selection of the surface-associated communities from a relatively similar community of free-living bacteria.

3.2. *P. tricornutum* exudate enrichments

Outdoor raceways and mesocosms are open to the environment and thus under constant exposure to invading microbes (*e.g.* bacteria and eukaryotes) that may not all be directly utilizing algal exudates. We were interested in refining the bacterial communities from the outdoor samples to those members able to subsist solely on *P. tricornutum*-derived fixed-carbon. Thus, we generated two exudate enrichments with source bacterial communities from the *P. tricornutum* monoalgal outdoor mesocosms (Exudate Enrichment "M") or the polyalgal outdoor raceways (Exudate Enrichment "P") inoculated into axenic *P. tricornutum* laboratory batch cultures. After several transfers, these exudate enrichments were sampled for community analysis by capturing the total community onto 0.2 μm filters. *P. tricornutum* cell densities were measured at $6.2 \times 10^5\,ml^{-1}$, and bacterial abundances at $2.9 \times 10^6\,ml^{-1}$. Many of the outdoor culture community members were lost during the enrichment process, with only a few ASVs retained in the exudate enrichments (Fig. 3A). An additional 174 and 164 ASVs emerged in Exudate Enrichments M and P, respectively, indicating these community members were below the level of detection in the outdoor cultures, but gained a competitive advantage after being transferred into the laboratory. By sequence abundance, the majority of the outdoor pond ASVs were lost in the exudate enrichments (Fig. 3B). Although relatively few ASVs were retained from the outdoor ponds to the exudate enrichments, these ASVs comprised ~25% of the outdoor culture abundance. In Exudate Enrichment M, these ASVs were further reduced in relative abundance to only 10%, while the retained ASVs in Exudate Enrichment P increased in relative abundance to two-thirds of the total.

Although both exudate enrichments experienced a loss of ASV richness compared to their corresponding outdoor cultures, Exudate Enrichment M exhibited a decrease in phylogenetic diversity (Faith's PD [43], from 16.1 to 9.7), whereas Exudate Enrichment P did not (Faith's PD from 11.2 to 11.5). The stability of phylogenetic diversity for Exudate Enrichment P was surprising given that the source community originated from a polyalgal raceway, while Exudate Enrichment M was cultured with *P. tricornutum* only. For Exudate Enrichment M, many taxonomic families were lost from the mesocosms (Fig. 3C), suggesting either a lack of fitness for growing exclusively on algal exudates or

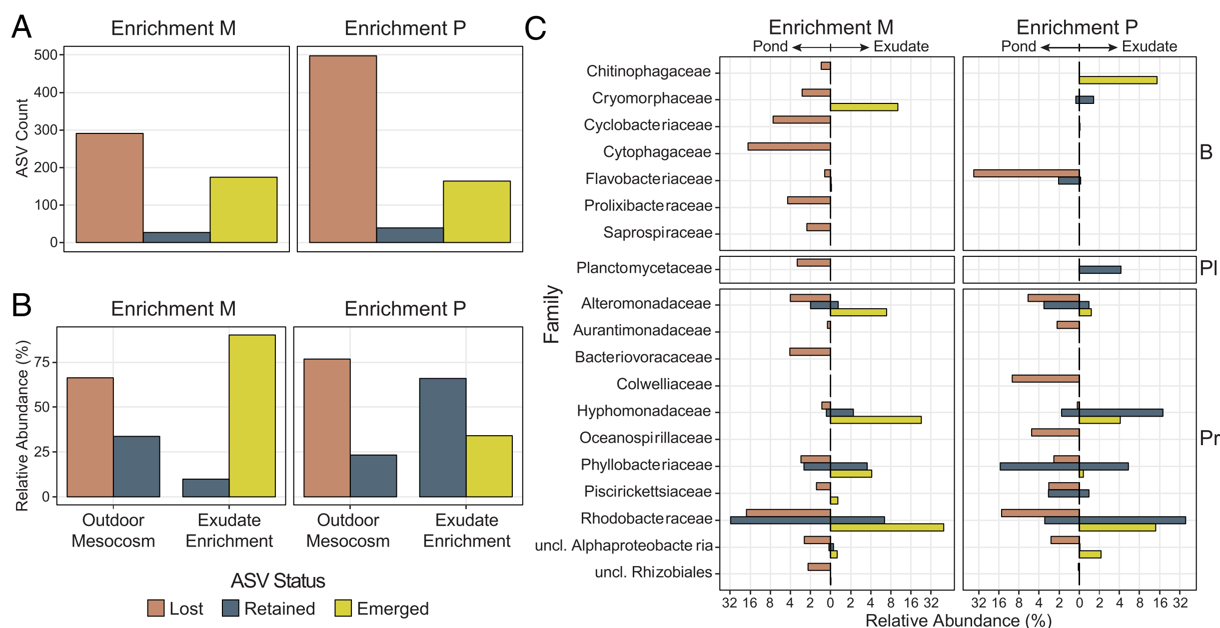

Fig. 3. Bacterial community succession from outdoor samples to exudate laboratory enrichments (source for Exudate Enrichment M was the monoalgal *P. tricornutum* mesocosm, and source for Exudate Enrichment P was the polyalgal *P. tricornutum* and *M. salina* raceway). A) Total counts of unique ASVs grouped by their status as identified only in the source outdoor algal mesocosm (Lost), identified in both the outdoor mesocosm and exudate enrichment (Retained), or identified only in the exudate enrichment (Emerged). B) Percent relative abundance of ASVs, grouped by their status in either the outdoor mesocosm or exudate enrichments. C) ASV status grouped by taxonomic family. Bars to the left of center are relative abundance in the outdoor mesocosm, and to the right of center are relative abundance in the exudate enrichments. Families are grouped on the right by phylum (B, Bacteroidetes; Pl, Planctomycetes; Pr, Proteobacteria).

"bottle effects" providing a disadvantage to potentially slower-growing taxa [44]. From Exudate Enrichment M, these "lost" families included Cytophagaceae, Cyclobacteriaceae, Prolixibacteraceae, Bacteriovoracaceae and Saprospiraceae, and from Exudate Enrichment P Colwelliaceae, Oceanospirallaceae and the Flavobacteriaceae marine clade. Other families exhibited losses of specific genera, but they were replaced by other genera within the same family. For example, in Exudate Enrichment M, the *Dyadobacter* genus (Cryomorphaceae) was relatively abundant in the outdoor ponds but was lost and replaced with an *Owenweeksia* ASV in the exudate enrichment. On occasion, this was seen at the genus level as well, with one *Marinobacter* (seq157) lost and replaced with another emergent *Marinobacter* (seq80) in Exudate Enrichment P. These two sequences were 97.4% identical, and this microdiversity likely represents adaptations of this species to different environments [45].

Another difference between the two exudate enrichments was a shift in the relative abundance of retained taxa (*i.e.* those present in both the original outdoor sample and the laboratory enrichment). Less than 10% of the reads in Exudate Enrichment M were retained ASVs, but Exudate Enrichment P retained ASVs made up 66% of its community (Fig. 3B). The combination of retained and emerged ASVs in the two exudate enrichments led to converging communities from a mean JSD of 0.62 between the outdoor cultures to 0.19 between the indoor exudate enrichments. Exudate enrichments M and P communities shared 92 ASVs (of 201 and 203 total ASVs, respectively), a majority of which emerged during growth in the laboratory (79 and 65 for M and P, respectively) and potentially indicate bacterial taxa able to best adapt to niches specific to *Phaeodactylum* exudates.

Overall, enrichments for microbial communities able to subsist on algal exudates led to a convergent selection of specific taxa, even when starting with different source bacterial inocula. Many of the ASVs were below the level of detection in the outdoor algal ponds yet emerged in the exudate enrichments. The emergence of these taxa from the microbial seed bank of the algal pond may be attributed to environmental differences between the outdoor and indoor systems such as algal density and nutrient availability [46] or the stochastic loss of other bacterial members during the enrichment process opening previously occupied niches [47].

3.3. Enrichments for phycosphere attachment

3.3.1. Microbiome remodeling between enrichment stages

We hypothesized that phycosphere-associated bacteria may be the most influential in modulating algal metabolism and metabolite exchange due to their physical proximity to the algal cells [48], and that the algal host might in part select for the specific microbial community members that attach. In order to examine this phenomenon, we used a methodology to artificially enrich for algal-attached bacteria by washing algal cells obtained from the exudate enrichments, recovering those algal cells with any attached bacteria, and inoculating into fresh media in replicate: five from the monoalgal Exudate Enrichment M, and six from the polyalgal Exudate Enrichment P. Total fractions from these phycosphere enrichments were collected on 0.2 μm filters to collect algae and both algal-attached and free-living bacteria. *P. tricornutum* cell densities were measured at $6.2 \times 10^5\,ml^{-1}$, and bacterial abundances at $3.1 \times 10^6\,ml^{-1}$. Due to the stricter conditions of these phycosphere enrichments, we expected to see a reduction in the number of ASVs and taxonomic groups present. Indeed, each phycosphere enrichment exhibited lower richness than its parent exudate enrichment, down to 64 and 92 unique ASVs in Phycosphere Enrichments M and P, respectively. Total taxonomic richness also decreased from 201 and 203 in the exudate enrichments to means of 30.6 and 30.3 in Phycosphere Enrichments M and P, respectively. This reduction indicates that although many taxa in the exudate enrichments could subsist on algal-derived exudates, only a subset exhibited attached or transiently attached lifestyles.

Although a large majority of the exudate enrichment ASVs were lost in the phycosphere enrichments (Fig. 4A), in terms of relative abundance, they were a relatively minor contribution to the exudate enrichments (Fig. 4B). Several dozen ASVs emerged in both phycosphere enrichments but remained relatively low in abundance. On the other hand, the relatively few exudate enrichment ASVs that were retained in

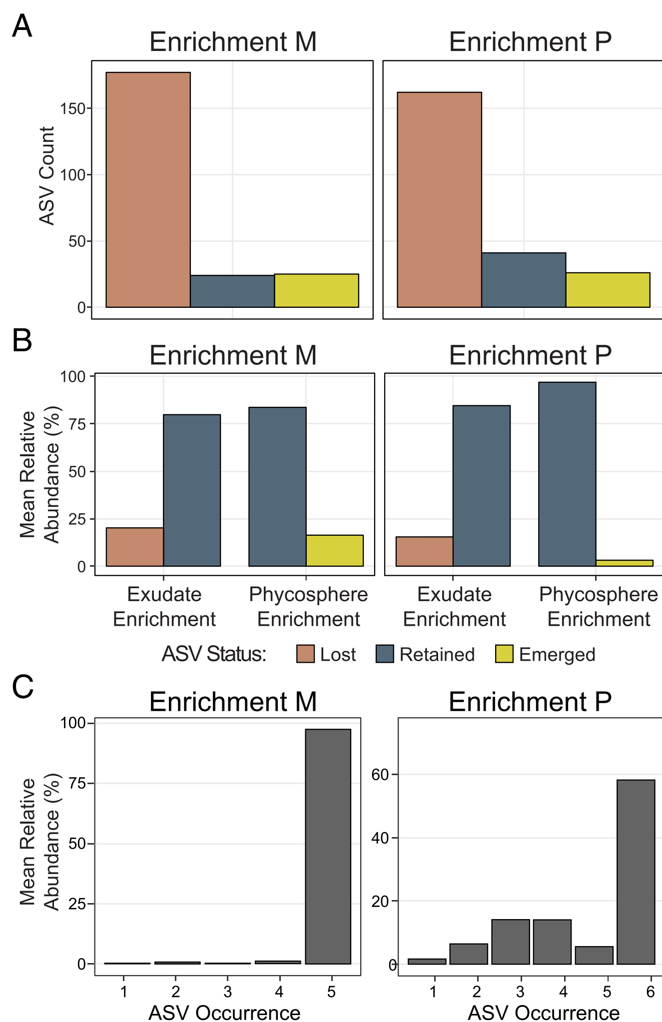

Fig. 4. Bacterial community succession from laboratory exudate enrichments to phycosphere enrichments; A) Total unique ASV counts grouped by their status as found only in the exudate enrichment (Lost), in both the exudate and phycosphere enrichments (Retained), or only in the phycosphere enrichment (Emerged). B) Percent relative abundance of ASVs, grouped by their status in either the exudate or phycosphere enrichments. An ASV must be absent from all replicates to have a lost status, however, only needs to be identified in one phycosphere enrichment replicate to be classified as retained or emerged. Phycosphere enrichment abundances are the mean of all replicates where the ASV was identified. C) ASV occurrence count (number of replicates an ASV was identified in) among the phycosphere enrichment replicates, and y-values are the mean percent relative abundance.

the phycosphere enrichments were on average > 75% of both exudate and phycosphere enrichments. This large overlap in the core microbial communities of both the exudate and phycosphere enrichments shows that the major community shift during the exudate enrichments occurred by removing numerous low-abundance taxa, and enriching for phycosphere association did not substantially shift the overall composition further. Moreover, it suggests that the more abundant taxa in both enrichment types have the capacity to attach to the algal cell surface.

3.3.2. Enrichments reveal alternative outcomes

Given the convergence of the exudate enrichments from different source samples towards similar community compositions, we expected further convergence in the phycosphere enrichments towards similar composition, particularly within replicates from the same source community. Contrary to our expectations, the mean JSD distance between Phycosphere Enrichments M and P was 0.21, which did not indicate a further convergence than that seen in the exudate enrichments (JSD 0.19). The abundance patterns of ASVs within replicates of Phycosphere Enrichment M were highly similar (mean coefficient of variation, CV, of 37.5%), but replicates of Phycosphere Enrichment P were more variable (mean CV of 116.0%). The greater variation of ASVs within Phycosphere Enrichment P was accompanied by a higher frequency of ASV loss between replicates, resulting in a lower frequency of occurrence (Fig. 4C). In terms of sequence abundance, ASVs found in all replicates for each enrichment set exhibited marked differences: they comprised > 97% of reads from Phycosphere Enrichment M but < 60% from Phycosphere Enrichment P. Remarkably, although Phycosphere Enrichment M had a higher proportion of "emerged" ASVs (Fig. 4B), they emerged in every one of the replicates. Conversely, the differences seen in Phycosphere Enrichment P were not due to differentially emerging ASVs among replicates, but rather a differential loss of retained ASVs among the replicates so that nearly 40% of the community members were found in 4 or less of the 6 replicates.

The consistent enrichment of community members in all Phycosphere Enrichment M replicates led to small pairwise JSD distances and close clustering of points in a principal coordinate analysis (Fig. 5A). The higher variability of Phycosphere Enrichment P replicates, however, led to divergence into two distinct clusters. One of these clusters, P1, was in fact more similar to the Phycosphere Enrichment M samples, and the other cluster, P2, was more dissimilar. This is exemplified quantitatively by the M and P1 clusters exhibiting mean JSD distances of 0.168 among one another, and P2 and the other two clusters with JSD distances of 0.305 and 0.264 for M and P, respectively.

To further dissect the process leading to the divergence of the phycosphere enrichments, we examined specific differences at the individual ASV level between the M cluster and the two P clusters. Of the 76 total Phycosphere Enrichment ASVs, 22 were found in at least one replicate of each cluster, and only 10 were common to all enrichments and replicates (Fig. 5B). Three ASVs appeared to drive the differentiation of the M and P1 clusters from P2, belonging to the genera *Owenweeksia* and *Loktanella*. For *Owenweeksia* (seq2779), this ASV was on average 9.2% of the Exudate Enrichment M community, but only 0.02% for Exudate Enrichment P, indicating seq2779 emerged in abundance in the P1 replicates, yet was entirely lost in the P2 replicates. Two abundant *Loktanella* ASVs displayed exclusion patterns where only one was dominant in a sample. The seq2790 *Loktanella* ASV was only 0.1% and 0.6% of the Exudate Enrichment M and P communities, respectively, dropping to 0% in the P2 communities. Seq2790, however, rose in abundance to between 5% and 26% in each of the M and P1 phycosphere enrichments. Conversely, the *Loktanella* seq2777 ASVs was only abundant in samples where seq2790 was in relatively low abundance. This pattern of mutual exclusion can arise if functional similarity between closely related taxa leads to niche competition [49]. Several ASVs had similar relative abundance in all phycosphere enrichments, including *Labrenzia* (seq81), *Hyphomonas* (seq110), *Marinobacter* (seq80), *Phaeobacter* (seq87) and *Oceanicaulis* (seq88).

4. Discussion

4.1. Selection of P. tricornutum and M. salina microbiomes

P. tricornutum is a very well-studied model organism for basic and applied research [50]. To our knowledge, this is the first published dataset of *Phaeodactylum tricornutum*-associated bacteria in outdoor mesocosms and raceways, with only one previous study documenting the bacterial community associated with *P. tricornutum* laboratory cultures [51]. Given that there is an increasing interest in microbiome impacts on the physiology of organisms ranging from algae to humans [52], our goal was to assess the influences of environment and host on bacterial community assembly. Additionally, we chose to compare *P.*

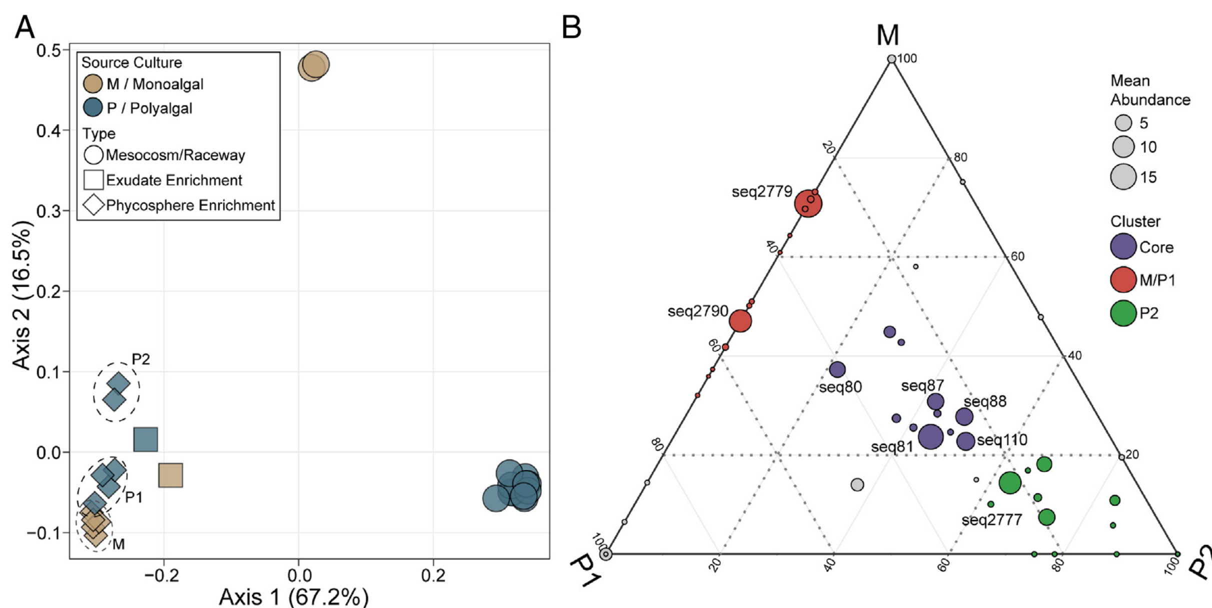

Fig. 5. A) Principal Coordinate Analysis of Jensen-Shannon distances of bacterial community compositions between the outdoor algal raceways/mesocosms (circles), exudate enrichments (squares) and phycosphere enrichments (diamonds). Dashed lines encircle the three phycosphere enrichment clusters (M, P1 and P2). B) Ternary plot showing the relative abundances of bacterial taxa among the three clusters of phycosphere enrichments. The location of taxa represents the relative abundance between the three clusters, and the circle size represents their mean abundance across all phycosphere samples. Three groupings of bacteria are highlighted; those that are core or roughly equal proportions in all phycosphere enrichment clusters, or those bacteria that signify either the M/P1 or P2 clusters.

tricornutum-associated bacterial communities with those of *M. salina* as the latter is a model biofuel alga [53], has a better characterized microbiome [54,55], and has been grown in outdoor ponds adjacent to or in co-culture with *P. tricornutum* [56].

All of the samples in the current study were numerically dominated by Proteobacteria and Bacteroidetes, regardless of algal host, fraction type or treatment, which is similar to that seen in natural marine systems [57,58]. The ratio of these two phyla, however, was different in the outdoor raceways and mesocosms compared to the smaller scale laboratory enrichments (Fig. 3C). Regardless of algal host, Bacteroidetes were the most relatively abundant in the free-living outdoor communities, whereas Proteobacteria were dominant in the outdoor algal-attached fractions and in the smaller-scale indoor enrichments. Transfer of the bacterial communities to smaller vessels grown indoors statically (without shaking) as well as culture propagation introduced an unknown selective disadvantage for these Bacteroidetes, which have been shown to grow slower than some Proteobacteria. The Bacteroidetes lost from the outdoor ponds were mostly from the marine clade of Flavobacteriaceae, which are often numerically dominant where high-molecular weight dissolved organic matter (DOM) is abundant, as in ocean algal blooms or well-mixed, air-bubbled algal mesocosms [59]. Their incubation in glass tubes or the composition of algal exudates under different nutrient levels and a 12/12 h light/dark cycle with constant photosynthetically active radiation may be in part responsible for their loss in the laboratory. Perhaps the comparatively low surface area of liquid exposed to the atmosphere in these cultures prevented outgassing of certain antibacterial volatile compounds, to which Flavobacteriaceae may be more susceptible [60]. Another possibility is that the unaerated culture conditions led to algal CO_2 limitation, which either led to the algal cells producing different organic matter compounds or which might directly inhibit the growth of Flavobacteriaceae, as the latter have been shown to incorporate inorganic C [61].

At the phylum level, large differences between the attached and free-living communities were observed (Fig. 2B). The bacterial communities attached to *M. salina* cells were enriched for *Methylophaga*, *Oceanicaulis*, *Pseudomonas*, *Dyadobacter* and an unclassified Rhodobacteraceae ASV with only a single nucleotide difference to various *Loktanella*, *Ruegeria* and *Silicibacter* 16S rRNA gene sequences. *Methylophaga* species are active DMS degraders [62], and there is evidence they cleave C1 compounds from methylated sugars prevalent in DOM [63]. *Dyadobacter* species have been found to induce extracellular polysaccharide production in diatoms [64], can utilize algal-produced isoprenes [65], and are even highly abundant in the *Arabidopsis* phyllosphere [66]. The bacterial communities attached to *P. tricornutum* were primarily enriched for *Rhodopirellula*, *Novosphingobium* and *Haliea* ASVs. Genome sequences of *Rhodopirellula* isolates indicate a capacity to degrade sulfated polysaccharides, major constituents of algal cell walls [67], and *Rhodopirellula* species have been found to associate with macroalgae [68]. Sulfated compounds are primary components of *P. tricornutum* cell walls [69], whereas analysis of *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana* [28]), a close relative of *M. salina*, revealed sulfate to be a minor component of *Microchloropsis* cell walls [70]. Although there were several genera in common between the phycosphere-associated communities of both algal hosts, they were often comprised of different ASVs. In fact, only four ASVs were found at > 1% relative abundance in the attached communities of both hosts: a *Marinobacter*, *Rhodopirellula*, an unclassified Rhodobacteraceae matching *Rhodovulum*, and the highly-abundant uncultivated Bacteroidetes ASV mentioned previously. These results indicate that the differences between the attached and free-living communities within an algal species were influenced at least partially by the host rather than by extrinsic factors such as environmental variables or medium composition which were consistent between parallel mesocosms.

4.2. Community development

A long-standing question in host-associated bacterial community assembly is how the relative contributions of the host, the environment, or the community itself shape the phylogenetic composition of the assembled community [71]. Our results show that host selection played a role in the establishment of the bacterial communities in outdoor algal ponds. Laboratory culturing of these communities to enrich for exudate-utilizing and phycosphere-associating bacteria (and removal of ancillary members) led to similar yet not entirely predictable community compositions. In the outdoor ponds, there was often a strong partitioning of phycosphere-associated and free-living bacteria, and these

differences were often algal species-specific (Fig. 2B). Indoor laboratory enrichments, featuring the inoculation of axenic algal cultures with bacterial communities acclimated to outdoor culture conditions, allowed the examination of host selection under several enrichment processes and controlled laboratory conditions. Enrichment steps winnowed the associated communities to fewer bacterial taxa, presumably those most suited for growth in the algal phycosphere. In this process, the enrichment communities converged towards similar compositions revealing core algal-associated bacterial taxa. The multiple outcomes from the phycosphere enrichments suggest mutually-excluding, closely-related taxa drive alternative stable states.

Numerous factors may influence the capacity for host selection of its microbiome, whether through active means (*e.g.* control of attachment or release of antibiotics) or through changes in the micro-environment, leading to habitat filtering. The algal-mediated chemical environment is very different between the outdoor ponds and indoor laboratory enrichments. The change in algal metabolism upon transitioning to indoor cultures contributes towards selecting against bacteria previously favored and introduce a "competitive lottery" for the establishment of new, dominant community members that differed among replicates [72,73]. Further, absent or low immigration rates and environmental variability, characteristics of standard laboratory cultivation, both magnify priority effects [74]. Nonetheless, this and other studies demonstrate the influence of the algal host in directing bacterial community composition to a greater degree than the initial community composition or other environmental factors [17,24].

Alternative to the competitive lottery hypothesis, or in combination with it, it is possible that the algal host was actively selecting for the community members through the production of bioactive compounds. In this study, we did not measure the metabolite pool released by *P. tricornutum*, and such studies are generally lacking (however, see [75]). We briefly discuss potential roles of such compounds, using as an example the antibiotic polyunsaturated fatty acid eicosapentaenoic acid (EPA), since it has been documented as being produced by *P. tricornutum* [76]. It is likely that *P. tricornutum* cells produced different bioactive compounds in outdoor raceways compared to indoor laboratory cultures. In addition, outdoor raceways are usually contaminated by other algal species whereas the indoor laboratory cultures were monoalgal, so *Phaeodactylum*-produced compounds in the tubes would not be masked by compounds produced by other algae. Indoor cultures were also incubated under lower light levels and with less aeration, potentially leading to stressful conditions. These cells may have been producing EPA (or another compound) to suppress competing bacterial heterotrophs, thereby creating antibacterial micro-environments or by encouraging the detachment and subsequent scattering of antibiotic-susceptible bacteria. The outcome of algal antibiotic production could therefore be toxicity and suppression of certain bacterial taxa, or an increase in fitness through detachment and colonization of new axenic algal cells.

Unlike the exudate enrichments, changes in the bacterial communities of the phycosphere enrichments were not primarily due to the emergence of new, previously low-abundance taxa. Rather, it was the loss of community members that allowed other, already abundant community members to thrive, although different members were favored in different replicates. This variation, which involved the emergence of alternating (but closely-related) bacterial strains, suggests divergence towards two phylogenetically (and perhaps functionally) similar alternative stable states. This selection was compounded by the added constraint of algal attachment. Bacterial attachment to the algal surface may be a transient interaction [8], and the bottlenecks introduced at the time of culture passage would only ensure the passage of bacteria that were attached at that time. These effects of drift and dispersal can introduce opportunities for new bacteria to fill vacant niches and, along with selective pressures exerted by the host, contributed to the variability seen in some enrichments.

4.3. Implications for microbiome engineering

Recent publications have highlighted the potential use of a microbiome engineering approach for improving the yield and resilience of industrial scale algal growth efforts [77]. Host selection and microbial community processes must be taken into consideration when attempting to engineer bacterial communities to lend robustness and resilience to large-scale algal production ponds [78]. In open raceway ponds, it is likely that the washing procedures employed after biomass harvesting and before algal re-inoculation do not sufficiently eliminate the bacterial consortia of the previous batch culture. The subsistence of algicidal bacteria, or bacteria that kill algal mutualists, would alter the trajectory and predictability of productivity within that pond. The results demonstrated here suggest that algal chemophysiology could significantly influence the bacterial species composition of its associated microbiome. These dynamics may, in turn, dictate whether or not a particular microbiome can cope with a given stress. Ultimately, a prediction of how algal productivity will be affected by engineering applications can only be obtained by examining the specific biotic and abiotic factors controlling bacterial community assembly, such as association with the algal phycosphere and the concentration and identity of DOM. Synergistic work to culture these bacteria will provide the genomic underpinnings of their interactions with both algae and each other, and facilitate the use of metabolic models and metabolite analyses to more clearly demonstrate the ecological feedbacks determining algal productivity.

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Author contributions

Conception and design (JAK, TS, XM), Analysis and interpretation of the data (JAK, CW, XM), Drafting of the article (JAK, XM), Critical revision of the article for important intellectual content (CW, MT), Provision of study materials (AS, PZ, TL), Collection and assembly of data (JAK, TS, DN, XM).

Conflicts

No conflicts, informed consent, human or animal rights applicable.

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