**Title:**

**Carbon resource richness shapes bacterial competitive interactions by alleviating growth-antibiosis trade-off**

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**Abstract**

1. Antibiosis and resource competition are major drivers shaping the assembly, diversity and functioning of microbial communities. While it is recognised that competition is sensitive to environmental conditions, it is unclear to what extent this mediated by the availability of different carbon resources.
2. Here we used a model laboratory system to directly test this by exploring how carbon resource richness and identity shape resource competition and antibiosis between plant probiotic *Bacillus amyloliquefaciens* and phytopathogenic *Ralstonia solanacearum* bacteria. We found that while sugars typically promoted *B. amyloliquefaciens* growth, organic and amino acids increased the production of both bacillaene and macrolactin antibiotics and the direct inhibition of *R. solanacearum*. In contrast, when multiple different carbon resources were available, *B. amyloliquefaciens* could efficiently grow and produce antibiotics at the same time.
3. Together, these results suggest that high carbon resource richness allows concurrent expression of growth- and antibiosis-related traits, potentially altering bacterial competitive dynamics and plant growth promotion in microbial communities.

**Key words:** antibiosis; competition; gene expression, resource complexity; trade-off

**Introduction**

Competition is a fundamental component of microbial community ecology and is thought to be responsible for driving the assembly, diversity, coexistence, functioning and evolution of microbial communities (Foster & Bell 2012; Mallon *et al.* 2015; Jousset *et al.* 2016; Mumford & Friman 2017; Mallon *et al.* 2018). Microorganisms may compete for shared limited resources or directly antagonize each other via antimicrobials (Hibbing *et al.* 2010; Ghoul & Mitri 2016; Hu *et al.* 2016). These two forms of competition are not mutually exclusive. Instead, both antibiosis and resource competition often take place at the same time and the observed net antagonism between interacting microbes is likely to be determined as the sum of the two competitive interactions (Foster & Bell 2012). Simultaneous expression of multiple competition-related traits could be however limited by trade-offs and environmental conditions (Andersson & Hughes 2010). For example, if producing antimicrobials is costly, the fitness of antibiotic producer could be reduced via resource competition especially when the availability of nutrients and the proportion of susceptible cells in the population is low (Czárán, Hoekstra & Pagie 2002; Kinkel, Bakker & Schlatter 2011). Moreover, most bacteria can selectively use substrates from a mixture of different carbon sources. The presence of preferred carbon sources often prevents the expression, and often also the activity, of other catabolic systems that enable the use of secondary substrates (Görke & Stülke 2008). As a result, bacterial preference to certain resources could constrain the expression of antibiotics leading to growth-antibiosis trade-offs (Schlatter & Kinkel 2015). Resource richness, *i.e.,* the number of available resources in a given environment, may in this context determine the metabolic costs of between growth and secondary metabolism (Görke & Stülke 2008; Takeuchi *et al.* 2009; Handel *et al.* 2013).

*Bacillus amyloliquefaciens* strain T-5 (Tan *et al.* 2013) isolated from tomato rhizosphere has been reported as an efficient biocontrol agent against soil-borne pathogen *R. solanacearum* bacteria(Wei *et al.* 2011)*.* In this study, we investigated how carbon resource type and combinations (carbon resource richness) affect the competitive interactions between the *B. amyloliquefaciens* T-5 and *R. solanacearum*. In order to infer the effect of resource richness and identity on resource competition and antibiosis, we first determined the growth and antibiotic production of *B. amyloliquefaciens* in the presence of different types of single carbon resources including sugars, amino acids and organic acids. In addition to growth, we also measured to what extent the strength of antibiosis against *R. solanacearum* was affected by specific carbons. We then explored how increasing the richness of carbon resources in the growth media affected *B. amyloliquefaciens* the expression of these two competitive traits. We hypothesized that 1) both carbon type and identity could affect *B. amyloliquefaciens* growth and antibiotic production in single carbon resource environments and that 2) the presence of multiple carbon resources would enable the expression of both growth- and antibiosis-related traits by alleviating potential trade-offs.

**Materials and methods**

**Bacterial strains and culture conditions**

We used *Bacillus amyloliquefaciens* T-5 (CGMCC accession No. 8547, China General Microbiology Culture Collection Center) and the phytopathogenic bacterium *Ralstonia solanacearum* QL-Rs1115 (Wei *et al.*, 2011), tagged with the pYC12-mCherry plasmid (Tan *et al.* 2016), as our focal bacterial species. *Bacillus amyloliquefaciens* T-5 can suppress the growth of *R. solanacearum* QL-Rs1115 by producing various antibacterial secondary metabolites (Tan *et al.* 2016). Both bacteria were kept at -80°C in glycerol stocks and experimental stocks were prepared as follows: *B. amyloliquefaciens* T-5 was grown at 30 °C in tryptic soy broth (TSB; 15 g L-1 tryptone, 5 g L-1 soy peptone and 5 g L-1 NaCl, pH=7.0) and *R. solanacearum* QL-Rs1115 strain at 30 °C on nutrient broth (NB, 10.0 g L-1 glucose, 5.0 g L-1 peptone, 0.5 g L-1 yeast extract, 3.0 g L-1 beef extract, pH=7.0) for 24 h and prior to each experiment, cells were suspended in sterile 0.85 % NaCl buffer and adjusted to a density of 107 cells mL-1.

**Extraction, purification and identification of antibacterial compounds produced by *B. amyloliquefaciens* T-5**

We used high-pressure liquid chromatography (HPLC) for the identification of antibacterial compounds produced by *B. amyloliquefaciens* T-5. For the preparation of samples, *B. amyloliquefaciens* T-5 was grown for 36 h in 250 mL Erlenmeyer flasks with 50 mL of Landy medium containing 20 g L-1 glucose, 5 g L-1 L-glutamic acid, 0.5 g L-1 MgSO4, 0.5 g L-1 KCl, 1.0 g L-1 KH2PO4, 0.15 mg L-1 FeSO4, 5.0 mg L-1 MnSO4, 0.16 mg L-1 CuSO4 and 2 mg L-1 L-phenylalanine (Landy *et al.* 1948) in a rotary incubator (180 rpm) at 30 °C. Bacterial cultures were then centrifuged (12000 × *g* for 8 min at 4 °C) and partial purified in an Amberlite XAD-16 resin (Alfa Aesar, a Johnson Matthey Company, Ward Hill, MA, USA) column (15 g) by washing with 500 ml of distilled water and eluting with 150 mL of 100% methanol. Finally, eluates were dried in a rotationevaporator, dissolved in 1.5 mL of a 1:1 (v/v) methanol: water solution and passed through 0.22 μm filter.

HPLC was performed with an HPLC 1200 device (1200 series, Agilent, Santa Clara, CA) as follows. Briefly, 10 μl of samples were injected onto an HPLC column (XDB-C18, 4.6 mm × 250 mm, 5um, Agilent) and the run was performed with a ﬂow rate of 0.5 mL min-1 with two solvents: A (0.1% v/v HCOOH) and B (CH3CN containing 0.1 %, v/v HCOOH). The two solvents were mixed with different volume ratios and run 30 mins with following running times during the gradient elution. We used initial concentrations of 30:70 % for solvents A and B for the first 5 mins, and then increased the relative concentration of solvent B to 45 % for the next 5 mins and slowly increased the relative concentration of solvent B from 45% to 100 % for the last 20 mins. After initial column equilibration for 5 mins, a fraction collector (Analyt FC, G1364C, Agilent, Santa Clara, CA) was used to collect the pure compounds by using the time and peaks mode. The injections were performed repeatedly to collect sufficient quantities of antimicrobial compounds. The fractions were then lyophilized and the residues were dissolved in 500 μl of methanol for mass spectrometry (MS) analysis and antibiotic activity assays. The molecular weight and formula of each secondary metabolite was determined by using a liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) system (1200 series, Agilent, Santa Clara, CA, and ESI-MS, 6410 Triple Quad LC/MS, Agilent, Santa with a C18 column (4.6 × 250 mm, 5 μm) with a flow rate of 0.5 mL min-1. We used the same mobile phase as in the HPLC purification step. For MS analysis, the electrospray needle was operated at a spray voltage of 4.5 KV and the capillary temperature was set at 300 °C. The MS analysis was conducted by electrospray ionization in positive ion mode and the mass spectra were acquired in an m/z range of 50-1200 at a scan rate of 500 atomic mass units (amu)/s. Antagonistic activity of purified antibacterial compounds was measured with agar disc diffusion method where a 50 μl aliquot of filtered supernatant or the purified elutes were applied into a 6 mm well on NA plates inoculated with 107 cells mL-1 of *R. solanacearum* pathogen.

**Growth and antibiotic production of *B. amyloliquefaciens* T-5 in single carbon resource treatments**

We grew the bacteria in OS minimal medium supplemented with 48 different carbon resources (see Appendix S1: Table S1 in Supporting Information) covering a range of sugars, organic acids and amino acids typically found in tomato root exudates (Wei *et al.*, 2015). Bacteria were inoculated into 96-well microtiter plates containing 200 µL OS minimal medium (Schnider-Keel *et al.* 2000) supplemented with 5 g L-1 of the respective carbon sources. We chose to normalize carbon source concentration in g L-1 instead of mM in order to minimize biases due to different molecule size. Plates were sealed and incubated for 36 h with agitation (170 rpm) at 30°C. Changes in bacterial densities were measured as optical density at 600 nm wavelength with a spectrophotometer (Spectra Max M5; Molecular Devices, Sunnyvale, CA). Culture medium supplemented with the corresponding carbon source without bacteria was used as a blank. Each treatment was replicated three times. Any OD600 value greater than 0.05 was scored as positive *B. amyloliquefaciens* T-5 growth on a given carbon source (Supporting Information, Appendix S1: Table S1). In the case of positive growth, antibiotic production and antagonistic activity against *R. solanacearum* were measured with reverse phase HPLC and agar disc diffusion method, respectively. Three replicates were used for each treatment.

**Assembly of different carbon resource combinations**

We selected eighteen carbon resources that could be utilized by *B. amyloliquefaciens* T-5 (Supporting Information Appendix S1: Table S1) to assemble a richness gradient ranging from single carbon resource (18 treatments in duplicate) to nine-carbon resource (eight treatments without replicates) and eighteen-carbon resource combinations (1 treatment with four replicates). Within all multi-carbon combinations (richness 9 and 18), each carbon resource was present in equal concentrations (0.56 g C l-1 and 0.28 g C l-1, respectively), while each carbon resource was present total of 10 times across all combinations. We grew the bacteria in OS minimal medium supplemented with different carbon sources at a uniform total concentration of 5 g l-1. Even though the energy content of different carbon media is likely to differ depending on the specific substrates (Figure S2A), these differences will be averaged out in multi carbon resource environments, and hence, increasing carbon diversity should not affect the mean energetic value of the media.

**Measuring the effect of resource richness on the *B. amyloliquefaciens* T-5 growth, antibiotic production, antibiotic gene expression and *R. solanacearum* inhibition**

The growth (biomass) and antibiotic production of *B. amyloliquefaciens* T-5 was measured in each resource combination using the same methodology as for the single resource experiments described above. We selected two time points for the measurements that corresponded to late exponential phase (18h) and past stationary phase (36h) of *B. amyloliquefaciens* growth in our experimental system. At both time points, three replicates were destructively harvested and used to measure *R. solanacearum* inhibition and antibiotic gene expression. To test the effects of carbon resource richness on *R. solanacearum* inhibition, we prepared a cell-free (filtered at 0.22 µm) supernatant of each *B. amyloliquefaciens* culture after 18h and 36h and then directly tested the antimicrobial activity of the supernatant against *R. solanacearum.* Briefly, 106 cells *R. solanacearum* mL-1 were grown in 96-well microplates, with each well containing 20 μl of supernatant in 180 μl of Nutrient Broth. *Ralstonia solanacearum* density was measured on the basis of the constitutive mCherry fluorescence signal expression (excitation: 587 nm, emission: 610 nm) after 36 h at 30 °C (Wei *et al.* 2015).

We used quantitative reverse transcription-PCR (qRT-PCR) to quantify the expression of two genes linked to the production of bacillaene (*baeB*) and macrolactin (*minA*) under each single carbon resource and their combinations (primer sequences are listed in the Supporting Information Appendix S1). Briefly, the total RNA was isolated from *B. amyloliquefaciens* cultures after 18h and 36h of growth at 30 °C by harvesting cells by centrifugation at 4 °C (10 min, 5,000 × g). RNA was extracted using RNAiso Plus kit (TaKaRa, Dalian, China) and reverse-transcribed into cDNA in 20 μL reverse transcription mix following the manufacturer’s instructions (TaKaRa, Dalian, China). The levels of bacillaene and macrolactin transcription were measured using an SYBR Premix Ex Taq (Perfect Real Time) kit (TaKaRa, Dalian, China). Reactions were carried out on an ABI 7500 system under the following conditions: cDNA was denatured for 10 s at 95°C, followed by 40 5-cycles at 95°C and a 34s cycle at 60°C. For the quantification, plasmid standards curves were generated from the cloned target genes as described previously (Cao *et al.* 2011). The levels of gene expression were expressed as gene copy concentration (Whelan, Russell & Whelan 2003).

**Data analysis**

We analyzed effects of carbon source type and richness on *B. amyloliquefaciens* growth and antimicrobial activity (production and gene expression of bacillaene and macrolactin and direct pathogen inhibition) using separate generalized linear models (GLM) for each response variable. In the single resource experiment, we explained each dependent variable with the type (chemical class) of carbon resources (a three-level factor: sugars, organic acids and amino acids). In the resource combination experiments, we explained the dependent variables with carbon resource richness as a continuous predictor. To explore potential carbon resource identity effects, we built another model where the carbon identity was sequentially fitted before richness. In order to assess potential trade-offs between *B. amyloliquefaciens* growth (biomass) and antibiosis, we explained antibiosis (the sum of the bacillaene and macrolactin peak area at 36h measurement time point) with the main effect of biomass (OD600 after 36h) and with the interaction between the biomass and carbon resource richness (2 levels: single and multiple resources). A significant, negative main effect of biomass indicates a trade-off, while a significant interaction indicates that trade-offs are altered by resource richness. For all the data, the average of measurement replicates was used and data shown in all figures is based on true replicates. When the assumption of normality and homogeneity of variances was not met, non-parametric analyses (Figure 1) or log10 transformed data (antibiotic concentration and gene expression data) were used instead. All statistical analyses were performed with the SPSS BASE ver.22.0 statistical software (SPSS, Inc., Chicago, USA) and are included to the supplementary materials.

**Results**

**a) Identification of antimicrobial compounds underlying the inhibition of *Ralstonia solanacearum* by *Bacillus amyloliquefaciens* T-5**

The analysis of different HPLC fractions revealed that the two main antimicrobial compounds that inhibited *R. solanacearum* growth were bacillaene and macrolactin polyketides (Supporting information Appendix S1: Table S3 and Appendix S2: Figure S1). Therefore, we decided to focus on these two antibiotics in subsequent experiments.

**(b) Carbon resource type and identity affect the growth and antibiotic production of *Bacillus amyloliquefaciens* T-5**

*Bacillus amyloliquefaciens* reached the highest population densities when grown with sugars compared to amino acids or organic acids (F2,15=9.64, P=0.002; Fig. 1A). The growth of *B. amyloliquefaciens* was especially high with sucrose, D-mannitol, D-ribose, and D-mannose (Supporting information Appendix S2: Figure S2A). We detected both antibiotics in each of the tested resources. However, the level of antibiotic production depended on both carbon resource type and carbon resource identity (Fig. 1B-C, and Supporting information Appendix S2: Figure S2B-C). In general, bacillaene and macrolactin production were the lowest when *B. amyloliquefaciens* grew on sugars compared to amino acids or organic acids (effect of resource type: antibiotic production: F2, 15= 5.13, P= 0.02; Fig. 1B). Specifically, L-Arginine, L-Proline, L-Alanine, L-Histidine, myoinositol, D-ribose, lactic acid and citric acid promoted the production of both bacillaene and macrolactin (Supporting information S2: Figure S2B-C). In contrast, these antibiotics were barely produced when *B. amyloliquefaciens* T-5 was grown with sugars such as fructose, glucose, maltose, D-mannitol, D-mannitol and D-mannose (Supporting information Appendix S2: Figure S2B-C). We also found a strong positive correlation between *R. solanacearum* inhibition and bacillaene (R2=0.73, F1, 16=47.31, P<0.0001) and macrolactin peak area (R2=0.46, F1, 16=15.77, P=0.001; Supporting information Appendix S2: Figure S3), which confirms that pathogen suppression could be well explained by these two antibiotics. Together, these results suggest that sugars mainly promoted *B. amyloliquefaciens* T-5 growth while both amino and organic acids supported the production of bacillaene and macrolactin antibiotics.

**(c) Effect of carbon resource richness and identity on *B. amyloliquefaciens* T-5 growth and antimicrobial activity**

Increasing carbon resource richness promoted the growth (18h: F1, 25=16.94, P=0.0004; 36h: F1, 25=8.25, P =0.0008; Fig. 2A) and antimicrobial activity of *B. amyloliquefaciens* (*R. solanacearum* inhibition; 36h: F1, 25=4.39, P =0.04; Fig. 2B). Moreover, carbon resource richness increased the expression of both bacillaene (F1, 25=10.94, P=0.003) and macrolactin (F1, 25=14.71, P=0.0008, Fig. 2C) antibiotic genes at 18h time point. We also found the production of bacillaene was significantly improved with increasing carbon resource richness (F1, 25=4.15, P=0.05). However, no significance was observed between the production of macrolactin and carbon resource richness. The measurement time point did not change the relationship between carbon resource richness and antimicrobial activity, which indicates that antimicrobials were produced both at late exponential and past stationary growth phases (Supporting information S3: Table S3).

**(d) High carbon resource richness alleviates trade-offs between *B. amyloliquefaciens* T-5 growth and antimicrobial activity**

We found clear evidence for a growth-antibiosis trade-off when *B. amyloliquefaciens* was grown in single carbon resources (Fig. 3). However, no significant correlation was found in more complex resource environments (9 or 18 carbon combinations; Fig. 3). Together these results suggest that increasing the number of different types of available carbons allowed *B. amyloliquefaciens* to both grow and produce antibiotics at the same time.

**Discussion**

In this study, we assessed whether resource competition (inferred as biomass production) and antibiosis are affected by the type and presence of multiple carbon resources. We observed that different types of resources induced different physiological responses: monosaccharides and disaccharides promoted the growth of *B. amyloliquefaciens* T-5, while both amino- and organic acids promoted the production of antibiotics. In single carbon treatments, we found a clear trade-off between growth and antibiotics production: depending on the specific resource, bacteria exerted either efficient growth or produced lots of antibiotics but were inefficient at doing them both at the same time. In contrast, when *B. amyloliquefaciens* was grown in the presence of multiple carbon resources it was able to grow efficiently and produce high amounts of antibiotics, *i.e.*, to concurrently perform functions that were individually promoted or suppressed by different types of carbons. Together these results suggest that increasing the availability of different types of resources allows concurrent expression of multiple bacterial traits, which could potentially alter bacterial competitive dynamics and plant growth promotion in microbial communities.

The observed relationships between growth and antibiotics could be explained by physiological constraints. The simple monosaccharides and disaccharides (specifically, sucrose, D-mannose, D-ribose and D-mannitol) that can be broken down fairly easily promoted *B. amyloliquefaciens* T-5 growth most clearly. In contrast, both amino and organic acids promoted the production of bacillaene and macrolactin. One explanation for this is that amino acids are required to synthesize and compensate the metabolic costs of antibiotic production (Händel *et al.*, 2013) and could thus be important for the production of non-ribosomal polyketides (Cane, Walsh & Khosla 1998) such as bacillaene and macrolactin. Moreover, the presence of multiple different carbon resources could reduce the need for synthesizing several enzymes *de novo* (Fujita 2009), which could help the expression of multiple other functions. Together these results suggest that the environmental carbon availability is an important factor in shaping *B. amyloliquefaciens* antimicrobial activity and growth. Interestingly, no clear difference was found between the 9 and 18 carbon resource richness levels, which suggests that inclusion of more than one resource type (sugars, amino and organic acids) was already sufficient to allow simultaneous growth and antibiosis. It has previously been shown that selective use of different resources (catabolic repression) can prevent the expression and activity of other catabolic systems that enable the use of secondary substrates (Görke & Stülke 2008). Potentially, a similar mechanism may have triggered the expression of multiple traits in our study system.

We also found that some sugars (sucrose, myoinositol and D-ribose) supported efficient production of antibiotics and that, in some rare cases (e.g. Lactic acid), enhanced antibiotic production did not lead to clear pathogen suppression. Moreover, occasionally a clear inhibition was observed without a concomitant increase in bacillaene or macrolactin antibiotic production (e.g. L-Glutamine). The lack of direct pathogen inhibition despite high levels of macrolactin production could have been due to potentially higher instability or weaker antimicrobial activity of the polyketide molecule synthesized in these given carbon environments. Alternatively, in some cases, antibiotics might have been produced only when the cells were actively growing and expressing antibiotic genes. Clear pathogen inhibition in the absence of polyketide production suggests that some other unidentified antimicrobials could have been produced under the tested conditions (Chen *et al.*, 2007, 2009). Together these results suggest antibiotic production is a complex biochemical process and that polyketide antibiotic synthesis can be maintained with different types of carbons indicative of metabolic redundancy of antibiotic production.

Understanding how resource complexity shapes bacterial competitive interactions has important implications for assessing the functioning of microbiomes (Mallon *et al.* 2018). For example, understanding the balance between competitive and facilitative interactions could be crucial for determining ecosystem functioning and stability (Coyte, Schluter & Foster 2015). Our results suggest that the relative strength of resource competition and antibiosis is heavily affected by the number and type of available carbon resources. The specific *B. amyloliquefaciens* strain we used can be introduced to soils in order suppress plant-pathogenic *R. solanacearum* (Huang *et al.* 2014; Wang *et al.* 2017). The results of the present study suggest that manipulating resource diversity may be a potential tool to improve pathogen suppression by allowing biocontrol bacteria, such as *B. amyloliquefaciens*, to both efficiently compete for resources and produce high levels of secondary metabolites that will directly inhibit the pathogen. In practice, this could be achieved by using organic fertilizers that provide biocontrol agent’s growth and functioning, prebiotics that promotes the production of certain metabolites, or by selecting crops that exudate several carbons resources including sugars, amino and organic acids. One caveat of this approach is that the pathogen might also benefit from certain carbon resources (Wei *et al.* 2015) thereby increasing the likelihood of infections (Eisenreich *et al.* 2010). It is also possible that enhanced antibiotic production by *B. amyloliquefaciens* could cause unwanted collateral damage to commensal bacterial communities in the rhizosphere. More work is thus needed to understand how resource availability and the antimicrobial activity of biocontrol bacteria shapes competitive interactions and plant growth promotion in more complex plant rhizosphere microbiomes.

In conclusion, here we show that carbon resource availability could be a key abiotic parameter driving bacterial interactions by affecting the relative strength of resource competition and antibiosis. While the application of beneficial microorganisms has been suggested as one of the most promising methods for safe and sustainable crop-management practices (Francis, Holsters & Vereecke 2010; Bhattacharyya & Jha 2012), our results show that, in order to achieve this, we need to understand to what extent environmental conditions promote or constrain the expression of desired biocontrol traits via trade-offs.

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**Figure Legends**

**Figure 1.** The effect of different carbon resources (amino acids, organic acids and sugars) on *B. amyloliquefaciens* T-5 strain growth (biomass) (A), and bacillaene (ba) and macrolactin (ma) antibiotics production (B) . Different lowercase letters above the boxplots indicate for significant mean differences between carbon resource types (Duncan’s test; alpha = 0.05) and individual observations show the bacterial performance on specific carbon resources. Each point is the mean of 3 measurement replicates.

**Figure 2.** The relationship between *B. amyloliquefaciens* T-5 strain growth (biomass) (A), *R. solanacearum* inhibition (B), antibiotic gene expression (C) and the production of antibiotics (D) as a function of carbon resource diversity (1, 9 or 18 multi-carbon combinations). In panels A and B, black triangles and white circles denote for *B. amyloliquefaciens* growth after 18 and 36 hours, and in panels C and D, the expression and production of bacillaene (black triangles) and macrolactin (white circles) antibiotics after 36 h of *B. amyloliquefaciens* growth. Antibiotics production data were log10 transformed prior to statistical analysis. Each point is the mean of 3 measurement replicates.

**Figure 3.** The relationship between *B. amyloliquefaciens* T-5 strain antibiotic production and growth (biomass) in simple (white symbols) and diverse (blue symbols) carbon resource environments. Diverse carbon resource environments include both 9 and 18 carbon combination and significant, a negative correlation was observed only in the simple carbon resource environment. Each point is the mean of 3 measurement replicates.