Vol. 73: 245–258, 2014
doi: 10.3354/ame01719

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online December 5

Carbon:phosphorus homeostasis of aquatic bacterial assemblages is mediated by shifts in assemblage composition

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ABSTRACT: Several studies have noted a disparity between the stoichiometric regulation of bacterial assemblages and populations. In response to phosphorus availability, assemblages of bacteria often exhibit greater flexibility in their biomass carbon (C) to phosphorus (P) ratios (C:P) than axenic populations, some of which are homeostatic. We hypothesized that assemblages are inherently non-homeostatic as the result of resource-driven shifts in dominance between more homeostatic strains at low resource C:P ratios and highly flexible strains when P is scarce relative to C. We enriched 6 assemblages of heterotrophic bacteria from 4 lakes in Minnesota, USA, using chemostats with varying supply C:P ratios and measured the bacterial biomass C:P ratios. The initial assemblage cultures exhibited non-homeostasis in biomass C:P across treatments, but there was no significant effect of lake trophic state on the strength of homeostasis. This suggests that the non-homeostatic physiology was abundant in each of the lakes. Using the initial assemblage chemostat cultures as a species sorting filter, we subsequently cultured the high-P and low-P selected fractions at varying supply C:P ratios and found that all of the low-P selected fractions were non-homeostatic, and that the high-P selected fractions were strongly homeostatic in 3 of the lakes. These results demonstrate that multiple stoichiometric regulation strategies were present among *in situ* bacterial communities and confirm that high P availability can select for homeostatic bacterial strains. The divergence in stoichiometric regulation strategies was coupled with changes in the assemblage composition, which highlights the role of ecological selection in the stoichiometric homeostasis of assemblages.

KEY WORDS: Heterotrophic bacteria · Phosphorus · Stoichiometry · Homeostasis · Lakes

INTRODUCTION

A fundamental feature of biology is homeostasis, or the ability of an organism to maintain a consistent physiological state despite variability in the environment. The strength of homeostasis differs among taxa; for example, birds and mammals exhibit strong homeostasis in body temperature whereas other vertebrates show weaker temperature regulation. With respect to homeostasis and elemental composition, many organisms are confronted with a fundamental imbalance between the chemical composition of their resources and their biomass stoichiometry. Plants and phytoplankton encounter stoichiometric imbalance when the availability of individual nutrients is variable across space and time (Hall et al. 2005, Yu et al. 2011) and grazers or predators are subject to imbalance due to variability in the nutrient and energy content of their prey (Elser & Hassett 1994, Hood & Sterner 2010). In response to nutrient imbalance, many primary producers exhibit weak homeostasis and alter their biomass chemistry to reduce their nutrient demands (Rhee 1973, Klausmeier et al. 2004), whereas many heterotrophs

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exhibit strong homeostasis (Sterner & Elser 2002, Makino et al. 2003, Persson et al. 2010). The stoichiometry of single species has been examined for a diverse suite of organisms (Sterner & Elser 2002, Elser et al. 2003, Persson et al. 2010), but comparatively few studies have examined the response of whole assemblages to resource imbalance (Tezuka 1990, Makino & Cotner 2004, Fanin et al. 2013). Because resource availability influences both species abundance and biomass stoichiometry, the elemental composition of assemblages may respond to imbalance more readily than populations of single species. This distinction is particularly important over longer timescales or under conditions where biomass turnover is rapid (i.e. chemostats).

Heterotrophic bacteria are responsible for most of the remineralization of organic carbon (C) in aquatic ecosystems (Biddanda et al. 2001, Cotner & Biddanda 2002) and affect the availability of dissolved nitrogen (N) and phosphorus (P) (Kirchman 1994, Danger et al. 2007). As consumers of multiple independent resources (e.g. dissolved organic C and inorganic nutrients), heterotrophic bacteria are likely to encounter elemental imbalance, particularly in unproductive ecosystems (Cotner & Biddanda 2002). The biomass C:N:P stoichiometry (C:N:$P_{biomass}$) and strength of homeostasis determine how bacteria respond to the availability of multiple elements and the rates at which they can assimilate or regenerate these elements (Hall et al. 2011). Models of consumer-driven nutrient recycling based on phagotrophic consumers (Sterner 1990, Elser & Urabe 1999) predict that strongly homeostatic organisms with low C:$P_{biomass}$ should regenerate inorganic P at low C:P_{supply} (C limitation) and remineralize C at high C:P_{supply} (P limitation). Although some strains of bacteria exhibit strict homeostasis at low C:$P_{biomass}$ (Makino et al. 2003, Godwin 2013), strains with varying degrees of flexibility in C:N:$P_{biomass}$ can assimilate excess C and P, serving to mediate imbalance between biomass and resources. To date, there have been no strains described that exhibit strong homeostasis at high C:$P_{biomass}$ (Scott et al. 2012, Godwin 2013), suggesting that strong homeostasis is associated with low C:$P_{biomass}$.

Studies investigating the ecological stoichiometry of heterotrophic bacteria show an apparent discrepancy between the C:P homeostasis of single strains and that of assemblages. Several strains of heterotrophic bacteria from the class *Gammaproteobacteria* (*Escherichia coli*, *Pseudomonas* sp., and *Vibrio* sp.) are homeostatic and have low C:$P_{biomass}$ (Bratbak 1985, Makino et al. 2003, Løvdal et al. 2008). In contrast to the viewpoint that bacteria are P-rich and strongly homeostatic, assemblages of bacteria from lakes adjust their C:$P_{biomass}$ in response to resource stoichiometry and often have higher C:$P_{biomass}$ than single strains (Tezuka 1990, Makino & Cotner 2004). Recent work with bacterial strains isolated from lakes has shown that C:$P_{biomass}$ can be higher than previously assumed and that some strains have flexible C:$P_{biomass}$ (Scott et al. 2012). Although stoichiometric regulation has been characterized for few assemblages, the available data suggest that assemblages are mostly non-homeostatic (Makino & Cotner 2004, Danger et al. 2008, Fanin et al. 2013).

Makino et al. (2003) demonstrated strong C:P homeostasis in *Escherichia coli* and hypothesized that if assemblages were composed of only homeostatic strains with different C:$P_{biomass}$, resource-dependent shifts in the relative abundance of strains would lead to non-homeostasis in the assemblage. This hypothesis has been implicated in subsequent studies demonstrating non-homeostasis of assemblages (Danger et al. 2008, Fanin et al. 2013), but the assumption of strong homeostasis at the species level is seldom tested directly (Kaiser et al. 2014). In contrast, in assemblages where a gradient of stoichiometric regulation is present, both physiological acclimation and resource-dependent changes in assemblage composition could determine the aggregate stoichiometry. This dynamic seems probable since availability of inorganic N and P causes shifts in microbial community composition (Haukka et al. 2006), and individuals strains of bacteria isolated from lakes exhibit a range of stoichiometric regulation (Scott et al. 2012).

Because assemblages appear to be stoichiometrically flexible and are composed of strains with varying degrees of plasticity, we hypothesized that within a single assemblage of bacteria, the strains that are dominant under low C:P_{supply} ratios are more strongly homeostatic than the strains that are dominant at high C:P_{supply} ratios. We sought to answer 3 questions related to this hypothesis: (1) Does the strength of homeostasis in an assemblage depend upon ecosystem productivity? (2) Are homeostatic strains dominant at low resource supply ratios? (3) Do shifts in assemblage composition explain non-homeostasis observed in assemblages? We addressed these questions using selection experiments in 4 lakes of varying productivity where we enriched natural assemblages of bacteria at a range of C:P_{supply} in continuous cultures, and subsequently characterized the degree of homeostasis in the assemblages selected under the highest and lowest P availability. If multiple stoichio-

metric strategies were present within *in situ* bacterial communities, and the strength of homeostasis was linked to competitive ability for C and P, then the high-P and low-P selected fractions should exhibit divergent strength of homeostasis. These experiments show that *in situ* bacterial communities from different trophic environments contain strains with a range of stoichiometric regulation and that homeostasis can be dominant under high P availability.

MATERIALS AND METHODS

Inoculum assemblages

The 4 source lakes for this study spanned a gradient of productivity and nutrient availability within Minnesota, USA. Lake Superior is a large, deep, oligotrophic lake with low productivity (Biddanda et al. 2001) and very low concentrations of dissolved and particulate phosphorus (Sterner 2011). Christmas Lake is a mesotrophic lake and Lake Owasso is a eutrophic lake (Biddanda et al. 2001, Stets & Cotner 2008); both are located in southeastern Minnesota. Lake Leverson is a highly productive shallow lake in western Minnesota (Theissen et al. 2012). Samples of water were collected from the surface layer of the lakes in sterilized acid-washed bottles. Within 24 h of collection, the samples were filtered through a sterilized Whatman GF/B filter (nominal retention size 1.0 µm) to exclude protist grazers and most phytoplankton. Samples from each lake were filtered through a 0.2 µm polycarbonate filter for measurements of total dissolved phosphorus and soluble reactive phosphorus (APHA 1995). Particulate C, N, and P in the bacterial-sized fraction were measured for samples collected on Whatman GF/F filters, using methods described below for cultures.

Growth media

Basal microbiological medium was prepared following Tanner (2002) using 18.2 megaohm (MΩ) deionized water (Milli-Q Nanopure). All glassware was soaked in 10% hydrochloric acid and rinsed with deionized water to remove trace contamination of phosphate. All chemical stocks were American Chemical Society reagent grade or equivalent. Glucose was supplied at 23.88 mmol C l^{-1} as the sole source of carbon and energy and ammonium chloride was supplied at 18.69 mmol N l^{-1} to ensure nitrogen sufficiency. Additional minerals, vitamins, and trace metals were supplied at concentrations described in Tanner (2002). Phosphorus was added as potassium phosphate at 4 levels (3 levels in Lake Owasso summer and fall), to create molar C:P ($C:P_{supply}$) ratios from 100:1 (239 µmol P l^{-1}) to 3162:1 (7.55 µmol P l^{-1}). In a previous study using bacterial isolates from lakes (Scott et al. 2012), threshold element ratios (Sterner & Elser 2002) for C versus P limitation ranged from 138:1 to 302:1. In our study, the lowest $C:P_{supply}$ treatment (100:1) likely represents weak C limitation. The medium was buffered between pH 7.2 and 7.4 using 11 mmol l^{-1} N-(tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid (Lake Owasso summer and fall) or 11 mmol l^{-1} 3-(N-morpholino)propanesulfonic acid (MOPS). Although only a fraction of the *in situ* bacterial assemblage is easily culturable using artificial media (Staley & Konopka 1985, Eilers et al. 2000, Page et al. 2004), we chose to use a defined medium to improve control over the resource chemistry. Similarly, although glucose selects for a subset of the *in situ* assemblage, a single C source with high energy content simplifies the relationship between $C:P_{biomass}$ and $C:P_{supply}$.

Initial assemblage cultures

The bacterial-sized fraction of lake water for each experiment was used to inoculate triplicate batch cultures at each level of $C:P_{supply}$. Filtered lake water (5 ml) was added to 100 ml of medium and the cultures were incubated at 22 to 24°C on a rotary shaker until turbid growth developed in all flasks (optical density at 600 nm > 0.05 cm^{-1}). Of each batch culture, 10 to 20 ml was used to inoculate triplicate chemostats at each level of $C:P_{supply}$. Chemostats, medium reservoirs, and tubing were acid-soaked, rinsed with deionized water, and sterilized by autoclave prior to use. Polypropylene 100 ml chemostats were continually aerated and mixed with 0.2 µm filtered air. The chemostats were maintained in a dark incubator at the same temperature as the source lake (16 to 27°C). For each lake, the chemostats were diluted at a uniform rate of 1 d^{-1} (0.218 d^{-1} for Lake Superior) for 9 complete turnovers prior to harvesting samples for analyses.

High-P and low-P selected fractions

Following the initial assemblage chemostat cultures, samples were collected for biomass analyses, as described below. The initial assemblage cultures

grown at the highest C:P_{supply} (1000:1 or 3162:1) are referred to as low-P selected assemblages and the cultures grown at the C:P_{supply} of 100:1 are referred to as high-P selected assemblages (Fig. 1). The bacterial abundance in the high- and low-P selected assemblages was determined as described below. To isolate the strains that were abundant at high or low P availability, samples from the high-P and low-P selected assemblages were diluted in sterile medium to approximately 100 cells. These bottlenecked assemblages, termed high-P and low-P selected fractions, became the inocula for the second phase of batch cultures at each level of C:P_{supply} (Fig. 1). The batch cultures were used to inoculate triplicate chemostats at each level of C:P_{supply} to quantify the degree of homeostasis in the selected communities. The high-P and low-P selected chemostats were sampled after 9 complete turnovers.

Cellular phosphorus, carbon and nitrogen analyses

Triplicate samples from each chemostat culture were filtered onto acid-rinsed Whatman GF/F filters using low vacuum pressure (<100 mm Hg). Following the batch culture enrichment, retention of cells on the GF/F filters was greater than 95%. The filtered samples were rinsed with deionized water and frozen at −20°C until analysis. Filters were digested with 25 g l^{-1} potassium persulfate at 121°C for 30 min (APHA 1995) to liberate organic phosphate. Following digestion, the phosphorus content was determined using the ascorbic acid molybdenum blue method (APHA 1995). Filter blanks were included in each run of analyses and used to correct the phosphorus content of the samples. Triplicate samples from each chemostat were collected onto pre-combusted Whatman GF/F filters as described for phos-

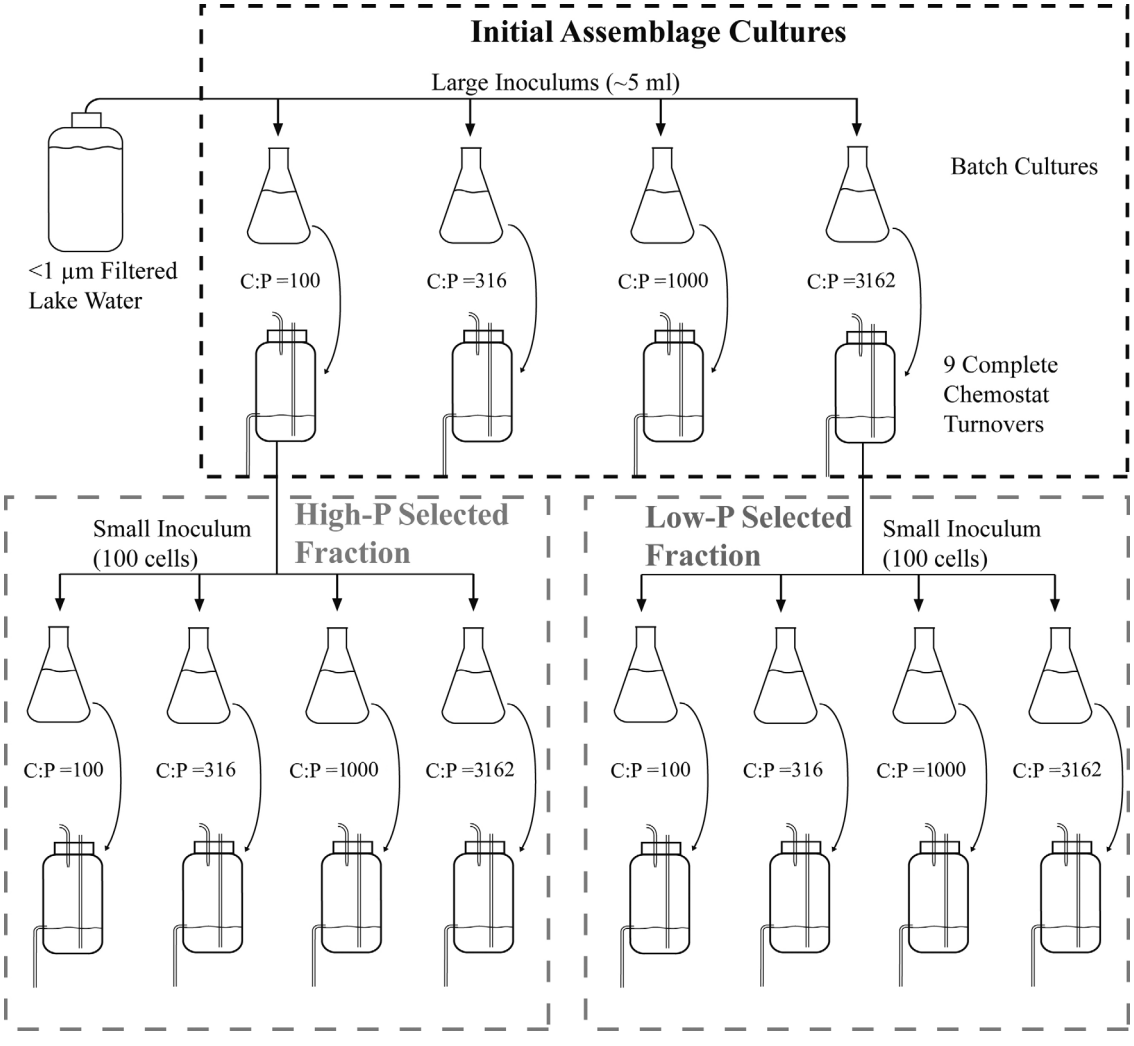

Fig. 1. Schematic representation of selection experiments and chemostats

phorus. The filters were frozen at −20°C and then dried at 60°C for 7 d prior to measuring the carbon and nitrogen content using a CHN analyzer (Perkin-Elmer 2400CHN) with combustion at 925°C. Acetanilide was used as the primary standard and a zooplankton standard was used to assess complete recovery of organic C and N. Filter blanks were used to correct the carbon and nitrogen content for each run of filters.

Bacterial abundance

Samples from each chemostat were preserved for microscopy with formaldehyde (final concentration 3.7%, w/v) and were stored at 4°C until analysis. Samples were diluted in 188 mmol l^{-1} sodium pyrophosphate (0.2 µm filtered) and sonicated to disperse clumps of cells (Velji & Albright 1993). Duplicate samples from each chemostat were stained with acridine orange, filtered onto black polycarbonate membrane filters (Millipore Nucleopore, 0.2 µm pore size), and mounted to slides for microscopy (Hobbie et al. 1977). Cell counts were performed manually at 1000× magnification using an Olympus BX40 epifluorescence microscope. At least 10 fields and 300 cells were enumerated on each filter.

Bacterial assemblage composition

To examine shifts in assemblage composition as the result of the selection treatments, the chemostat cultures were analyzed by automated ribosomal intergenic spacer analysis (ARISA) (Fisher & Triplett 1999). Samples for assemblage composition were not available for two of the Lake Owasso experiments (summer and fall). Samples of the chemostat assemblages from Lake Owasso (spring), Lake Superior, Lake Leverson, and Christmas Lake were preserved for ARISA analysis by suspending the pelleted cultures (1 ml) in lysis buffer (50 g l^{-1} sodium dodecyl sulfate in 120 mmol l^{-1} phosphate buffer, pH 8.0) and freezing at −20°C (Ghosh & LaPara 2007). The samples were further lysed by 3 cycles of freezing (−20°C) and thawing (22°C) and digestion with Proteinase K at 56°C. The nucleic acids were isolated and purified using the DNeasy Blood and Tissue Kit (Qiagen). The intergenic region was amplified following Nelson et al. (2010) using the primers ITSf (labeled with HEX) and ITSreub (Cardinale et al. 2004), using GoTaq polymerase and buffer (Promega).

Sample fragment lengths were resolved by capillary electrophoresis using a 3730xl sequencer (Applied Biosystems) with the size standard Map Marker 1000 (Bioventures) at the University of Minnesota Genomics Center. Sizing was performed using the PeakScanner software v. 1.0 (Applied Biosystems). The fragment peak areas were manually checked for each sample to ensure that the peak fluorescence was greater than the crossover fluorescence from the size standards. Fragment lengths below 156 bp or greater than 1000 bp were excluded prior to binning the fragment sizes from each experiment using the Interactive Binner source code for R statistical software (Ramette 2009). Peak sizes within ±1 bp were binned as a single peak and a manual check was performed for each experiment to ensure that the automated alignment was sufficient. Individual peaks representing at least 0.5% of the total peak area for a sample were used to perform non-metric multidimensional scaling (NMDS) using the package Vegan for R (Oksanen et al. 2011). Ordination was performed using the Bray-Curtis distance metric with 2 NMDS dimensions. Confidence ellipses (95%) were computed with the Vegan package. Differences in composition between the high-P and low-P selected fractions in each experiment were evaluated using Analysis of Similarity (ANOSIM) tests using the Vegan package. Due to low sample fluorescence, the following single replicate chemostats were excluded from analysis: Christmas Lake initial assemblage 316:1, Christmas Lake low-P selected assemblage 100:1, Lake Leverson high-P selected 1000:1, Lake Owasso spring initial assemblage 100:1, Lake Superior high-P selected assemblage 316:1, and 2 replicates from the Lake Superior low-P selected assemblage 3162:1.

Statistical analyses

The mean cellular C, N, and P measurements were used to calculate a single estimate of the molar $C:N:P_{biomass}$ for each chemostat culture. Samples that were below detection limits were excluded from figures and statistical analyses. Two samples from the Lake Superior assemblage cultures were below detection for biomass P (low-P selected, $C:P_{supply}$ of 3162:1) and one sample from Lake Owasso (fall) was below detection for N (low-P selected, $C:P_{supply}$ of 316:1). Mean stoichiometry (C:P, N:P, and C:N) and cellular P content from the initial assemblage cultures were compared within and among lake samples using analysis of covari-

ance (ANCOVA) with the lake samples as a fixed effect and $\log_{10}$ C:P_{supply} as the quantitative treatment. A significant effect of C:P_{supply} indicates that the assemblage biomass was non-homeostatic and a significant effect of lake sample indicates that the initial assemblage cultures differed in their mean ratio. A significant interaction between lake sample and C:P_{supply} indicates that the initial assemblage cultures had different responses to C:P_{supply}. The initial assemblages and selected assemblages were not compared using inferential statistics due to nested dependence of the replicates. In the high-P and low-P selected fractions, the biomass stoichiometry data ($\log_{10}$ transformed) and cell abundance were analyzed using a 2-way ANCOVA with selection treatment (high-P or low-P selected) as a fixed effect and C:P_{supply} ($\log_{10}$ transformed) as the quantitative treatment. Significant effects of C:P_{supply} indicate that the assemblage biomass was non-homeostatic, and a significant effect of selection treatment indicates that the high-P and low-P selected fractions differed in their mean ratio. A significant interaction between selection treatment and C:P_{supply} indicates that the high-P and low-P selected fractions had different responses to C:P_{supply}. The degree of stoichiometric flexibility (1/H′) (Sterner & Elser 2002) for each set of chemostats was calculated as the slope of the linear regression of $\log_{10}$ C:$P_{biomass}$ versus $\log_{10}$ C:P_{supply}.

RESULTS

Initial assemblage cultures

The lakes exhibited a wide range of dissolved phosphorus concentrations and *in situ* seston stoichiometry (Table 1). Christmas Lake and Lake Superior had low concentrations of dissolved P and relatively high C:$P_{biomass}$ and N:$P_{biomass}$ in the bacteria-sized fraction of the seston. Lake Leverson had a high concentration of dissolved P and low C:$P_{biomass}$ and N:$P_{biomass}$ in the bacteria-sized fraction of the seston. In the initial assemblage chemostat cultures, cell abundance decreased in response to higher supply C:P (ANCOVA, $p < 0.0001$) and there was no significant difference among the initial assemblage cultures from different lakes. The initial assemblages exhibited non-homeostasis of C:$P_{biomass}$ across levels of C:P_{supply} (Table 2, Fig. 2) and there were differences in C:$P_{biomass}$ among the lake samples (Table 3, Fig. 3). There was no significant interaction between lake samples and C:P_{supply}, which indicates that there was no difference in the strength of homeostasis among lake samples (i.e. they were similarly non-homeostatic). At low C:P_{supply}, the initial assemblage cultures from Lake Superior had lower C:$P_{biomass}$ than the other lake samples. The Christmas Lake initial assemblage cultures showed the highest C:$P_{biomass}$ under P limitation and also the greatest range of

Table 1. Dissolved P concentrations and stoichiometry of the bacteria-sized fraction of seston for the 4 source lakes (collected on GF/F filters). –: data not available

Lake Sample	Total dissolved phosphorus (µM)	Soluble reactive phosphorus (µM)	Seston carbon (µM)	Seston phosphorus (µM)	Seston C:N:P
Lake Owasso (summer)	–	–	15.18	0.181	84:29:1
Lake Owasso (fall)	–	–	–	–	–
Lake Owasso (spring)	0.49	0.28	–	0.104	–
Lake Superior	0.23	<0.05	13.84	0.062	223:25:1
Lake Leverson	1.55	0.50	22.23	0.583	38:7:1
Christmas Lake	0.54	0.31	6.92	0.065	106:24:1

Table 2. Biomass stoichiometry for selection experiments. C:N:$P_{biomass}$ ranges are for individual chemostats

Lake	Initial assemblage C:N:$P_{biomass}$ range	High-P selected C:N:$P_{biomass}$ range	Low-P selected C:N:$P_{biomass}$ range
Lake Owasso (summer)	102:21:1–218:29:1	89:19:1–183:38:1	70:14:1–448:82:1
Lake Owasso (fall)	93:17:1–381:54:1	80:16:1–399:54:1	63:14:1–430:61:1
Lake Owasso (spring)	59:14:1–325:38:1	50:12:1–565:78:1	59:15:1–437:55:1
Lake Superior	50:12:1–396:63:1	45:13:1–1,530:239:1	53:15:1–519:60:1
Lake Leverson	66:16:1–306:45:1	67:17:1–862:101:1	42:11:1–1396:166:1
Christmas Lake	83:20:1–910:118:1	76:17:1–820:73:1	68:15:1–2440:314:1

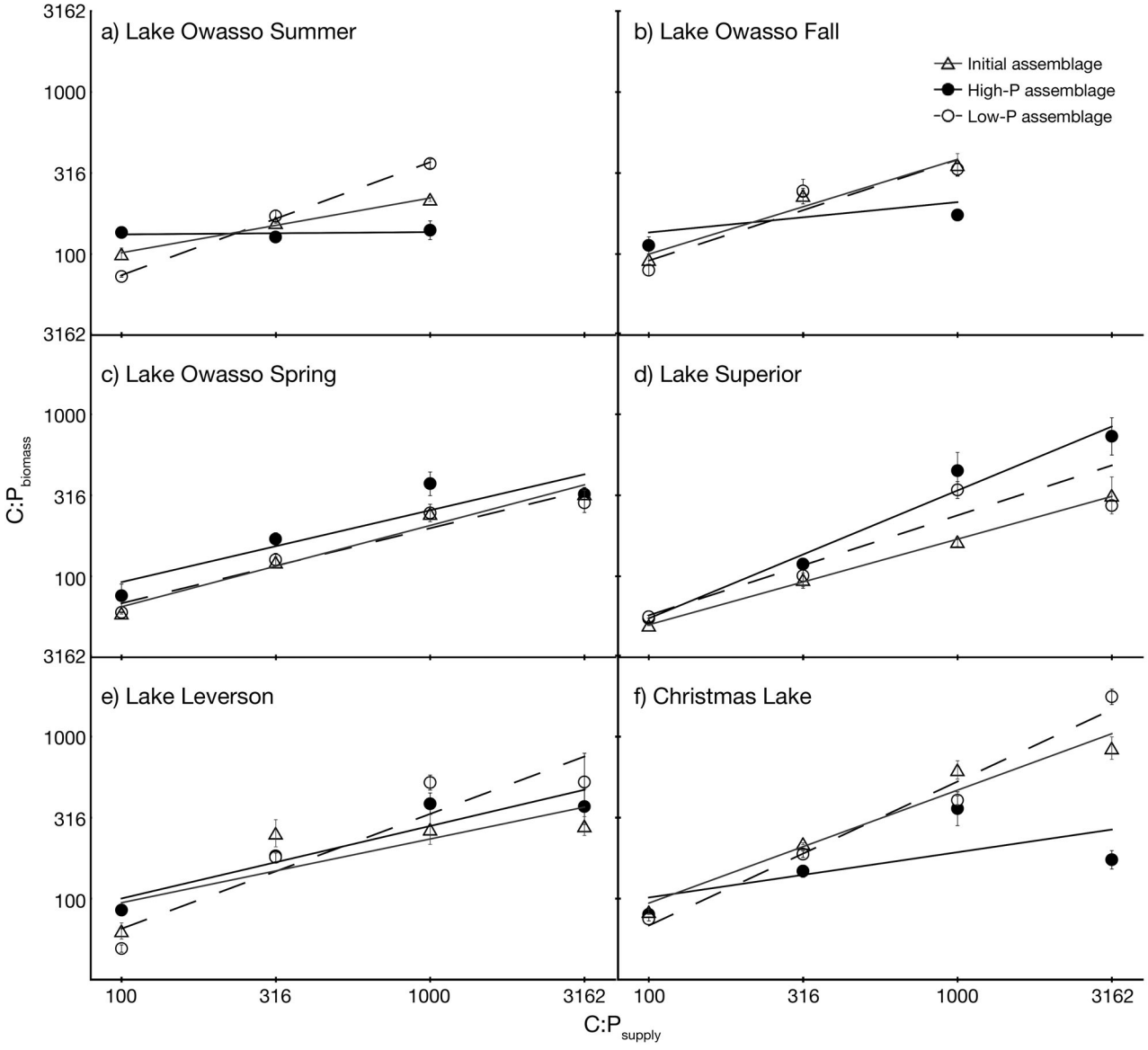

Fig. 2. Assemblage $C:P_{biomass}$ ratios across $C:P_{supply}$ treatments. (a) Lake Owasso in summer, (b) Lake Owasso in fall, (c) Lake Owasso in spring, (d) Lake Superior, (e) Lake Leverson, and (f) Christmas Lake. Error bars denote ± SE. Lines represent the linear regressions of $\log_{10}$ biomass ratio against $\log_{10}$ supply ratio. Both axes are log-scaled

$C:P_{biomass}$ (Table 2). In the Lake Leverson initial assemblage cultures, $C:P_{biomass}$ increased rapidly between $C:P_{supply}$ of 100:1 and 316:1, but showed little change from $C:P_{supply}$ of 316:1 to 3162:1. The slope of stoichiometric regulation (Sterner & Elser 2002) ranged from 0.338 to 0.697 (Fig. 3), with Lake Superior and Christmas Lake having the steepest slope and Lake Leverson the shallowest.

The $N:P_{biomass}$ of the initial assemblages behaved similarly to the $C:P_{biomass}$, exhibiting non-homeostasis in response to $C:P_{supply}$ (Table 3), with significant differences among lake samples in both the mean ratio and the strength of homeostasis. The initial assemblages from Lake Leverson and Lake Owasso (summer) were more homeostatic in $N:P_{biomass}$ than the other assemblages. The $C:N_{biomass}$ of the initial assemblage cultures was less variable than $C:P_{biomass}$ and $N:P_{biomass}$, but non-homeostatic in response to $C:P_{supply}$. Mean initial assemblage culture $C:N_{biomass}$ differed among lake samples and was lower in Lake Superior across all levels of $C:P_{supply}$ (3.0:1 to 10.5:1) compared to the other lakes (3.5:1 to 13.9:1). The absence of a significant interaction indicates that the initial assemblage cultures were similarly non-homeostatic in $C:N_{biomass}$. Cellular P quotas of the initial assemblage decreased in response to higher

$C:P_{supply}$ and differed among lake samples. In all of the initial assemblage cultures except Lake Owasso (summer), the increase in $C:P_{biomass}$ under P limitation was accompanied by a decrease in P quota (Fig. 4). Among all of the initial assemblage cultures, P quotas ranged from 0.02 to 1.23 fmol cell^{-1}. In all of the initial assemblage cultures except Lake Leverson, mean C quotas increased in response to increased $C:P_{supply}$ and ranged from 2.17 to 112 fmol cell^{-1}.

Table 3. ANOVA results (p-values) for the initial assemblage chemostats

Parameter	$C:P_{supply}$	Lake	Interaction
$C:P_{biomass}$	$<1\times10^{-15}$	$<1\times10^{-4}$	0.089
$N:P_{biomass}$	$<1\times10^{-15}$	<0.001	0.014
$C:N_{biomass}$	$<1\times10^{-15}$	$<1\times10^{-6}$	0.167
P cell^{-1}	$<1\times10^{-11}$	0.005	0.579

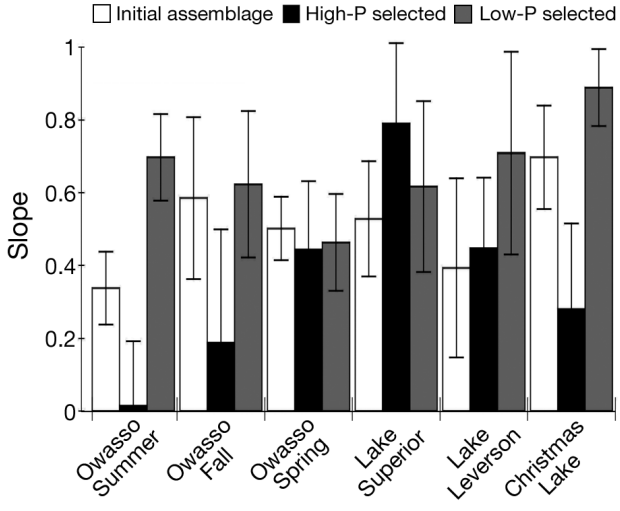

Fig. 3. Slopes of log $C:P_{biomass}$ versus log $C:P_{supply}$ for each experiment, with treatment conditions as categories. Error bars represent the 90% CI for the mean slope

High-P and low-P selected assemblages

In the high-P and low-P selected assemblages, $C:P_{biomass}$ responded to $C:P_{supply}$ (Table 4). In 3 of the experiments (Lake Owasso summer, Lake Owasso fall, and Christmas Lake), the significant interaction between selection treatment and $C:P_{supply}$ indicates that the low-P and high-P selected fractions differed in their response to $C:P_{supply}$ (Fig. 3, Table 4). In these experiments, the high-P selected fractions of the assemblage were more homeostatic and had a smaller range of $C:P_{biomass}$ than the low-P selected fractions. The homeostatic high-P selected assemblages from Lake Owasso (summer and fall) and Christmas Lake had lower mean $C:P_{biomass}$ than the low-P selected fractions (Table 2). The low-P selected fraction from Christmas Lake showed the greatest range of mean $C:P_{biomass}$. In the experiments without a significant interaction between $C:P_{supply}$ and selection treatment (Lake Owasso spring, Lake Superior, and Lake Leverson), both the high- and low-P selected fractions were similarly non-homeostatic in $C:P_{biomass}$ and resembled the response of the initial assemblage cultures (Fig. 2).

In the high-P and low-P selected fractions, assemblage $N:P_{biomass}$ increased with increasing $C:P_{supply}$ (Table 4). In 3 of the experiments (Christmas Lake, Lake Owasso summer, and Lake Superior), the significant interaction between selection treatment and $C:P_{supply}$ indicates that the response of $N:P_{biomass}$ to $C:P_{supply}$ differed between the high-P

Table 4. ANOVA results (p-values) for the selected fractions chemostats

Parameter	Effect	Lake Owasso			Lake Superior	Lake Leverson	Christmas Lake
		Summer	Fall	Spring			
$C:P_{biomass}$	$C:P_{supply}$	$<2\times10^{-5}$	$<1v10^{-3}$	$<7\times10^{-7}$	$<2\times10^{-7}$	$<5\times10^{-6}$	$<1\times10^{-7}$
	Selection	0.071	0.591	0.119	0.045	0.931	<0.002
	Interaction	$<3\times10^{-5}$	0.043	0.878	0.353	0.178	$<4\times10^{-4}$
$N:P_{biomass}$	$C:P_{supply}$	$<7\times10^{-5}$	$<8\times10^{-3}$	$<8\times10^{-6}$	$<4\times10^{-8}$	$<7\times10^{-6}$	$<1\times10^{-6}$
	Selection	0.114	0.557	0.201	0.024	0.723	0.013
	Interaction	$<9\times10^{-6}$	0.313	0.478	0.020	0.195	$<2\times10^{-3}$
$C:N_{biomass}$	$C:P_{supply}$	0.085	$<9\times10^{-3}$	$<5\times10^{-3}$	0.324	$<2\times10^{-4}$	$<4\times10^{-4}$
	Selection	0.276	0.643	0.536	0.271	0.610	0.056
	Interaction	0.370	0.070	0.580	0.551	0.495	0.220
P cell^{-1}	$C:P_{supply}$	0.019	0.516	$<2\times10^{-4}$	$<2\times10^{-3}$	0.076	$<5\times10^{-4}$
	Selection	0.050	$<3\times10^{-3}$	0.874	0.707	0.838	0.879
	Interaction	0.034	0.058	0.151	0.027	0.959	0.406

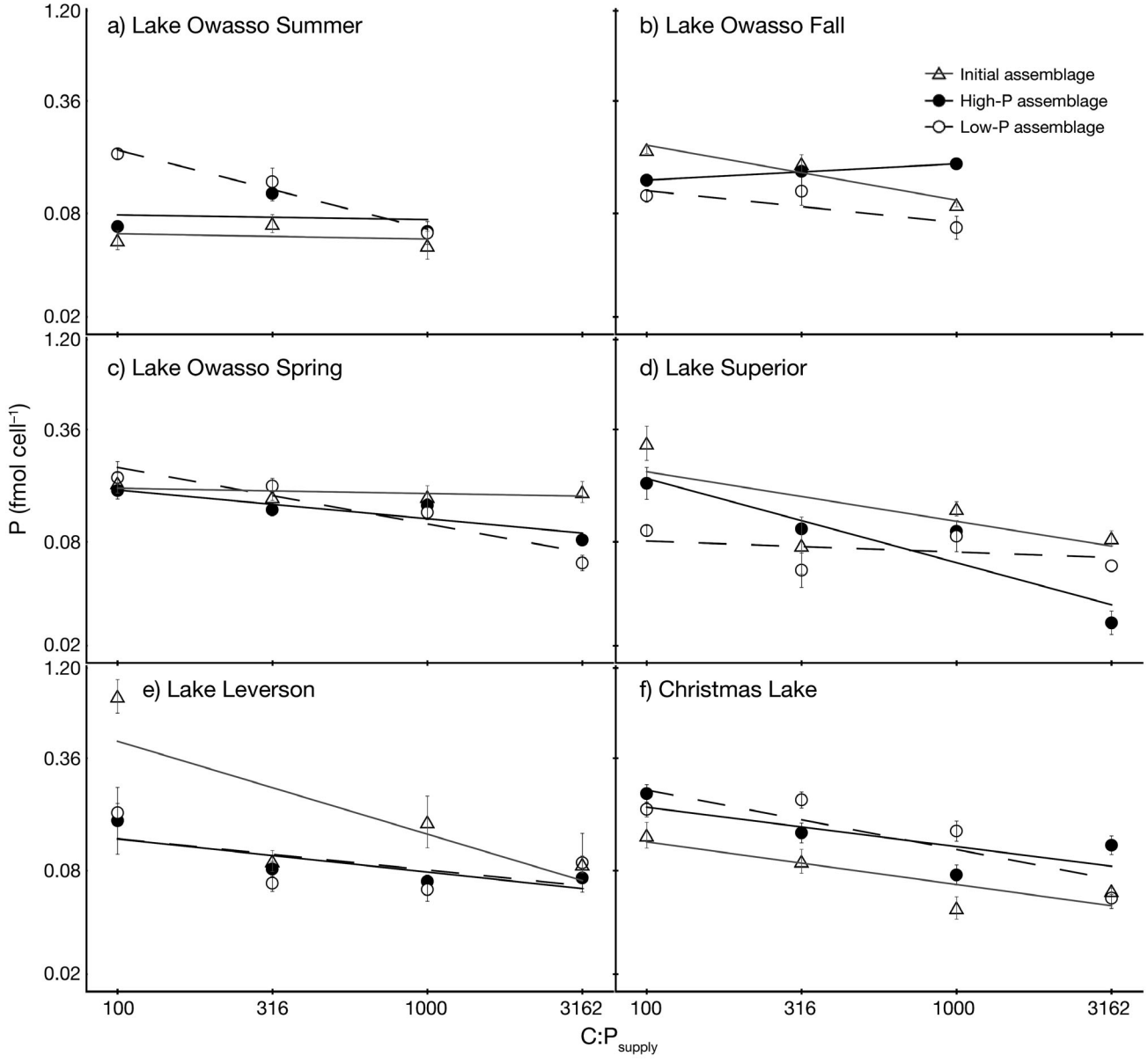

Fig. 4. Cellular P content of assemblages across $C:P_{supply}$ treatments. (a) Lake Owasso in summer, (b) Lake Owasso in fall, (c) Lake Owasso in spring, (d) Lake Superior, (e) Lake Leverson, and (f) Christmas Lake. Error bars denote ± SE. Lines represent the linear regressions of $\log_{10}$ phosphorus quotas against $\log_{10}$ supply ratio. Both axes are log-scaled

selected and low-P selected fractions. Biomass C:N was less variable than $C:P_{biomass}$ and $N:P_{biomass}$ and increased with $C:P_{supply}$ in the experiments from Lake Owasso (fall and spring), Lake Leverson, and Christmas Lake. Across the high-P and low-P selected fractions, increasing $C:P_{supply}$ led to a decrease in cellular phosphorus quotas (Fig. 4), although this was significant in only 4 experiments (Lake Owasso summer, Lake Owasso spring, Lake Superior, and Christmas Lake). Mean carbon quotas increased in response to increasing $C:P_{supply}$ in all of the low-P selected fractions, although the increase was significant only in Christmas Lake, Lake Leverson, and Lake Owasso fall (ANOVA, $p < 0.05$). In the Christmas Lake experiment, the mean carbon quota of the low-P selected fraction increased from 14 to 116 fmol cell^{-1} as the $C:P_{supply}$ increased. In contrast, the mean carbon quota of the high-P selected fraction for Christmas Lake ranged only from 19 to 29 fmol cell^{-1}

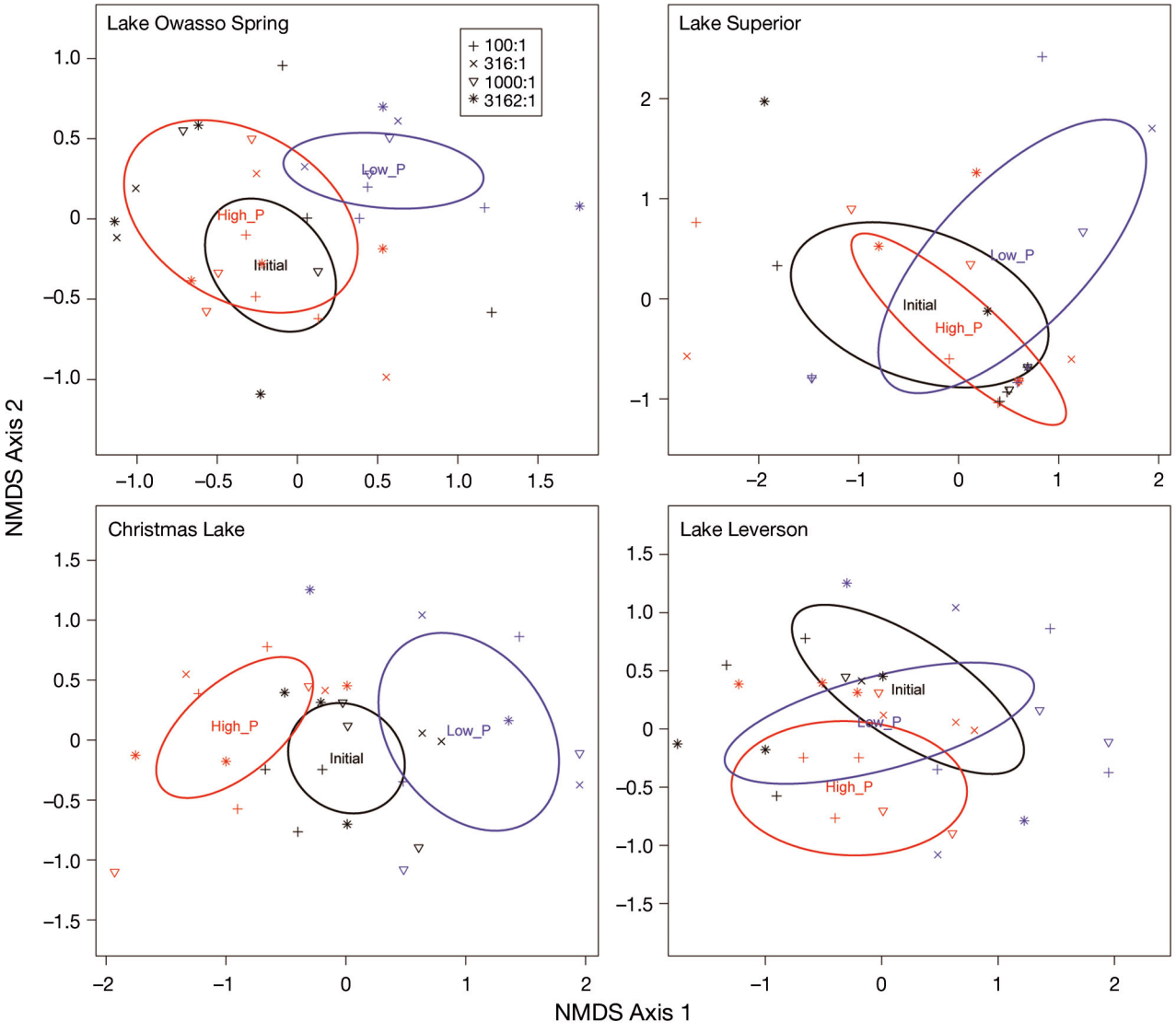

Fig. 5. Non-metric multidimensional scaling (NMDS) ordinations of assemblage ARISA profiles. Ellipses represent the 95% confidence region for the initial assemblages (black), high-P selected assemblages (red), and low-P selected assemblages (blue). Symbols represent C:P_{supply} levels

Assemblage composition

The high-P and low-P selected fractions exhibited divergent ARISA profiles both within and between selection treatments (Fig. 5). In the experiments from Lake Owasso (ANOSIM, p = 0.086), Lake Superior (p = 0.079), and Lake Leverson (p = 0.783), there was no significant divergence in assemblage composition between the initial, high-P selected, and low-P selected fractions. In the experiment from Christmas Lake, the high-P and low-P selected fractions exhibited significantly different composition (ANOSIM, p = 0.001).

DISCUSSION

The selection experiments were designed to test our hypothesis that P-dependent shifts in assemblage composition mediate the stoichiometric regulation of bacterial assemblages. We used the data from these experiments to describe 3 aspects of the stoichiometry of assemblages. The first conclusion from this work is that although non-homeostasis was observed in each of the initial assemblage cultures, low C:P_{supply} can select for homeostatic strains within these assemblages. The second conclusion is that assemblages of bacteria enriched from different

trophic environments have similar, though not identical, stoichiometric responses to imbalance in $C:P_{supply}$. The third conclusion is that the stoichiometric behavior of assemblages is partly explained by shifts in assemblage composition, driven by resource availability. These conclusions are interrelated and provide insights into the stoichiometry of bacterial assemblages.

Selection for stoichiometric strategies

Three of the experiments (Lake Owasso summer and fall, and Christmas Lake) exhibited divergent stoichiometric regulation in $C:P_{biomass}$ under high-P and low-P selection. The high-P treatment effectively selected for more homeostatic strains and the low-P treatment selected for strains with similar stoichiometric regulation and P quotas to the initial assemblage cultures. In the Christmas Lake experiment, the divergence in stoichiometric regulation between the selection treatments was matched by shifts in assemblage composition. This result supports our hypothesis that homeostatic strains can be dominant under high P availability. Several physiological tradeoffs could explain the apparent competitive advantage of strong homeostasis at low $C:P_{supply}$. Although the high-P and low-P selected fractions exhibited divergent stoichiometric strategies, the 2 fractions had similar P quotas under P limitation (Fig. 4). This suggests that aspects of competitive ability other than a low P quota, such as P affinity or carbon metabolism, could influence the outcome of competition in these experiments.

In 3 of the lake samples, the strength of homeostasis was not significantly different between the high-P and low-P selected fractions of the assemblages. High P availability did not select for homeostatic strains in these lakes. Our hypothesis predicted that homeostatic strains should be more abundant in lakes with high P availability (low $C:P_{supply}$). The initial assemblage from eutrophic Lake Leverson (slope 0.39) was more homeostatic than the initial assemblage from ultra-oligotrophic Lake Superior (slope 0.53). In these lakes without divergence following selection, both the high-P and low-P selected fractions were non-homeostatic and resembled the initial assemblage cultures, suggesting that $C:P_{supply}$ did not select for more homeostatic strains or that they were not abundant in the initial assemblage cultures. The ARISA results support this conclusion in that there were not substantial shifts in assemblage composition that were related to $C:P_{supply}$. This could be the result of stronger selective pressure for P compared to C, insufficient time for competitive exclusion, or a low relative abundance of homeostatic strains in the initial cultured assemblage. Previous work using individual strains of bacteria isolated from lakes found threshold element ratios between 138 and 302:1 (Scott et al. 2012), suggesting that the lowest $C:P_{supply}$ ratio used in the present study (100:1) likely led to C limitation. However, under the high-P treatment, the $C:P_{biomass}$ of the assemblage cultures (range 42 to 102:1) was generally lower than the $C:P_{supply}$ of 100:1, indicating that the bacteria experienced weak C limitation in this treatment. Differences in the strength of C limitation or the carbon competitive ability of the strains present among the experiments might explain the variable outcomes of selection at low $C:P_{supply}$. Additionally, the uniformly low dilution rate used within each study could have enabled strains with lower maximum growth rate to remain dominant.

Strength of homeostasis in assemblages from different environments

The initial assemblage cultures from each lake showed different degrees of stoichiometric flexibility, although these differences were not significant. This suggests that local *in situ* community composition is not the main determinant of the stoichiometric response of the culturable assemblage. The responses of the initial assemblage cultures and the selected fractions ranged from strong homeostasis (slope of 0.014) to weak homeostasis (slope of 0.889, Table 2). Previous studies investigating the C:P stoichiometry of bacterial assemblages or enrichment cultures (Bratbak 1985, Goldman et al. 1987, Jürgens & Güde 1990, Tezuka 1990, Makino & Cotner 2004) have found widely varying degrees of non-homeostasis in $C:P_{biomass}$, with slopes from 0 to 0.9. These studies were not performed with a single experimental design, and some of this variation is attributable to differences in culture conditions. For example, Makino & Cotner (2004) cultured an assemblage from Lake Owasso at multiple chemostat dilution rates and found that a 3-fold increase in dilution rate decreased the slope of stoichiometric regulation from 0.39 to 0.07. The effect of dilution rate on homeostasis has also been observed in other populations (Chrzanowski & Kyle 1996, Makino et al. 2003), where increasing the dilution rate relative to the maximum growth rate of bacteria leads to stronger homeostasis in $C:P_{biomass}$ and increased P quotas. The uniform cul-

ture conditions and dilution rate used for the present study allowed for comparison of the strength of stoichiometric regulation among assemblages. Since the dilution rate was uniform (excluding Lake Superior), and similar to *in situ* growth rates, these results show that non-homeostasis is prevalent in the *in situ* bacterial communities of lakes.

The lack of a significant difference in slopes among the initial assemblages, together with the fact that some strains isolated from lakes exhibit non-homeostasis (Scott et al. 2012), suggests that the disparity between the stoichiometry of populations and assemblages could be attributable to the prevalence of non-homeostatic strains in natural assemblages. Such plasticity could arise if bacteria in plankton environments experience temporal variability in both C and P availability and imbalance, which should select for plasticity in biomass composition. Bacteria can store surplus cellular P as polyphosphate (Jahid et al. 2006, Kornberg 1995) and surplus C as poly-β-hydroxybutyrate and glycogen (Preiss 1984, Thingstad et al. 2005), each of which would influence $C:P_{biomass}$. These adaptations to resource variability should be most important in non-equilibrium conditions, especially habitats where the availability of C and P is spatially or temporally variable. In contrast to chemostat culture, variable resource ratios should select for non-homeostatic strains that can store the surplus C or P (Hood & Sterner 2010), but might not lead to exclusion of homeostatic strains (Sommer 1985).

Implications for assemblage stoichiometry

The experiments presented here show that the $C:N:P_{biomass}$ and P content of aquatic heterotrophic bacteria are highly flexible, in contrast to the common assumption that bacteria and assemblages are homeostatic in their $C:N:P_{biomass}$ and uniformly rich in P. In response to P limitation, the non-homeostatic assemblages increased their $C:N:P_{biomass}$ by decreasing their P quotas and simultaneously increasing their C and N quotas. Although the phosphorus quotas in the high-P and low-P selected fractions generally decreased under P limitation, these patterns showed only partial correspondence with changes observed in $C:P_{biomass}$ and $N:P_{biomass}$ (Figs. 2 & 4). This indicates that flexible C and N quotas were partially responsible for the plasticity observed in $C:P_{biomass}$ and $N:P_{biomass}$. The apparent disparity between stoichiometric regulation of assemblages and populations is also attributable to limited data for both types of cultures. The experiments presented here suggest that flexible biomass stoichiometry should be included when modeling the stoichiometry of *in situ* bacterial communities in lakes. Assemblages with variable $C:P_{biomass}$ and $N:P_{biomass}$ (e.g. Christmas Lake) would serve to buffer moderate imbalances in the C:P of dissolved resources, but would be expected to remineralize excess C when P is strongly limiting.

Chemostats are effectively an allochthonous system where the bacteria do not directly affect the supply of resources, but do affect the concentration and ratio available in their immediate environment. In natural systems, bacteria compete with primary producers for inorganic nutrients (Currie 1990, Cotner & Wetzel 1992, Danger et al. 2007) and consume organic C derived from phytoplankton, macrophytes and allochthonous sources, suggesting a dynamic feedback in which consumption of inorganic P by non-homeostatic bacteria could lead to decreased C availability, thus diminishing the severity of elemental imbalance. Such coupling of elemental cycling between phytoplankton and heterotrophic bacteria (Currie 1990, Cotner & Biddanda 2002), both of which are stoichiometrically flexible, could explain the modest variability in seston C:N:P (Cotner et al. 2010) despite considerable variation in the availability of dissolved organic C and dissolved P. Non-homeostasis in bacterial $C:N:P_{biomass}$ also has implications for trophic transfer of these elements within the 'microbial loop' (Azam et al. 1983). Compared to homeostatic consumers, non-homeostatic assemblages of bacteria and a non-homeostatic protist consumer (Grover & Chrzanowski 2006) would allow for increased efficiency in the transfer of C within the microbial loop (Mitra et al. 2014).

Acknowledgements. Comments by Jeffery Gralnick, Robert Sterner, Timothy LaPara, and 2 anonymous reviewers greatly improved this manuscript. This work was funded by NSF IGERT grant DGE-0504195 and NSF-IOS award 1257571 to J.B.C.

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Editorial responsibility: Tom Fenchel, Helsingør, Denmark

Submitted: June 3, 2014; Accepted: September 6, 2014
Proofs received from author(s): December 2, 2014