Closed microbial communities self-organize to persistently cycle carbon

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Cycles of nutrients (N, P, etc.) and resources (C) are a defining emergent feature of ecosystems. Cycling plays a critical role in determining ecosystem structure at all scales, from microbial communities to the entire biosphere. Stable cycles are essential for ecosystem persistence because they allow resources and nutrients to be regenerated. Therefore, a central problem in ecology is understanding how ecosystems are organized to sustain robust cycles. Addressing this problem quantitatively has proved challenging because of the difficulties associated with manipulating ecosystem structure while measuring cycling. We address this problem using closed microbial ecosystems (CES), hermetically sealed microbial consortia provided with only light. We develop a technique for quantifying carbon cycling in hermetically sealed microbial communities and show that CES composed of an alga and diverse bacterial consortia self-organize to robustly cycle carbon for months. Comparing replicates of diverse CES, we find that carbon cycling does not depend strongly on the taxonomy of the bacteria present. Moreover, despite strong taxonomic differences, self-organized CES exhibit a conserved set of metabolic capabilities. Therefore, an emergent carbon cycle emerges at the scale of a microbial consortia, instead of being supplied exogenously. Our study helps establish CES as model ecosystems to study emergent function and persistence in replicate systems while controlling community composition and the environment.

Microbial communities | carbon cycling | closed ecosystems | functional redundancy

Nutrient cycles are an important feature of ecosystems at all scales. The persistent cyclic flow of nutrients through ecosystems arises from a balance between complementary metabolic processes. How ecosystems are organized to facilitate this balance is an important question because cycling enables ecosystem persistence by continuously replenishing resources. As a result, global cycles of carbon (1) and nitrogen (2) are important organizing processes of life across the planet. On a smaller scale, microbial communities often exploit nutrient cycling to overcome local nutrient limitation from carbon fixation and respiration in microbial mats (3), to denitrification and nitrogen fixation in soils (4), sulfur oxidation and reduction in anoxic marine microbial communities (5), and nutrient cycling in periphytic consortia (6).

The importance of nutrient cycling for ecosystems means that a key problem in ecology is understanding how the cyclic flow of nutrients emerges from interactions between organisms in communities (7). Microbial communities, owing to their small size, rapid replication rates, and tractability in the laboratory, are powerful model systems for discovering the principles governing ecosystem organization and function. For example, a conserved succession of bacteria with predictable metabolic capabilities describes the degradation of particulate organic carbon in marine microbial communities (8). Complex bacterial communities propagated in the laboratory reveal emergent cross-feeding between predictable taxa (9), and simple assembly rules govern the stable composition of synthetic communities (10).

However, few quantitative studies have exploited the advantages of microbial communities in the laboratory to uncover the principles governing the assembly of communities that cycle nutrients. A primary roadblock to studying nutrient cycling in model microbial communities is experimental: Most existing approaches use batch (9) or continuous culture (11), where nutrients are supplied externally at high rates. In these conditions, nutrient cycling rarely occurs since the external supply of nutrients favors those strains that can most rapidly exploit the supplied resource (8, 9). The continuous and rapid dilution of these systems means that slower-growing taxa are quickly washed out (12), frequently resulting in the assembly of communities with taxa that either exploit the primary resource or are sustained via strong mutualistic or commensal interactions (9, 13). Nutrient cycles occur when some nutrients are regenerated by the community itself, instead of being supplied exogenously.

Stable nutrient cycling therefore requires a balance between the production of byproducts (e.g., CO\textsubscript{2} by respiration) and their consumption (CO\textsubscript{2} fixation by photosynthesis) in a closed loop. For these reasons, cycling can arise in batch culture when growth rates are slow (14). Similarly, Winogradsky columns, life on Earth depends on ecologically driven nutrient cycles to regenerate resources. Understanding how nutrient cycles emerge from a complex web of ecological processes is a central challenge in ecology. However, we lack model ecosystems that can be replicated, manipulated, and quantified in the laboratory, making it challenging to determine how changes in composition and the environment impact cycling. Enabled by a new high-precision method to quantify carbon cycling, we show that materially closed microbial ecosystems (CES) provided with only light self-organize to robustly cycle carbon. Studying replicate CES that support a carbon cycle reveals variable community composition but a conserved set of metabolic capabilities. Our study helps establish CES as model biospheres for studying how ecosystems persistently cycle nutrients.

Significance

Author contributions: L.M.J.A., K.H.P., and S.K. designed research; L.M.J.A. and K.H.P. performed research; L.M.J.A. and H.M. constructed the apparatus; L.M.J.A., K.H.P., Z.L., and S.K. analyzed data; and L.M.J.A., K.H.P., Z.L., and S.K. wrote the paper.

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where communities stratify along redox gradients from anoxic to oxic conditions, are important experimental tools that overcome the limitations of batch and chemostat methods to provide model systems for studying nutrient cycling (15–17). However, it remains a challenge to quantify nutrient cycling in these spatially structured communities. Here we seek to overcome some of the limitations of existing methods by establishing closed microbial ecosystems (CES) as model systems for understanding how communities are assembled to cycle nutrients. Therefore, we hope that CES can complement existing batch-culture, chemostat, and Winogradsky column-based approaches (18).

Closed Microbial Communities

Several groups, including Obenhuber and Folsome (19) and Taub and McLasky (20), have pioneered the use of CES as models for understanding the principles of emergent nutrient cycling. CES are milliliter-scale aquatic communities that are hermetically sealed and illuminated (19–23). Since no nutrients enter or leave a CES after assembly, persistence in these communities requires that nutrient cycles be sustained through photosynthesis. Complex CES have been shown to retain biological activity for decades in some cases (23). As such, CES are ideal model microbial ecosystems for understanding nutrient cycling (18). However, most work on CES to date has focused on applications to spaceflight (24) or population dynamics (22, 25, 26).

Previous efforts to use CES to understand how communities cycle nutrients were limited by low-throughput measurements of cycling (20) and did not apply sequencing methods to quantify community structure.

Here we take a top–down approach (9, 19) to assemble replicate CES, comprising diverse bacterial consortia derived from soil and a domesticated algal species. We develop a high-precision method for quantifying carbon cycling in situ to show that our CES rapidly and persistently cycle carbon. We utilize 16S ribosomal RNA (rRNA) sequencing and metabolic profiling to reveal the conserved metabolic features of CES that cycle carbon.

Carbon Cycling in Closed Microbial Communities

Carbon cycling arises in CES from the catabolic activity of photosynthetic and heterotrophic microbes. The complementary reactions of oxygenic photosynthesis and aerobic respiration consume (produce) and produce (consume) CO2 and O2, respectively (Fig. L4). Carbon cycling emerges from the photosynthetic conversion of CO2 into organic carbon, which is then either excreted by phototrophic microbes (27) or made available to bacterial decomposers via death of primary producers. The subsequent respiration of organic carbon by bacterial community members produces CO2, completing the cycle.

Carbon exchange between microbial phototrophs and heterotrophs is important in many ecosystems. For example, in marine microbial communities carbon transfer from autotrophs to heterotrophs is important for the microbial loop (28), which drives ecosystem productivity by coupling photosynthesis to the generation of bacterial biomass and growth at higher trophic levels. In addition, phototroph and bacterial biomass production also has substantial impacts on the uptake of other inorganic nutrients in these ecological contexts (29). High nutrient availability can result in competition for carbon in eutrophic environments, resulting in carbon limiting photosynthetic growth (30, 31).

Carbon cycling can be quantified by continuously measuring the production and consumption of O2 or CO2 in a CES subjected to cycles of light and dark (20). The dependence of photosynthetic O2 production (CO2 fixation) on light results in

![Fig. 1. Quantifying carbon cycling in closed microbial ecosystems. (A) Schematic of carbon cycling in closed ecosystems. Cycling occurs via photosynthesis utilizing light to fix CO2 to organic carbon, producing O2 (top arrow), and respiration that utilizes O2 and organic carbon to produce CO2. (B) Sketch of changes in total O2 or pressure (red line) and CO2 (green line) in a CES subjected to cycles of light and dark (blue line). Sketch assumes photosynthetic rate exceeds respiration rate during the light phase. r is the rate of increase of CO2 during the dark phase. f is the net decrease in CO2 during the light phase. Assuming respiratory and photosynthetic quotients of one, O2 dynamics mirror CO2. Since O2 is 30-fold less soluble in water than CO2, changes in pressure quantify changes in O2 and CO2 concentrations in a CES (SI Appendix). (C) A schematic of our custom cultivation devices for quantifying carbon cycling in CES using pressure sensors. CES of volume 20 mL are housed in glass vials (40 mL total volume), stirred at 450 rpm, illuminated by an LED, and held at 30 °C under feedback temperature control (SI Appendix). A high-precision pressure sensor is integrated into the hermetically sealed cap and a porous foam stopper (yellow) shades the sensor from illumination. (D) Pressure measurements (acquired once per second) in a CES subjected to 12 h–12 h light–dark cycles as indicated by yellow and gray shaded regions, respectively. Light intensity during the light phase is 150 μmol m−2s−1. Pressure rises and falls in response to light and dark as expected. The pressure stabilizes during the light phase, indicating that photosynthesis becomes CO2 limited. The change in pressure is proportional to r and f as labeled. Carbon cycling, computed from these quantities, is proportional to the amplitude of pressure oscillations (SI Appendix). Data in D are smoothed with a 1-min moving average. A change in pressure of 1.56 hPa (black line, right side) corresponds to a production/consumption of ~2 μmol of CO2 assuming pH 6.5 and photosynthetic/respiratory quotients of 1 (SI Appendix).]
oscillations in O$_2$ and CO$_2$ levels when subjected to diel cycles of light and dark (Fig. 1B). We define the carbon cycling rate as the number of moles of carbon cycled (fixed and respired) per light–dark cycle. To quantify carbon cycling, we estimate fixation and respiration rates from measurements of O$_2$ and CO$_2$ dynamics. Since photosynthesis occurs only during the light phase, we measure the rate of respiration during the dark phase (r, Fig. 1B and D). We assume that the respiration rate during the light phase is the same as that during the dark phase. Although this assumption can break down in some cases (32), our data show that the respiration rate is stable during the dark phase (SI Appendix, Fig. S6), suggesting that bacterial respiration does not change dramatically between light and dark phases. The amount of CO$_2$ fixed during the light phase is computed by measuring the net oxygen production (CO$_2$ fixed; $f$, Fig. 1B and D) during the light phase and accounting for the respiration rate to infer a total CO$_2$ fixed (SI Appendix). The amount of carbon cycled over a light–dark cycle is then the number of moles of inorganic carbon both fixed and produced. Assuming constant photosynthetic and respiratory quotients (ratio of O$_2$ production [consumption] to CO$_2$ consumption [production]) allows carbon cycling to be quantified by measuring either O$_2$ or CO$_2$ dynamics under cycles of light and dark (33).

As noted by Öbenhuber and Folsome (19), O$_2$ has 30-fold lower solubility in water than CO$_2$. As a result, when photosynthesis converts water-soluble CO$_2$ to lower-solubility O$_2$ in a sealed vessel, most of the O$_2$ leaves the liquid and goes into the gas phase, increasing the pressure in the head space. Similarly, if O$_2$ is consumed by respiration, this reduces the pressure in the head space of the vessel by converting the lower-solubility O$_2$ into higher-solubility CO$_2$. Therefore, O$_2$ dynamics in a sealed vessel can be quantified by simply measuring changes in pressure in the head space under cycles of light and dark (Fig. 1B). These changes in pressure can be used to quantify rates of photosynthesis and respiration in situ.

We developed a custom culture device to precisely measure changes in pressure in a CES subjected to cycles of light and dark. A schematic is shown in Fig. 1C. Each device housed a 20-mL CES in a 40-mL glass vial. The cap of the hermetically sealed vial was fitted with a high-precision, low-cost, pressure sensor developed for mobile devices (Bosch; BME280). In contrast to devices that measure CO$_2$ pressure measurements are higher sensitivity, lower cost, require no calibration, do not consume analyte, and are stable for months. The vial was illuminated from below by a light-emitting diode (LED) and fitted in a metal block that was held under feedback temperature control via a thermo-electric heating–cooling element (34). Thus, our custom culture devices permit real-time quantification of carbon cycling rates in many replicate CES while precisely controlling temperature and illumination. When we subjected the CES housed in our devices to cycles of light and dark (12 h–12 h), we observed increases and decreases in pressure, driven by the production and consumption of oxygen by photosynthesis and respiration during light and dark phases, respectively (Fig. 1D). Performing the same experiment with only water in the vial resulted in no pressure oscillations as expected (SI Appendix, Fig. S1), and concurrent measurements of O$_2$ and pressure in the vial confirmed that pressure changes reflected the production and consumption of O$_2$ and therefore CO$_2$ (SI Appendix, Figs. S2 and S3).

In addition, we considered the possibility that other gases (nitrogen, hydrogen, sulfide) might be produced and consumed by the microbial community, driving changes in pressure. Based on the availability of these compounds in our CES, and the metabolic capabilities of the taxa detected via sequencing (Dataset S6), we concluded that the production and consumption of other gases are not dominating the pressure changes we observe. Consistent with this conclusion, we observe a correlation between the total oxygen produced in our CES and the autotroph relative abundance (SI Appendix, Fig. S14). Therefore, the respiration rate (r) and net photosynthesis (f) can be quantified directly from continuous pressure measurements (Fig. 1D). The rate of carbon cycling in our CES is proportional to the amplitude of the light-driven pressure oscillations (SI Appendix).

We developed a custom culture device to precisely measure carbon cycling in variants of a previously studied synthetic CES (25, 26) composed of Chlamydomonas reinhardtii (UTEX 2244, mt+) and Escherichia coli (MG1655) over periods of several weeks. We found that the alga alone or algae with E. coli failed to persistently cycle carbon (Fig. 2C and SI Appendix, Fig. S4). We speculate that this failure arose from the production of starch by the alga (35), which cannot be utilized by E. coli. Therefore, we reasoned that increasing the metabolic diversity of the bacterial component of our CES might improve carbon cycling. To accomplish this, we turned to a top–down community assembly approach (9, 11) outlined in Fig. 24.

**Top–Down Assembly of Closed Microbial Communities**

To assemble communities, we sampled local soils, removed eukaryotes by applying drugs, and extracted bacterial communities using standard techniques (SI Appendix). We then combined these diverse bacterial populations with the domesticated soil-dwelling alga C. reinhardtii (Fig. 24). We used soil communities to initialize our CES for two reasons. First, since C. reinhardtii is native to soil, we reasoned that bacterial communities in soils might more fully recycle nutrients and resources produced by C. reinhardtii. Second, soils harbor substantial metabolic diversity (36) and we reasoned that higher-diversity starting communities would be more likely to form stable nutrient cycles in a CES. The resulting CES contained a diverse assemblage of bacteria and the alga. While we were unable to completely exclude photosynthetic bacteria from the soils, our sequencing measurements indicated that the alga dominated the photosynthetic component of our communities (SI Appendix, Fig. S13). We assembled eight CES using this method, four each from two soil samples (designated “A” and “B”), and inoculated them into a chemically defined freshwater mimic medium (37) that included organic carbon (glucose), nitrogen (ammonia), and phosphorous (phosphate; SI Appendix, Table S4) to facilitate the initial growth of the community. We sealed these communities in vials and placed them in culture devices like the one shown in Fig. 1C and subjected them to 12 h–12 h light–dark cycles for a period of ∼50 d.

A representative time series of pressure for one of these CES is shown in Fig. 2B. We observed an initial large decline in pressure (Fig. 2 B, Inset), which arose from the rapid bacterial respiration of glucose (this decline is not present in CES of algae alone; SI Appendix, Fig. S4). The pressure remains ∼10% below ambient for 5 to 8 d and then begins to rise (SI Appendix, Fig. S5), reflecting the timescale over which we expect algae to grow (38). The rising pressure reflects photosynthetic activity (O$_2$ production) by the algae before saturating after 8 to 10 d (SI Appendix, Figs. S2 and S5).

Once the pressure saturated, we observed stable pressure oscillations driven by light–dark cycles. In this regime, during each light phase, the pressure stabilized within 2 to 3 h of the illumination being turned on. Therefore, the autotrophs rapidly fix CO$_2$ early in the light phase before exhausting the inorganic carbon supply later in the light phase. After CO$_2$ is depleted during the early periods of the light phase, respiration and photosynthesis rates are balanced, resulting in stable pressure (O$_2$ levels) late
Fig. 2. Long-term carbon cycling in closed ecosystems composed of C. reinhardtii and soil-derived bacterial communities. (A) Top–down assembly of microbial CES. Soil samples are harvested and bacterial communities are extracted. Bacteria are then combined with the alga C. reinhardtii and inoculated into the custom culture devices described in Fig. 1C. Eight CES were assembled, four each from two soil samples (“A” and “B”), in defined minimal medium, and subjected to 12 h–12 h light–dark cycles (yellow/grey shaded regions) for ∼50 d while pressure was measured. Light intensity was 150 μmol m⁻² s⁻¹ during the light phase. (B) Pressure measurements performed once per second, smoothed by a 1-min moving average, for one of the eight CES. The initial large drop in pressure due to rapid respiration of supplied organic carbon (glucose) is shown in Inset. (C) The rate of carbon cycling (moles per day) for all eight CES is computed from pressure traces as described in SI Appendix. Carbon cycling rates are reported only after the initial transient phase (B, Inset) has ended. We assume respiratory and photosynthetic quotients of 1 and pH 6.5. Circles indicate CES from soil sample A and triangles those from soil sample B. The transient increase in cycling around 25 to 35 d coincides with a reduction in photosynthetic rates and an increase in respiration (SI Appendix, Fig. S7). Red and green traces are synthetic CES composed of C. reinhardtii and E. coli (mean of two replicates) and C. reinhardtii (single replicate; SI Appendix, Fig. S4) as shown in the key. Statistical errors in estimates of carbon cycling are smaller than the size of the markers. Key in C applies to D–F. At the end of the acquisition shown in C all eight CES were opened, samples were taken, and CES were diluted 1:20 into fresh media. CES were then sealed for an additional ∼18 d of light–dark cycles and carbon cycling was monitored. (D) Carbon cycling rates after the first dilution. Two additional dilution rounds were performed and cycling rates are shown in E and F as indicated by the black arrows. The average cycling rates at the end of each round do not differ significantly between rounds of enrichment (P values: 0.31, 0.87, and 0.053, two-sample t test between last measurement between rounds 1 and 2, 2 and 3, and 3 and 4, respectively) in the light phase. We infer that the respiration is the rate-limiting step in the carbon cycle in our CES and that light is not limiting carbon fixation. During the dark phases of each light–dark cycle, we observe a linear decrease in pressure with time, indicating a constant rate of respiration during the dark phase (SI Appendix, Fig. S6).

We observed stable pressure oscillations, with saturating pressure levels during the light phase and constant respiration rates during the dark phase, for a period of ∼50 d. During this period, we observe longer-timescale dynamics whereby the pressure (O₂) levels slowly drop after about 25 d (seven of eight CES; Fig. 2B and SI Appendix, Fig. S5). A detailed analysis of the O₂ dynamics reveals that this transient decline in pressure coincides with a slowing of the photosynthetic rates and an increase in the respiration rates (SI Appendix, Fig. S7). We speculate that this results from the death of a fraction of the algal population that supplies the bacterial community with additional organic carbon for respiration. After this transient decline, the photosynthetic rate stabilizes (SI Appendix, Fig. S7), indicating that a stable population of autotrophs is fixing carbon.
We estimated the rate of carbon cycling in each of our eight CES directly from pressure measurements, like the one shown in Fig. 2B, and the results are shown in Fig. 2C. We observe robust carbon cycling at rates of ∼10 to nearly 30 μmol Cd−1sm−2h−1 in all eight CES. The magnitude of this carbon cycling rate is a sizable fraction of the total organic carbon supplied to each CES at the outset (∼200 μmol; SI Appendix, Table S5) and the amount of nonvolatile organic carbon present in each CES at the end of the experiment (120 to 180 μmol; SI Appendix, Fig. S8). Therefore, in a period of between 4 and 20 d the amount of carbon cycled approaches the total carbon in the CES. In this sense, we conclude that the carbon cycling rate in our self-assembled CES is high. In contrast, in CES composed of algal consortia, the carbon cycling rate in our self-assembled CES is high. This is evident by the presence of bacterial phototrophs native to the soil (SI Appendix, Fig. S27 and section 6.5). By comparison, as shown in Fig. S25 and section 6.5), the median 41% decrease; SI Appendix, Figs. S11 and S28), but not in terms of diversity (SI Appendix, Fig. S22). This, and the results of the control experiments with and without illumination and added algae, suggests that illumination and the presence of algae result in a reorganization of the soil community (SI Appendix, Fig. S26C).

To quantify taxonomic variability across CES, we computed the Jensen–Shannon divergence (JSD) (40) between the relative abundances in each pair of CES at each round of enrichment. The JSD quantifies differences in community composition between two communities and varies between 0 for two identical communities and 1 for two communities that share no taxa in common. On average, the taxonomic composition differs more between CES (inter-CES) than it does for the same CES across rounds of enrichment (intra-CES; SI Appendix, Fig. S15), a result that is robust to using other community similarity metrics (SI Appendix, Figs. S16–S18). We also found that the JSD between CES from different soil samples did not decline across rounds of enrichment (SI Appendix, Fig. S19), indicating that the taxonomic differences between CES from different soil samples are retained throughout the enrichment process. Inter-CES divergences remained larger than intra-CES divergences even when we grouped taxa with only 90% 16S sequence similarity, indicating that there is no taxonomic similarity between CES even at higher levels of classification (SI Appendix, Figs. S20 and S21). To visualize community taxonomic composition, we embedded the JSD between all CES at all rounds into two dimensions using multidimensional scaling (MDS) (see SI Appendix, Fig. S24 for the stress of this embedding) and the result is shown in Fig. 3B. Note that the points corresponding to each instance of a CES remain largely separated from each other. Fig. 3B supports our assertion that the taxonomic composition diverges strongly from one CES to the next and that during enrichment these differences are retained. The differences between CES from soil sample A are larger than those from sample B (SI Appendix, Fig. S15), but in neither case did we observe CES converging to a shared taxonomic makeup
of the bacterial community. We conclude that the bacterial communities in our CES differ substantially in their taxonomic composition.

**Metabolic Characterization of Closed Microbial Communities**

The result that the taxonomic structure differs strongly from one CES to the next despite similar carbon cycling rates supports the idea that carbon cycling in our CES is accomplished by diverse but functionally redundant bacterial communities. Functional redundancy, where different community compositions drive similar metabolic function, is often observed in microbial communities (11, 39). Our observation of functional redundancy suggests that the metabolic capabilities of the assembled bacterial communities might be conserved across CES. Reasoning that the identity of the organic carbon compounds produced by *C. reinhardtii* (and the minor native autotrophs present from the soils) is likely similar across CES, we hypothesized that the carbon utilization capabilities of the assembled bacterial communities might be similar across CES.

To test this hypothesis we measured carbon utilization capabilities on diverse carbon sources for all CES after each round of enrichment. To accomplish this we used Biolog 96-well EcoPlates (41) that exploit a redox-sensitive dye to report respiration in the presence of 32 diverse carbon sources (including compounds excreted by *C. reinhardtii*, Table S6), each in triplicate. After each round of dilution we distributed aliquots of each CES into an EcoPlate. We then incubated the plates and measured dye absorbance, a proxy for carbon respiration, daily for a period of 4 d. Example absorbance time series are shown in Fig. 3C. For each replicate of each carbon source, we computed a rate of respiration for that carbon source (1/τ) by estimating Δlog(OD590)/ΔT, where OD590 is the absorbance signal reporting respiration (Fig. 3C and SI Appendix). The quantity 1/τ approximates the rate at which a CES utilizes a given carbon source.

**Fig. 3.** Divergent taxonomic structure and convergent metabolic capabilities across replicate CES. (A) Relative abundances measured by 16S rRNA amplicon sequencing of the bacterial taxa comprising the CES (y axis) for each round of dilution (x axis). Each exact sequence variant (ESV) is represented by a unique color, indicated in the legend. Only the ESVs that have a relative abundance of 5% or higher in at least one of the four dilution rounds for each CES are shown. Most ESVs belong to unique genera (SI Appendix, Fig. S21, where multiple ESVs having the same genus are combined). (B) The JSD of the relative abundances of all detected taxa at the ESV level is computed between all 32 CES, as described in SI Appendix. MDS is applied to the JSD to embed the data in two dimensions. The circles denote CES derived from soil sample A and the triangles denote CES derived from soil sample B; colors correspond to Fig. 2 color, indicated in the legend. Only the ESVs that have a relative abundance of 5% or higher in at least one of the four dilution rounds for each CES are shown. Most ESVs belong to unique genera (SI Appendix, Fig. S21, where multiple ESVs having the same genus are combined).
compound. We averaged $1/\tau$ across the three replicates for each carbon source at each round of enrichment in each CES (Fig. 3D). Each row of Fig. 3D shows the average $1/\tau$ (utilization rate) for a single carbon source and each column a profile for a CES. Comparing carbon utilization profiles across rounds reveals a convergence in the metabolic capabilities across our eight CES, with profiles becoming more similar across CES as the number of rounds of enrichment increases. For example, by the end of round 4 none of the CES utilize 2-hydroxy benzoic acid despite six of eight CES being capable of consuming the carbon source after round 1. Conversely, the enrichment process increases $1/\tau$ for other carbon sources (phenethylamine, putrecine, $\gamma$-amino butyric acid). We note that the carbon utilization profiles of the enriched CES, after round 4, differ strongly from those of E. coli (SI Appendix, Fig. S29), which itself fails to cycle carbon with C. reinhardtii (Fig. 2C), suggesting that the carbon utilization capabilities of the complex CES are important for stable carbon cycling.

To quantify the variation in the carbon utilization profiles across CES we computed the cross-correlation coefficient in carbon utilization between every pair of CES in each round (columns, Fig. 3D). The results are shown in Fig. 3E where we observe a steady and statistically significant increase from round 1 to round 4. This correlation measures the similarity between pairs of CES in their carbon utilization profiles, and thus the increase we observe quantifies the extent to which CES are converging over rounds of enrichment to a similar carbon utilization profile.

We speculated that the metabolic convergence we observe in Fig. 3E might be a consequence of carbon limitation in our CES. For example, if organic carbon is limiting and provided to bacteria by the algae either by excretion or by cell death, then the spectrum of carbon compounds provided by the algae would determine the carbon catabolic capabilities that the bacterial community must possess to utilize the available carbon. Indeed, a control experiment indicates that some of the compounds utilized by the assembled bacterial communities are excreted by C. reinhardtii (SI Appendix, Table S6 and Dataset S3). However, from the pressure data or metabolic profiling, we cannot determine the nutrient-limiting respiration in our CES. To address this question we performed an assay after each round of dilution to determine the nutrient-limiting respiration. We used a Microresp assay (SI Appendix) whereby small aliquots of each CES were dispensed into 96-well plates and supplemented with carbon, nitrogen, or phosphorous. We measured CO$_2$ production in each sealed well directly using a pH-sensitive dye and compared the results to control wells where no nutrients were added (SI Appendix, Fig. S30). We found respiration in our CES was in some cases carbon limited, in some cases phosphorous limited (predominantly in CES from soil sample A; SI Appendix, Fig. S30), or in some cases both (community A.1). A quantitative analysis of the nutrient budgets in our CES and literature values for the stoichiometry of biomass revealed that, given the excess phosphorous in the media, phosphorous limitation most likely arises from phosphate storage, either by bacteria (42) or by C. reinhardtii (43) and not the incorporation of phosphorous into new biomass (SI Appendix, section 7.2.2). These results suggest that sequestration may change the nutrient-limiting respiration in our CES, but that the metabolic convergence we observe (Fig. 3 D and E) is robust to limitation by other nutrients. A more complete accounting of the mechanisms governing nutrient limitation in these communities will require a detailed interrogation of respiratory coefficients, biomass stoichiometry, and carbon transfer between the autotroph and bacterial components.

Discussion

The primary results of our study are the demonstration that CES can be powerful model microbial ecosystems for studying nutrient cycling and the development of a high-resolution method for quantifying cycling in closed communities. Model systems have proved essential for advancing every area of biology, including from gene expression (44), to development (45), to evolution (46). However, we lack model systems to serve the same purpose at the level of the community or ecosystem (18). Since CES are closed, nutrient cycling is required for persistence. Therefore, CES constitute model systems for studying nutrient cycling at the level of the collective with the key property of permitting control of community composition, nutrient, and energy availability. Given this tractability, CES constitute model biospheres for understanding how communities are organized to satisfy the constraints placed on them by nutrient cycling and for learning how evolutionary processes impact this organization. One of the main limitations in the field was a lack of precise, long-term, in situ measurements of nutrient cycling. We have overcome this limitation and demonstrated that CES are amenable to quantitative measurements of nutrient cycling while interrogating community structure at the taxonomic and metabolic levels.

Our taxonomic and metabolic characterization of replicate CES showed that carbon cycling in CES can be sustained by diverse bacterial consortia that exhibit a conserved set of metabolic capabilities. The result points to the idea that the emergent functional property of carbon-cycling microbial communities is likely a conserved set of metabolic capabilities (39) that are robust to variation in the taxonomic structure of the system. However, some aspects of community function that we have not measured may depend on the taxonomic structure of the community, such as phosphorous sequestration. Ultimately, the functional aspects of the community that can be performed by diverse taxa likely depend on the phylogenetic conservation of the associated phenotypic traits. In this context, our data suggest that the conserved properties of carbon-cycling CES are likely carbon utilization pathways and the taxonomic diversity in our CES potentially reflects the weak phylogenetic conservation of carbon utilization phenotypes (47). It will be interesting to extend this study to understand the role of this taxonomic variability and metabolic convergence in determining the robustness of nutrient cycling to environmental perturbations such as changes in temperature or light levels. While previous studies have considered functional robustness in communities (48), our CES offer the advantages of real-time measurements of community function for many replicate consortia in the laboratory.

The fact that CES are hermetically sealed means that they differ markedly from natural communities where immigration can change the makeup of the community. Despite this difference, we propose that CES can act as model systems for understanding how nutrient cycling constrains the structure of a community. While immigrations can and do alter the taxonomic structure of communities in the wild, it is frequently observed that metagenomic structure is tightly coupled to abiotic factors (39), suggesting that the assembly of functional communities may be deterministic given specific environmental contexts (8, 26). In this case, provided a CES is initialized with sufficient metabolic diversity to satisfy the constraints on the system set by cycling, the final functional structure of the community may not depend strongly on whether or not immigrations are allowed to occur, a hypothesis that could be tested by opening CES and introducing invaders.

Nutrient cycling in wild microbial communities often involves recycling of a single nonsubstitutable nutrient such as sulfur (5) or carbon (49), with other essential nutrients available in excess. This is in contrast to CES where no nutrients are supplied exogenously and biomass generation requires cycling all nutrients at once. In our CES it remains unclear to what extent nutrients other than carbon are cycled, such as those primarily involved in anabolism (N, P, Fe). It may be that the generation times in our CES are long, yielding few cell divisions in the course of the
experiment. In this case carbon exchange could be utilized by algae and bacteria for maintenance energy. In this situation the cycling of nutrients such as N or P would be slow. In contrast, if generation times are short and many generations occur over the course of an experiment, nutrients such as N and P would need to be rapidly cycled to sustain cell division (50).

In addition, quantifying abundance dynamics and metabolite exchanges in our CES would reveal how specific ecological interactions endow these communities with stable cycling capabilities. Detailed data on abundance dynamics would also permit comparison between our experiments and the substantial existing body of theoretical work on closed ecosystems (51–54). In particular, because the energy available to the system is readily varied by changing light intensity, CES could be used to test the proposed role of energetics in determining community structure (55).

CES have a key role to play in future work understanding evolution at the level of the community. Simulations and directed evolution approaches have been used to ask whether and how ecosystem-level traits can be selected (56, 57). As with directed evolution in proteins or organisms, the target of adaptation by the ecosystem is typically stipulated by the experiment. For example, communities might be selected for the production (58) or degradation (56) of a particular compound. Prior work in this field has faced two problems. First, it has been challenging to perform selection in the laboratory on a community-level trait that cannot be optimized by adaptation of an individual member of the community (57, 59). For example, selecting a community for fast degradation of a compound can be simply selecting the strain that degrades that compound most rapidly. Second, community-level evolution requires a notion of heritability, whereby successive generations of a community retain emergent traits of the parent community. However, theoretical work suggests a way to circumvent these obstacles: When selection acts on interaction-dependent properties of the ecosystem, such as metabolite exchange between strains, individual traits evolve to improve community heredity (60). Consistent with this expectation is the proposal that communities mediated by competition or exchange of resources can behave as cohesive units exhibiting emergent traits that are transmitted between generations (61). However, experimentally selecting a community on the basis of an emergent function that relies on interactions between constituents is a challenge. Nutrient-cycling closed ecosystems would appear to be ideal systems to address this problem since carbon cycling requires cooperative metabolic processes. Moreover, nutrient cycles in CES are frequently observed to be rapidly cycled to sustain cell division (50).

Given these possibilities, we propose that CES, coupled with careful measurements of metabolic dynamics like those made here, constitute powerful model systems for the quantitative study of emergent nutrient cycling in the environment.

**Materials and Methods**

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