Running Head: Growth rate and flexible stoichiometry

Title: Growth rate and resource imbalance interactively control biomass stoichiometry and elemental quotas of aquatic bacteria

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affect the cycles of energy, carbon, and nutrients within ecosystems. Because the stoichiometry of a bacterium’s resources is not necessarily the same as the stoichiometry required for biomass and metabolism, bacteria are likely to experience stoichiometric imbalance and low relative growth rates in many ecosystems. Understanding the interactions among resource availability, growth rate, and biomass stoichiometry is key to understanding how biogeochemical cycles will respond to imbalance in the availability of organic carbon and inorganic nutrients (Elser et al. 2009, Peñuelas et al. 2012).

In a seminal paper describing the saturating relationship between resource availability and growth rate, Monod (1949) hypothesized that the biochemical composition of a bacterium is invariant when the cell is growing exponentially near its maximum growth rate ($\mu_{\text{max}}$). He reasoned that at $\mu_{\text{max}}$, all of the linked processes and reactions necessary for growth operate at some high specific rate, the mean concentrations of all metabolites and cell constituents will be high, and the mean biochemical composition of the cells will converge. One well-studied application of Monod’s prediction is the growth rate hypothesis (GRH), which predicts that for P-limited organisms, the demand for P-rich ribosomal RNA at rapid growth rates leads to a positive correlation between growth rate and biomass P content (Elser et al. 2000, Hessen et al. 2013). For heterotrophic bacteria, the growth rate hypothesis is supported by experiments that vary the growth rate of populations and communities in chemostat culture (Chrzanowski and Kyle 1996, Makino et al. 2003, Makino and Cotner 2004). However, comparisons among multiple species of bacteria have found either a weak correlation or no relationship between $\mu_{\text{max}}$ and P-content (Mouginot et al. 2014, Zimmerman et al. 2014, Godwin and Cotner 2015b), meaning that the predictive power of the GRH is limited for such comparisons.

Although the original iterations of the GRH were applied to the P-content of biomass, the GRH is frequently extended to predict that biomass C:P and N:P should decline with increasing growth rate. There is support for this extrapolation among phytoplankton, but the effect of $\mu:\mu_{\text{max}}$ on biomass stoichiometry is conditionally dependent upon resource imbalance (Goldman et al. 1979, Hillebrand et al. 2013, Garcia et al. 2016). In a recent meta-analysis of multiple phytoplankton species, Hillebrand and others (2013) showed that phytoplankton biomass N:P was not dependent on growth rate ($\mu$) when N-limited, but N:P decreased with increasing growth rate when P-limited. Some data from phytoplankton (Hillebrand et al. 2013, Garcia et al. 2016) suggest that stoichiometric flexibility could decrease linearly with increasing relative growth rate.

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(µ:µ$_{\text{max}}$, Figure 1 A-B), but the saturating relationship between internal nutrient quota and
growth rate (Droop 1973, Klausmeier et al. 2004) suggests that stoichiometric flexibility should
diminish rapidly with increasing µ:µ$_{\text{max}}$ (Figure 1 C). Alternatively, cells could remain
stoichiometrically flexible at higher growth rates by altering their nutrient acquisition rates, cell
size (Thingstad et al. 2005), or nutrient use efficiency (Sterner and Elser 2002) (Figure 1 D).

It is increasingly acknowledged that heterotrophic bacteria have as much flexibility in
C:P$_{\text{biomass}}$ and N:P$_{\text{biomass}}$ as phytoplankton (Chrzanski and Kyle 1996, Scott et al. 2012,
Godwin and Cotner 2015a) and many strains of bacteria increase their C quotas and cell size in
response to high resource C:P (Thingstad et al. 2005, Chrzanski and Grover 2008, Godwin
and Cotner 2015b). Previous studies that measured stoichiometric flexibility in bacteria did so
across a narrow range of relative growth rates (Makino et al. 2003, Scott et al. 2012, Godwin and
Cotner 2015b) and more comprehensive data are required to determine how flexibility changes
with growth rate. Additionally, it is unclear whether the predictions based on phytoplankton N:P
would translate to bacterial N:P or C:P. Because C is partitioned between growth and respiration
in bacteria, bacterial C:P flexibility could respond to growth rate differently than N:P (in bacteria
or phytoplankton). The Droop function can be used to describe the dependence of growth rate on
C quota in the same manner as for N or P, but the uptake and accumulation of cellular C are also
dependent on growth rate-dependent respiration, growth rate-independent respiration
(maintenance metabolism), and energetic costs associated with nutrient limitation (Thingstad
1987). Although respiration could diminish C quotas under some circumstances, its effect on
stoichiometric flexibility is uncertain.

Here we describe an experiment where we tested two hypotheses related to the three-way
interaction among resource stoichiometry, growth rate, and biomass stoichiometry. Hypothesis 1:
For individual strains, stoichiometric flexibility in C:P$_{\text{biomass}}$ and N:P$_{\text{biomass}}$ decreases non-
linearly with increasing growth rate (Figure 1 C). Hypothesis 2: C:P$_{\text{biomass}}$, N:P$_{\text{biomass}}$, cell quotas,
and cell size increase with resource C:P at low growth rates, but as the growth rate approaches
µ$_{\text{max}}$, quotas of C, N, and P increase, cell size increases, and the effect of resource C:P on quotas
decreases. To test these hypotheses, we cultured two strains of aquatic heterotrophic bacteria
with different growth rates, element quotas, and stoichiometric flexibility in chemostats using a
factorial design of varying dilution rate and resource C:P. We describe the interactive effect of
growth rate and resource stoichiometry on biomass C:N:P, elemental quotas, cell size, and metabolic rates.

METHODS

The two strains of bacteria used for the present study were previously described by Godwin and Cotner (2015b). The isolates were selected to provide a contrast in terms of stoichiometric flexibility. *Brevundimonas* strain D703 has highly flexible biomass stoichiometry, a low minimum cell P quota (0.013 fmoles cell$^{-1}$), and an apparent maximum growth rate of 0.159 h$^{-1}$ at 20ºC in the defined medium described below. *Achromobacter* strain D1207 has moderately flexible biomass stoichiometry, a higher minimum P quota (0.032 fmoles cell$^{-1}$), and an apparent maximum growth rate of 0.057 h$^{-1}$. Hereafter, we refer to the strains as *Achromobacter* and *Brevundimonas*. Both strains were preserved as glycerol stocks at -80ºC and revived for each culture. Bacteria were grown using basal microbiological medium (BMM, Tanner 2002) with glucose supplied as the sole carbon source at 23.88 mmoles-C L$^{-1}$. Inorganic phosphate in the medium was manipulated to achieve molar C:P$^\text{supply}$ ratios of 100, 316, 1,000, 3,162, and 10,000:1. By manipulating the concentration of phosphate in the medium, we were able induce P-limitation at high population densities and cause the bacteria to experience stoichiometric imbalance (Scott et al. 2012, Godwin and Cotner 2014, 2015a). For this reason, we denote levels of P supply as C:P$^\text{supply}$ ratios and also include the P concentration where appropriate. Previous studies using this system have show that bacteria switch from C- to P-limitation at C:P supply ratios between 100 and 300:1 (Scott et al. 2012, Godwin and Cotner 2015b). All other inorganic nutrients were supplied in excess relative to the stoichiometric demand for C and P (i.e. C:N=1.3:1).

Chemostat Cultures. Each strain was cultured in duplicate chemostats using a factorial design of C:P$^\text{supply}$ and dilution rates equivalent to 10, 20, 40, 60, 80, and 95% of their apparent $\mu_{\text{max}}$ (as measured in nutrient-replete batch cultures). *Brevundimonas* was also cultured at 5% of $\mu_{\text{max}}$. Each 100 mL polypropylene chemostat was inoculated with 50 mL of batch culture grown in medium with the same initial C:P$^\text{supply}$. The chemostats were mixed and aerated with sterile air and maintained at 20ºC in darkness. All chemostats were harvested at a single time point. At dilution rates equal to 40% of $\mu_{\text{max}}$ and greater, the chemostats were harvested after nine times the reciprocal of the dilution rate. To minimize potential wall growth at dilution rates of 20%
µ<sub>max</sub> or less, we monitored the optical density at 600 nm and once the cultures reached steady state biomass, we maintained the cultures for three times the reciprocal of the dilution rate prior to harvesting. Due to low biomass, we obtained limited data from cultures grown using the combination of rapid dilution rate and high C:P supply. In total, 133 independent chemostat runs were included in this study. We measured the residual phosphate in the supernatant of centrifuged chemostat cultures using the ascorbic acid molybdenum method (APHA 1995), and used a detection limit equal to the 99% confidence interval for a large number of process blanks.

**Biomass Elemental Analyses.** Samples of bacterial biomass were collected onto Whatman GF/F filters (0.7 µm nominal retention) and frozen at -20ºC prior to analysis for particulate C, N, and P. Filter samples for C and N determination were dried at 60ºC and analyzed using a Perkin Elmer 2400 CHN Analyzer with a zooplankton recovery standard. Filter samples for P determination were digested with acid persulfate and analyzed using the ascorbic acid molybdenum method (APHA 1995) with a spinach leaf recovery standard (NIST reference material). Process filter blanks were included in each run and were used to determine detection limits (99% confidence level). We have shown previously that small amounts of non-bacterial C and N from the medium are retained on the filters (<1% of the C and N measured on filters in this study), but there was no significant retention of P (Godwin and Cotner 2015a).

**Flow Cytometry.** Cells of *Achromobacter* formed small aggregates that could not be completely dispersed and therefore flow cytometry data are not presented for this strain. Samples from *Brevundimonas* were preserved with buffered formaldehyde to a final concentration of 3.7% and stored at 4ºC until use. Duplicate samples from each culture were diluted with sodium pyrophosphate and vortexed to improve cell dispersion (Velji and Albright 1993). Fluorescent beads (Spherotech) were added to each sample as an internal standard to enumerate cells. Bead stocks were assayed daily by epifluorescence microscopy. Samples were stained with SybrGreen (Invitrogen, Carlsbad, CA) to a final concentration of 0.05% and incubated for at least 10 minutes prior to analysis. Cytometry was performed using a Becton Dickson FACSCalibur with a 488 nm laser. Side scatter and fluorescence at 530 nm were used to identify populations of cells and beads. At least 9,000 cell events were recorded for each sample.

**Respirometry.** To measure the rate of O<sub>2</sub> consumption and CO<sub>2</sub> production, we incubated air-equilibrated samples from the chemostats in 6 mL gas-tight vials at 22ºC. At multiple time points up to 2 hours, we preserved the samples with mercuric chloride to a final concentration of

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0.015% and stored the vials at 4°C until analysis by membrane-inlet mass spectrometry (Kana 1994). The oxygen concentration was determined using the O₂:Ar method (Kana 1994) with air-equilibrated BMM medium as a reference standard. Carbon dioxide content was determined using a cryotrap at -140°C, which retained water vapor but allowed CO₂ to be measured. Flasks of BMM with mercuric chloride were saturated with calibrated gas standards of CO₂ and used to determine the pCO₂ in each sample. The total CO₂ in each sample was determined from pCO₂ and the estimated endpoint pH after the addition of mercuric chloride and pCO₂ from respiration. Respiration rates were calculated as the decrease in O₂ or increase in total CO₂ per unit time and normalized to cell abundance for Brevundimonas. The rate of biomass production at steady state was calculated as the product of the C quota and the dilution rate. The apparent bacterial growth efficiency was calculated as the ratio of biomass production to the sum of production and respiration.

**Batch Cultures.** We performed a parallel experiment to the chemostats, but using batch cultures grown in the same medium. The objectives of this experiment were to 1) characterize biomass stoichiometry at µ\text{max} (which is difficult in chemostats) and determine whether batch cultures could be used to provide a similar measurement of stoichiometric flexibility as the chemostats. The initial concentration of C and P would not be limiting in batch cultures, even at high C:P. But as population growth depletes the resources, C or P becomes limiting and the growth rate decreases in response. We sought to measure the biomass stoichiometry during the initial density-independent phase and at multiple points during density dependence. At each level of C:P\text{supply} used for the chemostats, we inoculated 24 cultures (100 mL) with varying volumes of frozen glycerol stocks and incubated the cultures at 22-24°C on an orbital shaker. Because multiple simultaneous cultures were used for this experiment, we consolidated these growth curves using simple models based on population densities (optical density at 600 nm, OD\text{600}). For each individual batch culture, consecutive OD\text{600} measurement pairs were used to compute instantaneous growth rates. Population growth was modeled as density-dependent for C:P\text{supply} of 3,162:1 and 10,000:1 and using a biphasic model with density-independent growth followed by density dependence for C:P\text{supply} of 100:1 to 1,000:1 (Appendix S1).

**Statistical Analyses.** For the chemostat culture experiment, a two-way analysis of variance (ANOVA) was used for each strain to examine the effects of growth rate, log C:P\text{supply}, and their interaction on the dependent variables. Biomass stoichiometric ratios were log-
transformed prior to ANOVA to improve normality. We were unable to test for an interaction between growth rate and medium C:P in the batch cultures because the range of population density and growth rate attained was different for each C:P treatment. In batch culture samples where the bacteria were growing at $\mu:\mu_{\text{max}}$ of 95% or more, we evaluated the effect of C:P supply by one-way ANOVA. All statistical analyses were performed using R version 3.2.3.

RESULTS

Chemostats. Biomass yields decreased with increasing C:P supply (i.e. decreasing P concentration) in both strains, but at low $\mu:\mu_{\text{max}}$ the biomass of *Brevundimonas* was highest at C:P supply of 316:1 (Appendix S1: Fig. S2). At C:P supply of 316:1 and greater, both strains depleted the available phosphate to low levels, depending upon $\mu:\mu_{\text{max}}$ (Appendix S1: Figs. S3 and S4). Biomass stoichiometry for *Brevundimonas* ranged from C:N:P of 47:9:1 to 618:86:1 and in *Achromobacter* it ranged from 42:8:1 to 290:35:1 (Figure 2). All three elemental ratios (C:P$_{\text{biomass}}$, N:P$_{\text{biomass}}$, and C:N$_{\text{biomass}}$) were strongly affected by C:P supply in both strains (Figures 2, Appendix S1: Figs. S5 and S6). Growth rate and the interaction between growth rate and C:P$_{\text{supply}}$ also had strong effects on the biomass stoichiometry of each strain, but in *Achromobacter* the interaction was significant for C:P$_{\text{biomass}}$ and N:P$_{\text{biomass}}$ only (p<0.05). In both strains, C:P$_{\text{supply}}$ had no significant effect on biomass stoichiometry at high $\mu:\mu_{\text{max}}$. At a C:P$_{\text{supply}}$ of 100:1, biomass ratios in *Achromobacter* were not significantly affected by growth rate, but for *Brevundimonas*, C:P$_{\text{biomass}}$ and C:N$_{\text{biomass}}$ ratios decreased in response to increasing $\mu:\mu_{\text{max}}$ (Figures 2, Appendix S1: Figs. S5 and S6).

In *Brevundimonas*, both P and C quotas were strongly affected by C:P$_{\text{supply}}$ and growth rate, but only the P quota showed a significant interaction between C:P$_{\text{supply}}$ and growth rate (Figure 2). At low $\mu:\mu_{\text{max}}$, P quotas decreased markedly between C:P$_{\text{supply}}$ of 100:1 and 316:1. At $\mu:\mu_{\text{max}}$ of 60% or greater, the P-quotas were substantially higher and increased with increasing C:P$_{\text{supply}}$. At every $\mu:\mu_{\text{max}}$, C quotas increased with increasing C:P$_{\text{supply}}$ and the C quota was generally higher at high growth rates. When analyzed across levels of C:P$_{\text{supply}}$, the correlation coefficient between C and P quotas was negative at 5% $\mu_{\text{max}}$, then increased to a strong positive correlation between 40 and 80% of $\mu_{\text{max}}$, and declined again at 95% $\mu_{\text{max}}$. Quotas of C and N were strongly correlated at every level of $\mu:\mu_{\text{max}}$ ($r^2>0.95$).

The flow cytometry characteristics of *Brevundimonas* were responsive to both C:P$_{\text{supply}}$ and growth rate (Appendix S1: Fig. S7). These changes in flow cytometry observations were
consistent with cell elongation in response to P limitation, as observed in a previous study with these strains (Godwin and Cotner 2015b). At $\mu:\mu_{\text{max}}$ of 5-40%, SybrGreen fluorescence (proportional to DNA content), side scatter, and forward scatter all increased with increasing $C:P_{\text{supply}}$. At $\mu:\mu_{\text{max}}$ of 60-95%, the flow cytometry characteristics were much less sensitive to $C:P_{\text{supply}}$. At $C:P_{\text{supply}}$ of 100 to 316:1, the cells became larger at faster growth rates. But, at higher $C:P_{\text{supply}}$ the cells became smaller at faster growth rates. At 5 to 20% $\mu:\mu_{\text{max}}$, both cell size and C quota increased in response to increasing $C:P_{\text{supply}}$ (Appendix S1: Figs. S9 and S10). At $60% \mu:\mu_{\text{max}}$, the cells remained the same size and the quotas of C, N, and P increased in response to increasing $C:P_{\text{supply}}$.

For *Brevundimonas*, both O$_2$ consumption and CO$_2$ production showed strong effects of $C:P_{\text{supply}}$, growth rate, and an interaction. At low growth rates, cell-specific respiration rates (both O$_2$ and CO$_2$) increased with increasing $C:P_{\text{supply}}$. At higher growth rates, the respiration rates were much higher and were less sensitive to $C:P_{\text{supply}}$. The apparent bacterial growth efficiency (BGE) ranged from about 30 to 80% (Figure 3) and was most sensitive to $C:P_{\text{supply}}$ at low $\mu:\mu_{\text{max}}$, decreasing from 60% at $C:P_{\text{supply}}$ of 100:1 to less than 40% at $C:P_{\text{supply}}$ greater than 1,000:1.

**Batch Cultures.** In batch cultures, both strains exhibited variable growth rates in response to population density and phosphorus availability (Appendix S1: Fig. S1 and Table S1). At high $C:P_{\text{supply}}$ neither strain was observed to grow at its apparent $\mu_{\text{max}}$. In early batch culture, when the growth rate was density-independent (at or near $\mu_{\text{max}}$), the bacterial biomass stoichiometry was not sensitive to resource stoichiometry. During density-dependent growth, the biomass stoichiometry was sensitive to both the resource stoichiometry and the growth rate. For both strains, the chemostats and batch cultures showed that $C:P_{\text{biomass}}$ decreased with growth rate and the effect was most profound at high $C:P_{\text{supply}}$ (Figure 4). In batch cultures at $C:P_{\text{supply}}$ of 100:1, the $C:P_{\text{biomass}}$ stoichiometry increased slightly with growth rate in *Brevundimonas* (p<0.01) and was not correlated with growth rate in *Achromobacter*. At every level of $C:P_{\text{supply}}$, decreasing growth rate in the batch cultures led to a significant reduction in the P quota of *Brevundimonas*. Carbon quotas also decreased at lower growth rates although this was only significant for $C:P_{\text{supply}}$ of 100 to 3,162:1.

Biomass stoichiometry (C:P, N:P, and C:N) did not differ between quickly-growing chemostat cultures (80-95% $\mu:\mu_{\text{max}}$) and batch cultures where the instantaneous growth rate was...
> 95% of $\mu_{\text{max}}$ (t-test $p>0.05$). At high growth rate, the median C:N:P_{biomass} was 73:14:1 for Brevundimonas (95% confidence interval: C:P_{biomass} of 68.5-77.4 and N:P_{biomass} of 12.8-14.2:1) and 70:13:1 for Achromobacter (95% CI: C:P_{biomass} of 62.6-76.7 and N:P_{biomass} of 11.8-13.7).

**DISCUSSION**

Here we show that relative growth rate strongly modified the relationship between resource stoichiometry and bacterial biomass composition. Overall, the physiology of the bacteria and their response to P availability was markedly different at low versus high $\mu:\mu_{\text{max}}$, with the most extreme biomass stoichiometry observed at the lowest growth rates. In the following sections, we discuss the effect of this interaction on biomass stoichiometry, element quotas, size, and respiration.

We hypothesized that for individual strains, stoichiometric flexibility decreases non-linearly with increasing $\mu:\mu_{\text{max}}$. This hypothesis was supported in both strains and the biomass stoichiometry was essentially homeostatic at $\mu:\mu_{\text{max}}$ greater than 50%. The non-linear pattern in Figure 2 (panels B and D) resembles the pattern depicted in Figure 1C and is consistent with the limited data for heterotrophic bacteria (Makino and Cotner 2004) and previous studies in phytoplankton (Goldman et al. 1979, Hillebrand et al. 2013). A recent chemostat study with the cyanobacterium *Synechococcus* found that under P limitation, N:P_{biomass} decreased linearly with growth rate between $\mu:\mu_{\text{max}}$ of 0 to about 70% (Garcia et al. 2016). Across a similar range of $\mu:\mu_{\text{max}}$, our data for C:P_{biomass} and N:P_{biomass} are distinctly non-linear (Figure 2 and Appendix S1: Fig. S5), which suggests that surplus accumulation of C and N was more pronounced in this heterotrophic bacterium.

The non-linear response of C:P_{biomass} and N:P_{biomass} to increasing $\mu:\mu_{\text{max}}$ is attributable to the saturating relationship between internal quotas and growth rate, as described by the Droop cell quota model (Droop 1974, Thingstad 1987, Klausmeier et al. 2004). At low $\mu:\mu_{\text{max}}$, the quota of any element that limits the growth rate approaches its minimum quota, so the quota of non-limiting elements can vary substantially in response to surplus availability (Chrzanowski and Kyle 1996, Vrede et al. 2002, Thingstad et al. 2005, Godwin and Cotner 2015b). At higher $\mu:\mu_{\text{max}}$, the quotas of all elements approach their values at $\mu_{\text{max}}$ and the quota of any single element becomes less flexible than at low $\mu:\mu_{\text{max}}$. As a result, the stoichiometric ratio of any two elements becomes less flexible at high $\mu:\mu_{\text{max}}$. The effective $\mu_{\text{max}}$ can be different for each element in the Droop model (Cherif and Loreau 2010, Garcia et al. 2016), which contributes to
the nonlinear pattern in biomass stoichiometry at increasing growth rates. Although we were unable to parameterize the Droop model for C and N in our experiment, our estimates of effective $\mu_{\text{max}}$ based on P quotas under P limitation (C:P$_{\text{supply}}$ > 100:1) were substantially lower than the actual $\mu_{\text{max}}$ for *Brevundimonas* (Appendix S1: Fig. S8).

It is clear that elemental ratios can overlook important trends in quotas. We hypothesized that C:P$_{\text{biomass}}$, N:P$_{\text{biomass}}$, cell quotas, and cell size would increase with resource C:P at low growth rates, but as the growth rate approaches $\mu_{\text{max}}$, quotas of C, N, and P would increase, cell size would increase, and the effect of resource C:P on quotas would decrease. Overall, the chemostat experiments support our hypotheses, but there were important deviations from our expectations. At low growth rates, P quotas were negatively coupled to C and N quotas in *Brevundimonas* and plasticity in C and N quotas was associated with an increase in cell size under P-limitation, which has been described previously for this isolate and other aquatic bacteria (Thingstad et al. 2005, Godwin and Cotner 2015b). In contrast, at high $\mu$:$\mu_{\text{max}}$, element ratios and cell size were constrained, which seems to suggest homeostatic composition. However, the C, N, and P quotas at high $\mu$:$\mu_{\text{max}}$ varied substantially in response to growth rate and C:P$_{\text{supply}}$ (Figure 2, Appendix S1: Figs. S9 and S10). This finding indicates strong positive coupling of C, N, and P quotas at high growth rates, which is predicted by the Droop model (Droop 1973).

There were strong effects of both growth rate and P availability on the relationship between cell size and C quota. Cell size is expected to increase with increasing growth rate (Vadia and Levin 2015, Garcia et al. 2016). Cell size increased somewhat with growth rate at C:P$_{\text{supply}}$ of 100:1 and 316:1, but the cells became smaller with increasing growth rate at higher C:P$_{\text{supply}}$. High C:P$_{\text{supply}}$ ratios tend increase bacterial cell volume and C quotas (Thingstad et al. 2005, Godwin and Cotner 2015b), but in *Brevundimonas* this effect was only apparent at low $\mu$:$\mu_{\text{max}}$ (Appendix S1: Fig. S9). At higher $\mu$:$\mu_{\text{max}}$, the cells changed their C quota substantially but cell size changed very little. Although we expected C and N quotas to increase at high $\mu$:$\mu_{\text{max}}$, the quotas were highest at $\mu$:$\mu_{\text{max}}$ of 60% (Figure 2 and Appendix S1: Fig. S8). Combined with the observation that cell size changed little above 60% $\mu$:$\mu_{\text{max}}$, it is apparent that the cells changed their C content independently of size. This behavior is dramatically different from the plasticity observed at low $\mu$:$\mu_{\text{max}}$ and the behavior of *Synechococcus* (Garcia et al. 2016). Importantly, the strong correlation between C and N quotas suggests that this surplus accumulation was not due to C-rich storage polymers (Sterner and Elser 2002).
The respiration rate of *Brevundimonas* increased with $\mu:\mu_{\text{max}}$, consistent with models of bacterial metabolism (Thingstad 1987, Russell and Cook 1995), but there was also a dramatic effect of C:P$_{\text{supply}}$ on respiration rate. At all $\mu:\mu_{\text{max}}$ below 50%, increasing C:P$_{\text{supply}}$ (decreasing P availability) led to a several-fold increase in the respiration rate and a decrease in bacterial growth efficiency. The increase in specific respiration rate at high C:P$_{\text{supply}}$ is similar to the respiration of excess C by phagotrophs consuming C-rich but nutrient-poor prey items (Frost et al. 2005, Jeyasingh 2007, Hessen and Anderson 2008). However, osmotrophic heterotrophs should not need to consume excess C at high resource C:P in order to obtain inorganic P. One explanation for this response is that under these conditions, the uptake and respiration of glucose were decoupled from the immediate growth demands of the bacteria (Russell and Cook 1995). A possible reason for this decoupling is that high-affinity P-uptake mechanisms increased the energetic demands of the bacterium (Rosenberg et al. 1979), leading to an increase in respiration rate relative to the growth rate-dependent rate (Tempest and Neijssel 1978, Thingstad 1987).

These mechanisms are heavily dependent upon intracellular ATP and phosphate, which would seem a contradictory strategy under P-limitation, unless ATP turnover is rapid (Russell and Cook 1995).

Experiments to characterize stoichiometric physiology have important design limitations. The present study shows that data from batch cultures and chemostats are comparable when growth rates approach $\mu_{\text{max}}$, but during density-dependent growth the two methods showed inconsistent agreement. Although batch cultures might yield the same physiological state as steady-state chemostat cultures, the transient nature of both resource concentration and growth rate in batch cultures makes it difficult to separate the effect of resource availability from that of growth rate. This methodological issue is important for characterizing the stoichiometric physiology of other osmotrophic organisms such as phytoplankton. Meta-analyses that combine data from batch cultures and continuous cultures require careful interpretation (Persson et al. 2010), but can also identify broad-scale patterns that are difficult to detect in individual studies (Hillebrand et al. 2013). Ongoing efforts to characterize stoichiometric flexibility in diverse taxa would benefit from robust high-throughput methods for measuring biomass composition.

Our study shows that resource imbalance controls bacterial biomass stoichiometry and element quotas at low growth rates, but when growth rate exceeds about 50% of $\mu_{\text{max}}$, resource imbalance has little impact on biomass stoichiometry and C, N, and P quotas increase.
proportionally in response to resource imbalance and growth rate. Based on this work and
previous studies, bacterial biomass can be modeled as a flexible pool of C, N, and P at low
growth rates. It is increasingly recognized that stoichiometric flexibility has important
implications for biogeochemical cycles (Galbraith and Martiny 2015). Although the majority of
bacterial isolates from lakes exhibit stoichiometric flexibility at low \( \mu : \mu_{\text{max}} \) (Scott et al. 2012,
Godwin and Cotner 2014), mass balance models typically assume a fixed stoichiometry for their
biomass. However, for bacteria growing at high \( \mu : \mu_{\text{max}} \), our results support the use of models
with homeostatic biomass stoichiometry.

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**FIGURE LEGENDS**

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Figure 1. Schematic plots of C:P biomass versus relative growth rate (µ:µmax) with different nutrient status scenarios (A-D, after Hillebrand et al 2013). In all plots, the shading represents varying degrees of nutrient imbalance, as denoted by the arrows. The dashed line represents the biomass stoichiometry under co-limitation and has a negative slope in panels B-D.

Figure 2. Biomass C:P as a function of C:P supply and relative growth rate (µ:µmax) for Achromobacter (A-B) and Brevundimonas (C-D). Lines are segmented fits to the mean values and the points are the replicate chemostat and the black dashed lines represent the Redfield ratio of C:P_biomass = 106:1. For each strain, the two-way ANOVA tests showed significant effects of C:P supply, µ:µmax, and an interaction (all p<0.01) for C:P_biomass. Cell quotas of C and P for Brevundimonas across C:P supply (E-F). The two-way ANOVA tests showed significant effects of C:P supply, µ:µmax, and an interaction (all p<0.01) for P quotas and significant effects of C:P supply and µ:µmax for C quotas (p<0.001). The significance of one-way ANOVA tests at each level of µ:µmax is denoted in the legends (*p < 0.05, **p<0.01, ***p<0.001).

Figure 3. Cell-specific O2 consumption rate (A), CO2 production rate (B), and bacterial growth efficiency (C) for Brevundimonas as a function of C:P supply, with separate symbols for levels of µ:µmax. The two-way ANOVA tests showed significant effects of C:P supply, µ:µmax, and an interaction for O2 consumption, CO2 production, and BGE (p<0.01).

Figure 4. Dependence of bacterial C:P_biomass on growth rate (µ, h⁻¹) at each level of C:P supply. Each panel contains data from both chemostats and batch cultures. Growth rates for batch cultures are predicted from the growth models described in Appendix S1.
A) *Achromobacter*

C:P supplying (µmoles–P L⁻¹)

- **µ:µmax**
  - 0.10*  
  - 0.20***
  - 0.40**
  - 0.60

B) *Achromobacter*

C:P supplying (µmoles–P L⁻¹)

- 100:1 (239)
- 316:1 (75.6)
- 1,000:1 (23.9)
- 3,162:1 (7.55)
- 10,000:1 (2.39)

C) *Brevundimonas*

C:P supplying (µmoles–P L⁻¹)

- **µ:µmax**
  - 0.05***
  - 0.10*  
  - 0.40
  - 0.60***
  - 0.80
  - 0.95

D) *Brevundimonas*

C:P supplying (µmoles–P L⁻¹)

- 100:1 (239)
- 316:1 (75.6)
- 1,000:1 (23.9)
- 3,162:1 (7.55)
- 10,000:1 (2.39)

E) *Brevundimonas*

P Quota (fmole cell⁻¹)

- **µ:µmax**
  - 0.05***
  - 0.10**

F) *Brevundimonas*

P Quota (fmole cell⁻¹)

- 0.05***
- 0.10**
- 0.40**
- 0.80*
Figure 3: Graphs showing the relationship between C:P supply (µmoles-P L\(^{-1}\)) and metabolic rates (O\(_2\) consumption and CO\(_2\) production) for different RGR treatments (5%, 10%, 20%, 40%, 60%, 80%, 95%).

**A)** O\(_2\) Consumption (fmoles O\(_2\) cell\(^{-1}\) h\(^{-1}\))

**B)** CO\(_2\) Production (fmoles CO\(_2\) cell\(^{-1}\) h\(^{-1}\))

**C)** Apparent BGE

The graph shows a trend where higher RGR treatments generally result in higher metabolic rates, especially at lower C:P supply levels. The data points are color-coded to represent different RGR treatments.