SHORT- VERSUS LONG-TERM RESPONSES TO CHANGING CO$_2$ IN A COASTAL DINOFLAGELLATE BLOOM: IMPLICATIONS FOR INTERSPECIFIC COMPETITIVE INTERACTIONS AND COMMUNITY STRUCTURE

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Increasing pCO$_2$ (partial pressure of CO$_2$) in an "acidified" ocean will affect phytoplankton community structure, but manipulation experiments with assemblages briefly acclimated to simulated future conditions may not accurately predict the long-term evolutionary shifts that could affect inter-specific competitive success. We assessed community structure changes in a natural mixed dinoflagellate bloom incubated at three pCO$_2$ levels (230, 433, and 765 ppm) in a short-term experiment (2 weeks). The four dominant species were then isolated from each treatment into clonal cultures, and maintained at all three pCO$_2$ levels for approximately 1 year. Periodically (4, 8, and 12 months), these pCO$_2$-conditioned clones were recombined into artificial communities, and allowed to compete at their conditioning pCO$_2$ level or at higher and lower levels. The dominant species in these artificial communities of CO$_2$-conditioned clones differed from those in the original short-term experiment, but individual species relative abundance trends across pCO$_2$ treatments were often similar. Specific growth rates showed no strong evidence for fitness increases attributable to conditioning pCO$_2$ level. Although pCO$_2$ significantly structured our experimental communities, conditioning time and biotic interactions like mixotrophy also had major roles in determining competitive outcomes. New methods of carrying out extended mixed species experiments are needed to accurately predict future long-term phytoplankton community responses to changing pCO$_2$.

KEY WORDS: Acclimation, artificial community, climate change, competition, dinoflagellate community, ocean acidification.

Future marine phytoplankton assemblages will be influenced by environmental changes that include transfer of fossil fuel-derived CO$_2$ from the atmosphere to the surface ocean. Because of the tremendous physiological variability found among microalgal taxa, it is likely that differential responses to consequent ocean acidification will provide a selective advantages or disadvantages,

resulting in "winners" and "losers" (Rost et al. 2008; Hutchins et al. 2009; Boyd et al. 2010). These two outcomes may be expressed either in terms of increased or decreased reproductive fitness (e.g., growth rates), or in terms of competitive success (relative abundance or biomass in mixed communities).

Experiments using natural communities have manipulated the partial pressure of CO$_2$ (pCO$_2$) in seawater to characterize algal physiological responses that may help us predict future phytoplankton community structure (Riebesell 2004; Kim et al. 2006; Hare et al. 2007; Feng et al. 2009, 2010). In contrast to these

ocean acidification simulation experiments that typically last at most a few weeks, although, phytoplankton populations in nature will encounter gradually changing conditions over time scales of years to centuries. The long-term evolutionary responses of phytoplankton groups whose competitive interactions ultimately determine algal community structure have only begun to be examined, so we can only speculate at present on the composition of these future "greenhouse" phytoplankton assemblages.

Aside from assemblage-level effects, there is also a limited amount of information available on potential long-term evolutionary responses of single species of phytoplankton to high pCO$_2$ (Collins and Bell 2004, 2006; Collins 2010; Müller et al. 2010; Crawfurd et al. 2011; Lohbeck et al. 2012). After 1000 generations of growth at high CO$_2$, a freshwater green alga revealed no adaptive evolutionary response of specific growth rate (Collins and Bell 2004). Müller et al. (2010) found little difference between short- and long-term effects of high CO$_2$ on two species of coccolithophores. The marine diatom *Thalassiosira pseudonana* revealed no evidence of genetic adaptation to high CO$_2$ over 3 months (Crawfurd et al. 2011), but another recent study documented increases in growth rates and calcification in coccolithophore cultures adapted to high CO$_2$ conditions (Lohbeck et al. 2012).

Dinoflagellates within the class Dinophyceae are important members of ocean ecosystems, especially in the coastal zone. These microscopic protists are typically motile with the aid of their two flagella, and many exhibit extensive diel vertical migrations within the water column. They are evolutionarily ancient organisms that exhibit diverse morphological forms, life history strategies, and modes of nutrition ranging from autotrophy to mixotrophy to heterotrophy (Smayda 1997, 2002). In particular, dinoflagellates have unique chromosomal attributes, including size, structure, and composition (Rizzo 2003). Some members of this class have environmental, economic and human health significance as they are capable of forming dense "blooms," as well as manufacturing potent neurotoxins that can be accumulated through trophic transfer in marine food webs (Fu et al. in press).

To address some of the unknowns regarding the long-term assemblage-level responses to changing CO$_2$ conditions, we used a novel experimental design that compared the outcome of competition in short-term (2-week) natural dinoflagellate community pCO$_2$ manipulations with the results of competition between the same species in artificial communities after conditioning to the same pCO$_2$ treatments in clonal cultures for 1 year. An objective of this work was to determine if short-term incubation experiments are reasonable proxies for predicting the effects of long-term processes on community structure, and thus address the question: Is the outcome of multispecies competitive interactions the same under short-term and long-term selection by pCO$_2$?
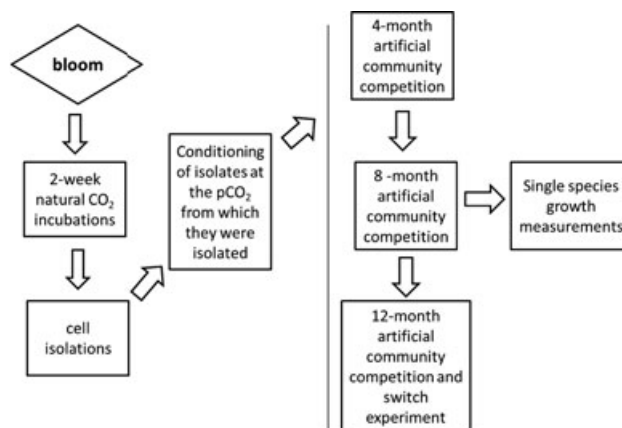

Figure 1. Flow chart showing the sequence of the major components of the year-long experiment.

Materials and Methods
EXPERIMENTAL DESIGN

A flow chart of the experimental design is shown in Figure 1, including collection and incubation of the natural bloom in a short-term (2 weeks) pCO$_2$ experiment, isolation of cells of all four species from each pCO$_2$ treatment, conditioning of the isolates at the pCO$_2$ from which they were isolated for 1 year, and recombining isolates into artificial communities to compete for 2-week periods following 4, 8, and 12 months of conditioning. Growth rates of each species were assessed during conditioning in unialgal cultures at the 8-month timepoint, as well as in mixed communities during the initial bloom experiment and the 12-month artificial community experiment. "Switch" competition experiments in which clones conditioned at each pCO$_2$ were competed in artificial communities at the other two pCO$_2$ levels were also performed after 12 months.

INITIAL pCO$_2$ INCUBATION EXPERIMENT

A mixed natural dinoflagellate bloom dominated by *Lingulodinium polyedrum*, *Prorocentrum micans*, *Alexandrium* sp., and *Gonyaulax* sp. at a total cell density of approximately 700 cells per mL was collected off Venice Beach, California, in September, 2009. This large regional bloom extended throughout the Southern California Bight region. Samples were collected near shore for both the initial incubations and all experimental dilution water used throughout the 12-month experiment.

The experiment was incubated in the laboratory at 18°C under 90 photons m^{-2} s^{-1} of cool white fluorescent illumination on a 14-h light : 10-h dark cycle. Triplicate sterilized 1 L polycarbonate bottles were gently bubbled (60 bubbles min^{-1}) using commercially prepared air/CO$_2$ mixtures (230, 433, and 765 ppm, Praxair Gas). Preliminary experiments verified that growth rates of cultures bubbled at this rate were not significantly different from those of unbubbled cultures (data not shown), and these methods

have been employed for other CO_2 experiments (Fu et al. 2007; Hutchins et al. 2007), including dinoflagellate studies (Fu et al. 2008, 2010). Filtered seawater was amended with L1/20 nutrient, vitamin, and trace metal concentrations (Guillard and Hargraves 1993), except NH_4Cl^+ was substituted for $NaNO_3^-$ and silicate was omitted. Nutrient concentrations at the Redfield ratio (by atoms) of 16 N : 1 P (Redfield 1958) were added initially to the incubation bottles, and replenished once at the 1 week dilution.

The CO_2-amended treatments were maintained in active growth using semicontinuous culture methods (Tatters et al. 2012). Each bottle was diluted to the original time-zero *in vivo* chlorophyll *a* fluorescence value after 1 week with nutrient-amended filtered seawater. Aliquots were removed initially, and after 1 and 2 weeks for examination of carbonate buffer system parameters and community structure using microscopic cell counts. Samples for cell counts were obtained at the 1 week timepoint (after dilution), and after the 2 week incubation, to calculate acclimated growth rates (1–2 week rates) and final abundances of all species.

CLONAL CULTURE ISOLATIONS

Three individual cells representing the four dominant genera were isolated from each incubation bottle at the end of the 2-week incubation of the natural community, and maintained in long-term culture (52 weeks) at the pCO$_2$ from which they were obtained under conditions of temperature, light, nutrients, CO_2 bubbling, etc., identical to the 2-week natural community experiment. Cultures were maintained in exponential phase using autoclave-sterilized enriched seawater growth medium with semicontinuous weekly dilutions based on specific growth rates within each bottle, calculated as in Tatters et al. (2012). The approximate number of generations during this time period was: *L. polyedrum* (48–62), *P. micans* (58–71), *Alexandrium* sp. (34–38), and *Gonyaulax* sp. (75–126).

ARTIFICIAL COMMUNITY COMPETITION EXPERIMENTS

The conditioned clonal cultures were recombined into artificial communities after 4, 8, and 12 months in the same relative proportions and cell densities as the original natural bloom assemblage. The 8- and 12-month experiments used triplicate communities of all four species, but because of logistical limitations, the 4-month experiment used only *L. polyedrum*, *P. micans*, and *Alexandrium* sp. in duplicate communities. Because *Gonyaulax* sp. was not included in this preliminary 4-month experiment and replication was different, it is not fully comparable to the other experiments. The dinoflagellates in the artificial community trials were allowed to compete under identical experimental conditions of light, temperature, nutrient availability, and pCO$_2$ for the same time period and diluted exactly as in the original natural bloom incubation.

Table 1. Measured seawater carbonate buffer system values, pH, and total dissolved inorganic carbon (DIC) in the initial 2 week incubation with the natural bloom sample, in the artificial community competition experiments at 4, 8, and 12 months, and in the single species cultures during long-term conditioning at the 8-month timepoint. Also shown are pCO$_2$ values calculated from the two measured parameters. For clarity, in the text pCO$_2$ values of 230 to 336 are referred to as "low," values of 433 to 506 are referred to as "medium," and values of 709 to 792 are referred to as "high."

Community	pH (NBS)	Total DIC	Calculated pCO$_2$
2 weeks			
Low	8.38	1971	230
Medium	8.15	2067	433
High	7.94	2162	765
8 months			
Low	8.25	2048	336
Medium	8.13	2125	469
High	7.96	2190	731
12 months			
Low	8.27	2011	305
Medium	8.14	2091	451
High	7.97	2174	709
8 months, cultures			
L. polyedrum			
Low	8.31	1987	280
Medium	8.14	2078	450
High	7.95	2216	760
P. micans			
Low	8.27	2022	310
Medium	8.10	2138	501
High	7.93	2224	786
Alexandrium sp.			
Low	8.28	2023	305
Medium	8.15	2150	448
High	7.93	2209	792
Gonyaulax sp.			
Low	8.30	1995	286
Medium	8.13	2118	466
High	7.94	2192	762

Samples were collected for cell counts and carbonate system parameters (Table 1) in all experiments.

SWITCH EXPERIMENT

A triplicated CO_2 switch experiment at the 12-month timepointconsisted of "switching" low CO_2-conditioned cell lines to medium and high CO_2 (low→medium and low→high), medium CO_2-conditioned cell lines to low and high CO_2 (medium→low and medium→high), and high CO_2-conditioned clones to low and medium CO_2 (high→low and high→medium). The same experimental bottles employed in the 12-month artificial community

experiments described above were used to provide appropriate controls of low→low, medium→medium, and high→high. Other than the switched pCO$_2$ treatments, protocols for these trials were exactly the same as in the other artificial community experiments.

ANALYTICAL METHODS
Cell counts and growth rates
Growth rates were measured for each species in mixed communities during the second week of the initial natural community incubation, and during the 12-month and switch artificial community experiments. Because of logistical limitations and the fact that dinoflagellates cannot be cryopreserved for subsequent growth rate measurements, growth rates were determined immediately for all isolates in unialgal culture only at the 8-month timepoint. These values are representative of their long-term steady-state exponential growth rates throughout the conditioning period. Final cell abundances of each species were measured in every natural or artificial community competition experiment. Algal cells were preserved in acidified Lugol's solution and enumerated using an Accu-Scope 3032 inverted microscope using the Utermöhl method (Utermöhl 1931).

Carbonate buffer system
Dissolved inorganic carbon analysis used a CM5230 CO$_2$ coulometer (UIC; King et al. 2011). pH was determined on freshly collected samples using a calibrated Orion 5-star plus pH meter using an NBS buffer system with three-point calibration. Experimental pCO$_2$ was calculated using CO$_2$SYS software as in Tatters et al. (2012) (Table 1). Because of unavoidable minor variability in calculated pCO$_2$ levels between experiments (largely from differences in batches of commercial gas mixtures), for clarity, throughout the text pCO$_2$ values of 230 to 336 ppm are referred to as "low," values of 433 to 506 ppm are referred to as "medium," and values of 709 to 792 ppm are referred to as "high."

Multivariate statistical methods
Multivariate analyses used the PRIMER v6 statistics package (Clarke and Warwick, 2001) with the permutational analysis of variance/multivariate analysis of variance (PERMANOVA) add-on. Final cell abundances for each species from replicate bottles were square-root transformed prior to community structure comparisons based on Bray–Curtis similarities and log-transformed species growth rates compared based on Euclidean distance measures. ANOSIM permutation tests were used to test the impact of differing pCO$_2$ competition levels on overall community structure and on relative abundance of the four species at the end of all incubations, and on their growth rates in the initial natural community, 12-month and switch experiments. These tests resulted in

R values and significance levels where $R = 0$ implies no difference among groups, and $R = 1$ suggests that group separation is so large that all dissimilarities among groups are larger than any dissimilarity within them (Clarke and Warwick 2001).

Data from the natural community experiment and from competition trials after 8 and 12 months were further combined for PERMANOVA analyses to test for significant differences among and within predefined groups in response to both the pCO$_2$ competition levels and the differing periods of conditioning to these pCO$_2$ concentrations. PERMANOVA resulted in Pseudo-F and significance levels, where Pseudo-F $= 1$ implies a large overlap among sample groups being compared, whereas Pseudo-F > 1 indicates little or no overlap between them (Anderson et al. 2008). PERMANOVA also allowed us to test for interaction between the factors pCO$_2$ competition level and conditioning period in forcing overall community structure.

For the 12-month switch competition trials, we used final cell abundances from all treatments to examine the comparative effects of differing pCO$_2$ competition levels and differing pCO$_2$-conditioning levels. Using a two-way crossed design for the ANOSIM routine, we tested the average effect on overall community structure and on the four individual species separately of pCO$_2$ levels during competition removing differences in conditioning pCO$_2$, and the average effect of conditioning pCO$_2$ removing differences in competition pCO$_2$ (Clarke and Warwick 2001).

Results
INITIAL pCO$_2$ INCUBATION EXPERIMENT
Community structure
The natural bloom composition at the time of collection was dominated by *L. polyedrum* (82.5%), followed by *P. micans* (9.0%), *Gonyaulax* sp. (4.8%), and *Alexandrium* sp. (3.7%) (Fig. 2A). Other phytoplankton species were also present but they comprised $< 1\%$ of total cell abundance within the community, therefore we considered only the four dominant species. Each of the pCO$_2$ treatments yielded a different dinoflagellate assemblage at the end of the 2-week incubation, indicating that community structure was strongly altered by pCO$_2$ (average $R = 0.97$ at $P = 0.004$, one-way ANOSIM; Table 2). Treatment-specific trends were consistent within the triplicate experimental flasks, as evidenced by the clustering of Bray–Curtis similarities for each group of replicates illustrated in a nonparametric, multidimensional plot (MDS; Fig. 3A).

Final relative abundance
After the original community incubation the final relative abundance of the dominant species *L. polyedrum* was highest in the low CO$_2$ treatment (90%) and declined progressively with rising pCO$_2$ at medium pCO$_2$ (73%) and high pCO$_2$ (67%). In contrast,
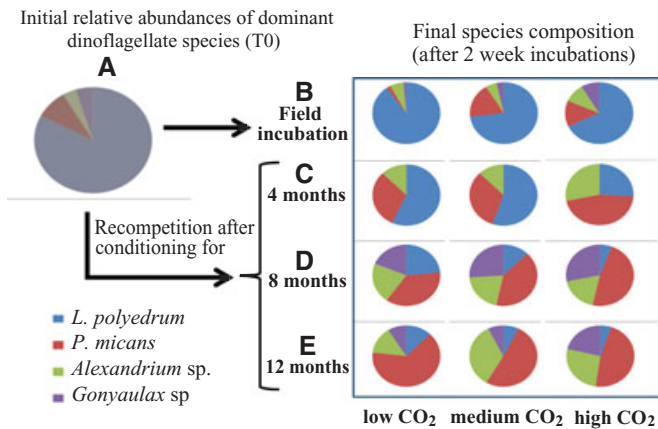
Figure 2. Relative abundance community composition graphs (A) from the original bloom sample, (B) at the end of the corresponding 2-week natural community pCO_2 incubation experiment, and at the end of the pCO_2-conditioned artificial community competition experiments at (C) 4 months, (D) 8 months, and (E) 12 months.

Table 2. Results of one-way ANOSIM comparing overall algal community structure across three different pCO_2 levels (low, ambient, and high) for each experiment after 0, 4, 8, and 12 months of conditioning of the clonal isolates at the three different pCO_2 concentrations. All global tests were run at 280 permutations except for the 4 months dataset that was limited to 15 permutations. P = significance level; ns = not significant at $P < 0.05$.

	R^1	P
Community structure		
0 months	0.97	0.004
4 months	0.67	ns
8 months	0.89	0.004
12 months	0.60	0.004

[1]R values typically range from 0 to 1, where R equal to 0 indicates community structures are not dissimilar among sample groups (H0 hypothesis), whereas R equal to 1 indicates strong dissimilarities among sample groups.

relative abundance increased progressively with increasing pCO_2 for *Gonyaulax* sp. (2%, 3%, and 8%). The relative abundances of the other two species were also highest at one or both of the two most elevated pCO_2 levels, although stepwise trends were less well defined (*P. micans* 2%, 17%, and 13%; *Alexandrium* sp. 6%, 5%, and 10%; Fig. 2B).

1- to 2-week growth rates

Growth rates of *L. polyedrum* in the natural community incubation declined moderately (global $R = 0.52$, $P = 0.029$; ANOSIM) as pCO_2 level increased (Fig. 4A). pCO_2 competition level had the strongest effect on growth rates of *P. micans* (global $R = 0.68$, $P = 0.007$), resulting in higher rates at high pCO_2 (Fig. 4A). In contrast, there was no significant effect of the three pCO_2 levels
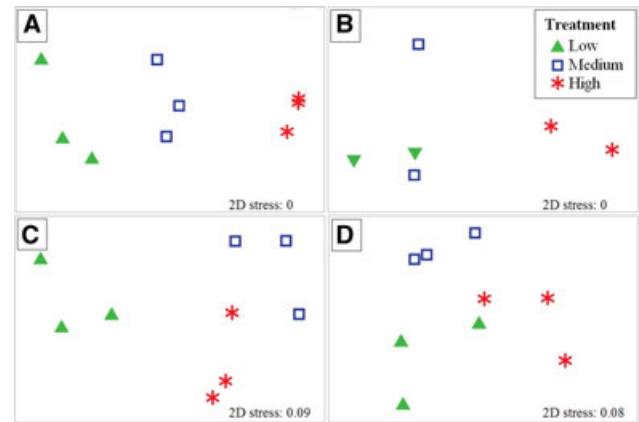

Figure 3. Cluster-diagram showing Bray–Curtis similarity for the dinoflagellate assemblages analyzed (A) after the initial 2-week natural community pCO_2 incubation, (B) after 4 months of conditioning, (C) after 8 months of conditioning, and (D) after 12 months of conditioning for each of the three pCO_2 treatments (low, ambient, and high).

on the growth rates of either *Alexandrium* sp. (global $R = 0.39$, $P > 0.05$) or *Gonyaulax* sp. (global $R = 0.04$, $P > 0.05$).

GROWTH RATES OF INDIVIDUAL CLONES

Growth rates of each clone were assessed in triplicate after 8 months of conditioning under their respective pCO_2 conditions. A striking observation was that growth rates of the dinoflagellates at all pCO_2 levels were in general much higher in the original natural community incubation (Fig. 4A) than in unialgal cultures (Fig. 4B), despite identical growth conditions. For *L. polyedrum*, clonal culture growth rates were 50% (low pCO_2), 32% (medium), and 27% (high) lower than in the mixed natural community incubation, whereas for *P. micans* these rates decreased by 65% (low), 53% (medium), and 82% (high). *Alexandrium* sp. also showed large decreases when brought into clonal culture, with growth rates that were 64% (low), 38% (medium), and 72% (high) lower compared to the original experiment. *Gonyaulax* sp. growth rates were least affected by unialgal culture conditions, with rates that were 33% lower (low), 44% higher (medium), and 14% lower (high) than in the natural community experiment (Fig. 4B).

The growth rates of some of the dinoflagellates in unialgal culture were affected by pCO_2, despite being lower overall than in the mixed natural community. Specific growth rates for *L. polyedrum* were 0.12 day^{-1} (low), 0.11 day^{-1} (medium), and 0.09 day^{-1} (high; Fig. 4B). The 22% growth rate decrease from low to high pCO_2 was significant ($P < 0.05$). Growth rates of *P. micans* were 0.11 day^{-1} (low), 0.13 day^{-1} (medium), and 0.14 day^{-1} (high; Fig. 4B), however the 15% increase from low to high pCO_2 was not significant ($P > 0.05$). Clones of *Gonyaulax* sp. grew the fastest at all CO_2 concentrations, ranging from
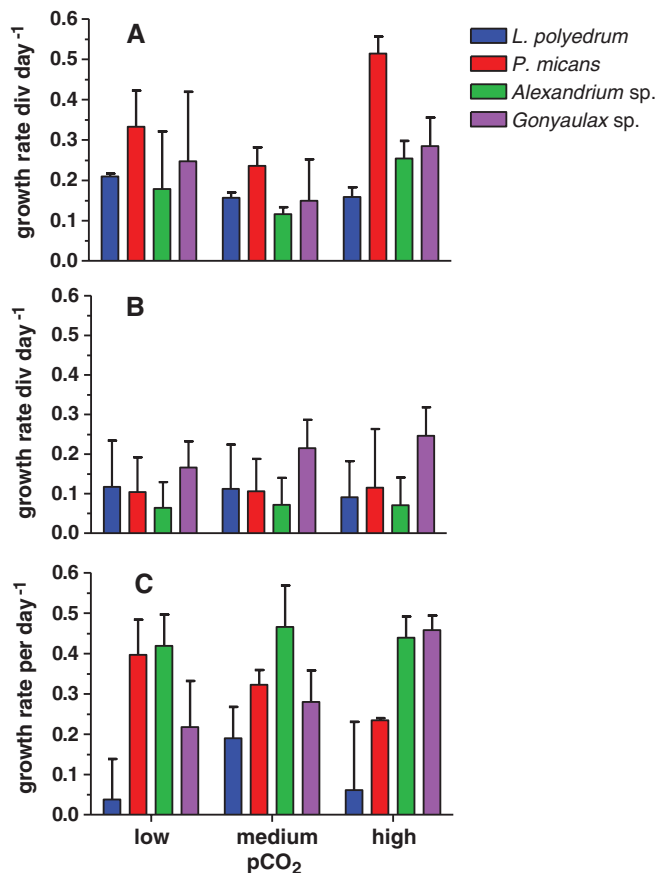
Figure 4. Specific growth rates of the four species in the three pCO_2 treatments, (A) during the second week of the initial natural community incubation experiment, (B) in the clonal isolates after 8 months of conditioning in unialgal cultures, and (C) during the second week of the 12-month artificial community recombination experiment. Error bars represent the standard deviations of triplicate bottles.

0.17 day^{-1} (low) to 0.22 day^{-1} (medium), and 0.25 day^{-1} (high; Fig. 4B). Growth rates were thus 32% higher at high versus low pCO_2 ($P < 0.001$). Growth of *Alexandrium* sp. was the slowest of the four dinoflagellates at all CO_2 concentrations, at 0.06 day^{-1} (low), 0.07 day^{-1} (medium), and 0.07 day^{-1} (high; Fig. 4B), and was not significantly different between any of the treatments ($P > 0.05$). Analysis of the growth rates of all species combined (i.e., independent of species) suggested a strong overall effect of pCO_2 on growth rate ($R = 0.704$, $P = 0.004$).

ARTIFICIAL COMMUNITY COMPETITION EXPERIMENTS
Final community structure
Community structure varied significantly in response to different pCO_2 levels in two out of the three artificial competition trials (Table 2). A trend of a steady decline of R-values with increasing

conditioning period was revealed as illustrated in side by side MDS plots for each of the experiments, from the natural community experiment (Fig 3A) to the 4-month (Fig. 3B), 8-month (Fig. 3C), and 12-month (Fig. 3D) artificial community competition trials. No conditioning resulted in the highest dissimilarities at the end of the initial natural community incubation, whereas increasing conditioning periods resulted in still significantly different, but less dissimilar, assemblages after 8 months (average $R = 0.89$ at $P = 0.004$) and 12 months (average $R = 0.6$ at $P = 0.004$). Our 4-month competition experiment with two replicates and a lower number of competing species (3) resulted in a limited number of permutation runs for the ANOSIM test (15) and was the only trial that did not yield a significance level (at $P < 0.05$) when overall community structure was compared. PERMANOVA (Type 1, sequential at 9999 permutations) analyses of three out of the four competition experiments (4-month trial excluded because of differences in design) indicated that both factors, pCO_2 level and conditioning time, interacted in forcing overall community structure (Pseudo-F $= 4.9$ at $P = 0.0001$). PERMANOVA test results further suggested the effect of different conditioning periods (Pseudo-F $= 161.8$ at $P = 0.0001$) was slightly stronger than that of differing pCO_2 competition levels (Pseudo-F $= 17.9$ at $P = 0.0001$).

Final relative abundance
The strongest effect of changed pCO_2 level was observed for the final abundance of *L. polyedrum* and *P. micans* (global $R = 0.54$ and 0.55, respectively; $P = 0.0001$; ANOSIM), with lesser but still significant effects for *Alexandrium* sp. and *Gonyaulax* sp. (global $R = 0.32$ and 0.24 and $P = 0.004$ and 0.033, respectively; Table 3A). Generally, conditioning time appeared to have a strong effect on the outcome of the competition trials for all four species with a relatively lower global R value of 0.44 ($P = 0.0001$) for *L. polyedrum* compared to 0.73 to 0.82 ($P = 0.0001$; ANOSIM) for the remaining three species (Table 3A).

L. polyedrum comprised a significantly larger percentage of the community at low versus medium or high pCO_2 levels in all three artificial community experiments (Fig. 2C–E). However, *L. polyedrum* became a significantly less successful competitor at all pCO_2 levels with progressively increasing conditioning time, from 0 to 4 to 8 and 12 months (Fig. 2B– E).

P. micans was an increasingly successful competitor relative to the other dinoflagellates with progressively longer conditioning periods (Fig. 2). The differing competition pCO_2 treatments affected *P. micans* relative abundance within each of the incubations, however, trends differed at the 12-month timepoint (highest abundance at low pCO_2) from those in the initial, 4- and 8-month trials (higher abundance at higher pCO_2). *P. micans* always increased in abundance at least twofold in each treatment for all

Table 3. Statistical results from two-way ANOSIM examining the effect of (A) differing competing pCO_2 level (low, ambient, and high) and preconditioning time (0, 8, and 12 months) on relative abundance changes for each of the four dinoflagellate species; and (B) the effect of pCO_2 competition level versus pCO_2 conditioning levels prior to the 12-month switch experiment. All global tests were run at 9999 permutations. P = significance level; ns = not significant at $P < 0.05$.

A

Factor	Competing pCO_2		Conditioning Time	
	R	P	R	P
L. polyedrum	0.54	0.0001	0.44	0.0001
Alexandrium sp.	0.32	0.004	0.73	0.0001
Gonyaulax sp.	0.24	0.033	0.81	0.0001
P. micans	0.55	0.0001	0.82	0.0001

B

Factor	Conditioning pCO_2 Level		Competing pCO_2	
	R	P	R	P
L. polyedrum	0.49	0.0003	0.14	ns
Alexandrium sp.	0.21	ns	0.12	ns
Gonyaulax sp.	0.16	ns	0.07	ns
P. micans	0.61	0.0002	0.56	0.0002

recombination trials. It was clearly the winner at high CO_2 after 4 months (Fig. 2C), and the dominant competitor at all CO_2 concentrations after 8 months of conditioning (Fig. 2D). At the 12-month timepoint, *P. micans* made up > 50% of the community at low and medium pCO_2, and closely approached that relative abundance at high pCO_2 (Fig. 2E).

   Gonyaulax sp. was always a winner in each artificial community trial (Fig. 2D, E), and similar to *P. micans*, its competitive success was increased by longer conditioning time (Table 3A). *Gonyaulax* sp. thus competed better in both the 8- and 12-month trials than in the original short-term natural community experiment. Its positive relative abundance trend with pCO_2 was conserved throughout the year-long experiment, with the highest final abundance always observed at high pCO_2 (Fig. 2).

   Alexandrium sp. increased in relative abundance during each recombination trial (Fig. 2) and, similar to *P. micans* and *Gonyaulax* sp., conditioning time positively affected its competitive ability. The clones conditioned to medium and high pCO_2 generally competed the best as reflected in the specific growth rate (Fig. 4A), but the significance level of this pCO_2 effect on competitive success was lower than for either *L. polyedrum* or *P. micans* ($R = 0.32$, $P = 0.004$, Table 3A). *Alexandrium* sp. competed more successfully after 4 months at high pCO_2 (Fig. 2C), similar to the original short-term incubation (Fig. 2B), and after 8 months it was a strong competitor under all pCO_2 concentrations (Fig. 2D). This species achieved a higher relative abundance after 12 months at medium CO_2, followed closely by high CO_2 (Fig. 2E).

1- to 2-week growth rates

When the individually conditioned clones were recombined in the 12-month artificial community experiment, in most cases their growth rates in mixed culture were substantially higher than in unialgal culture (Fig. 4C). The exception to this was *L. polyedrum*, which exhibited a range of growth rates that compared to the unialgal culture rates were 73% lower (low), 78% higher (medium), and 53% lower (high). In contrast, *P. micans* growth rates in coculture with the other species increased by 339% (low), 287% (medium), and 257% (high). *Gonyaulax* sp. growth rates exhibited a mixed response to coculturing, ranging from 30% lower (low) to 30% higher (medium) and 86% higher (high). *Alexandrium* sp. growth rates increased most dramatically in the mixed communities, with increases of up to 650% across all pCO_2 levels (compare Fig. 4B with Fig. 4C).

   Effects of pCO_2 on the growth rates of *L. polyedrum* were not significant in the 12-month artificial community experiment (global $R = 0.08$, $P > 0.05$; ANOSIM; Fig. 4C), unlike the natural community experiment (Fig. 4A) but similar to the 8-month unialgal culture measurements (Fig. 4B). pCO_2 competition level significantly affected the growth rates of *P. micans* (global $R = 0.49$, $P = 0.021$), with values that were inversely related to pCO_2 (Fig. 4C). This contrasted with the positive relationship in the original experiment (Fig. 4A), as well as with the lack of significant effect of pCO_2 in the unialgal cultures (Fig. 4B). In the 12-month artificial community experiment, increasing pCO_2 positively affected the growth rates of *Gonyaulax* sp. (global $R = 0.49$, $P = 0.043$), similar to the 8-month unialgal cultures (Fig. 4B), but unlike the original incubation experiment where its growth rates did not respond significantly to pCO_2 (Fig. 4A). At 12 months, the effects of pCO_2 competition level on *Alexandrium* sp. growth rates were not significant (global $R = 0.24$, $P > 0.05$), as was the case in both the natural community experiment and the unialgal cultures.

SWITCH EXPERIMENTS
Final community structure

The pCO_2 switch experiments at the 12-month timepoint (Fig. 5) showed that both pCO_2 level during competition (global $R = 0.60$ at $P = 0.0001$) as well as pCO_2 concentration during the conditioning period (global $R = 0.78$ at $P = 0.0001$) were
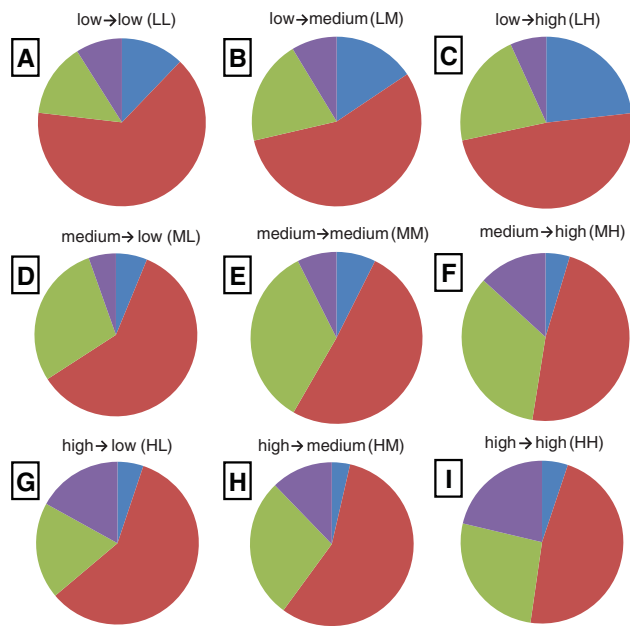
Figure 5. Relative abundance composition graphs after 2-week pCO_2 switch or reversion artificial community incubation experiments carried out at the 12-month timepoint, in which the isolates were allowed to compete at the two pCO_2 levels other than the one that they had been isolated from and conditioned to. Controls in which the competition experiment was conducted at the same pCO_2 to which the isolates were conditioned are the same data shown in Figure 2E, and are included again here for direct visual comparison with the switch treatment outcomes.

forcing factors on community structure (two-way ANOSIM test). CO_2 concentrations during conditioning thus appeared to have a slightly stronger effect on community structure than the pCO_2 levels the algae were exposed to during the 2-week competition.

Final relative abundance

Competition pCO_2 and conditioning pCO_2 levels had varying effects on final relative abundances during the switch experiment. There was a significant effect of prior pCO_2 conditioning on the dominance of *L. polyedrum* (global $R = 0.49$, $P = 0.0003$; Table 3B), but this was primarily a function of the cultures conditioned at low pCO_2 (pairwise ANOSIM test results not shown). *L. polyedrum* competed best in all treatments following conditioning at low pCO_2, and the clones conditioned at medium or high pCO_2 were relatively poor competitors in the pCO_2 switch experiments (Fig. 5D–F). Although its apparent relative abundance was somewhat lower in the low→low treatment than in the low→medium (Fig. 5B) and low→high (Fig 5C) treatments, its overall competitive success was not significantly different across the three pCO_2 levels in any of the switch experiments (global $R = 0.14$, $P > 0.05$; Table 3B).

The influence of prior conditioning pCO_2 on *P. micans* dominance was a significant influence at all three pCO_2 levels (global $R = 0.61$, $P = 0.0002$; Table 3B), and this was greater than for the primarily low pCO_2-conditioned treatment effect on *L. polyedrum* (pairwise ANOSIM test results not shown). Also unlike *L. polyedrum*, pCO_2 competition level during the artificial community incubations significantly influenced the relative dominance of *P. micans* (global $R = 0.56$, $P = 0.0002$; Table 3B). *P. micans* was still the most abundant species at low→medium (Fig 5B) and low→high (Fig 5C), but it did not dominate the community to the same degree as in the low→low treatment (Fig. 5A). For clones conditioned to medium pCO_2 levels, *P. micans* again was the dominant species after the incubation with the highest relative abundance attained at medium→low (Fig 5D). The medium→medium (Fig. 5E) and medium→high (Fig. 5F) pCO_2 treatments yielded approximately 50% *P. micans*, whereas its relative abundance was higher in the high→low (Fig. 5G) and high→medium (Fig. 5H) treatments than in the high→high (Fig. 5I).

Alexandrium sp. was a good competitor in all switch communities (Fig. 5), but statistical comparisons showed no significant effects of either prior pCO_2 conditioning level or the pCO_2 treatments during the competition experiments (global $R = 0.21$ and 0.12, respectively; $P > 0.05$; Table 3B). Relative abundance trends for *Gonyaulax* sp. indicated it was in general a less successful competitor in these switch experiments (Fig. 5), but like *Alexandrium* sp. neither conditioning history nor experimental pCO_2 levels (global $R = 0.16$ and 0.07, respectively; $P > 0.05$; Table 3B) influenced dominance trends.

1- to 2-week growth rates

In the 12-month switch experiment, growth rates were significantly affected by conditioning pCO_2 for all species (*L. polyedrum* global $R = 0.47$, $P = 0.002$; *P. micans* global $R = 0.56$, $P = 0.005$; *Alexandrium* sp. global $R = 0.42$, $P = 0.036$; *Gonyaulax* sp. global $R = 0.30$, $P = 0.037$; ANOSIM). In contrast, competition pCO_2 levels during the 2-week competition experiment only had a significant effect on growth rates of *L. polyedrum* (global $R = 0.48$, $P = 0.014$).

For *L. polyedrum*, there was a significant increase in growth rates for the cultures conditioned at low pCO_2 (LL) when grown in the communities incubated at medium (LM) or high (LH) levels ($p < 0.05$). There were no significant differences in *L. polyedrum* growth rates between any of three treatments for the medium or high pCO_2-conditioned cultures (Fig. 6A, $P > 0.05$). The same trends were observed for *Gonyaulax* sp. (Fig. 6D), but there were no clearly defined trends in growth rate across the three competition pCO_2 levels for *P. micans* (Fig. 6B) or *Alexandrium* sp. (Fig. 6C). In general, for each of the four species, pairwise comparisons from the switch experiment did not provide evidence
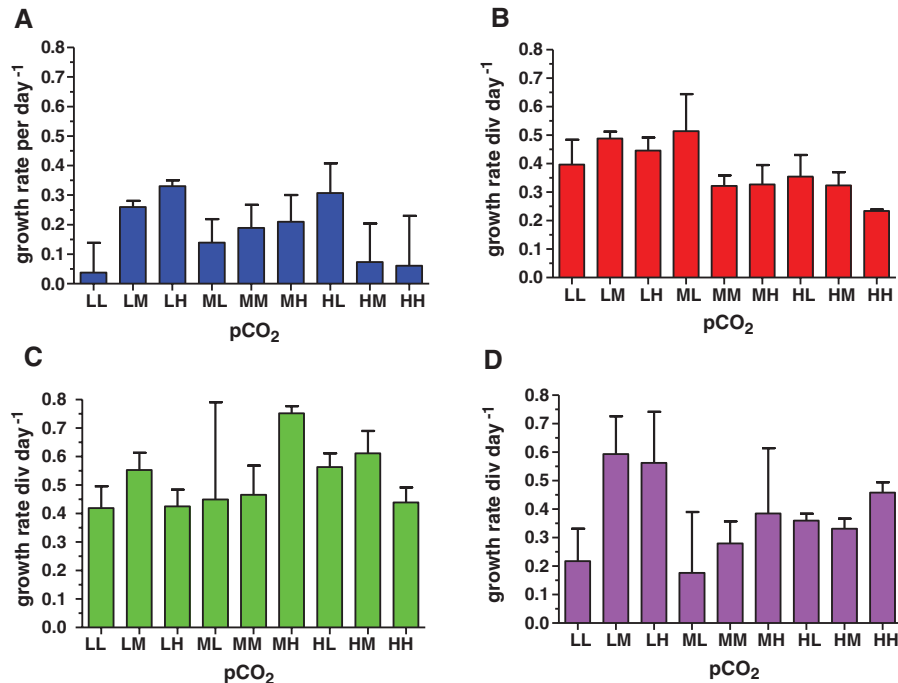
Figure 6. Specific growth rates for all four species during the second week of the 12-month switch experiments, including cultures conditioned at low pCO_2 and competed at low (LL), medium (LM), and high (LH) pCO_2; cultures conditioned at medium pCO_2 and competed at low (ML), medium (MM), and high (MH) pCO_2; and cultures conditioned at high pCO_2 and competed at low (HL), medium (HM), and high (HH) pCO_2. (A) *Lingulodinium polyedrum*, (B) *Prorocentrum micans*, (C) *Alexandrium* sp., and (D) *Gonyaulax* sp. Error bars represent the standard deviations of triplicate bottles.

Discussion

We initially examined competitive dynamics in a 2-week CO_2 incubation experiment using a natural community composed of four dominant dinoflagellate species. We then isolated clonal cultures of each species from these presumably genetically and phenotypically diverse populations, with the implicit assumption that by the end of the natural community experiment, the best-adapted variants of each species would have become dominant in each pCO_2 treatment. These clonal isolates were then conditioned separately for 1 year to the abiotic factor CO_2, without the adaptive evolutionary pressure of interactions between species. Periodically, these isolates were reassembled in artificial communities analogous to the original natural assemblage, to assess potential evolutionary influences of CO_2 conditioning on their competitive success at each pCO_2 level.

We employed artificial communities because enclosure artifacts can confound interpretation of long-term enclosed natural plankton assemblage experiments (Caron and Countway 2009). One natural plankton community mesocosm experiment spanning

several years suggested that it is virtually impossible to predict long-term trends in species abundances, because of progressively greater chaotic behavior over time (Benincà et al. 2008). Our novel approach of periodically recombining and competing individually conditioned isolates was intended to minimize some of the stochasticity inherent in long-term conditioning of the natural dinoflagellate assemblage, by providing greater predictability in a simplified format. However, clearly our artificial communities differ in many ways from the original natural community, despite being composed of the same four dominant species grown under the same set of environmental conditions. To better distinguish between the potential effects of this winnowing of a set of diverse populations down to a handful of clonal culture lines, and the selective effects of pCO_2 conditioning time, future studies using similar experimental designs could be improved by conducting an additional set of artificial community experiments directly after isolating the cultures.

The results of our 4-, 8-, and 12-month acclimated artificial community competition experiments had trends that were in some respects similar to, and other ways different from the outcome of the original mixed bloom incubations. Although the "winners" of the artificial community competitions in terms of competitive dominance were not the same as in the natural community experiment (i.e., *P. micans* instead of *L. polyedrum*), relative abundance

trends of individual species (i.e., *L. polyedrum*) with pCO$_2$ were often parallel to those in the original incubation. These observations provide some support for the trends in the responses of species abundance to pCO$_2$ changes in short-term natural assemblage experiments, while emphasizing that such brief experiments have limited predictive power for long-term community structure outcomes.

There are many possible reasons for the differences in final relative abundance between the original short-term natural community and the long-term conditioned artificial community experiments. The assumption that the outcome of competition between our sympatric dinoflagellate species hinges only on a single factor (i.e., pCO$_2$) may be unwarranted, as during the conditioning period the clones also may become acclimatized to and/or selected by other laboratory culture conditions. Statistical analysis suggested conditioning time was slightly more influential than pCO$_2$ levels in structuring the artificial communities, as indicated by the emergence of the "lab weed" *P. micans*, which competed progressively better over time and eventually dominated in all treatments. In fact, length of time in culture became progressively more influential than pCO$_2$ on overall community structure, suggesting the possibility that if we had extended the experiments long enough, the pCO$_2$ treatments might have eventually become irrelevant to the species composition of the assemblage. It is obvious that like short-term manipulations, long-term culture studies have artifacts of their own, and caution should be used when deriving information from older isolates because of accumulated culture selection artifacts. In fact, many phytoplankton clones used as model organisms for physiological, genetic, and evolutionary studies have been in culture for decades, suggesting that fresh isolates could increase the environmental relevance of laboratory studies.

A striking and unexpected result was that growth rates of several species in unialgal culture were significantly lower than in the natural and artificial community experiments. These large differences were not because of abiotic factors, as light, nutrients, temperature, and seawater chemistry were kept constant throughout all of the experiments. Thus, this effect must have been because of biotic factors directly associated with the presence or absence of the other species. In fact, these and other dinoflagellate species often occur in mixed-species blooms worldwide. (Allen 1941; Marasovic et al., 1995; Tahri-Joutei et al., 2000; Amorim et al., 2001). Our experimental results suggest that these multiple species associations are probably not simply because of coincidental similarities of environmental growth preferences, but could be a result of specific interactions between species.

Several possibilities may have contributed to differences in the growth rates of the dinoflagellates in unialgal versus mixed cultures. Many dinoflagellates exhibit facultative mixotrophic (phagotrophic) behavior (Burkholder et al. 2008; Caron 2000), but how the relative degree of autotrophy versus heterotrophy in

these organisms may be affected by future rising pCO$_2$ is unknown (Caron and Hutchins in press). All four of our species are potentially mixotrophic, and in fact *L. polyedrum*, *Alexandrium* sp., and *Gonyaulax* sp. have all been demonstrated to ingest *Prorocentrum* (Jeong et al. 2005). Growth rates of *Alexandrium* spp. (Jacobson and Anderson 1996) and other dinoflagellates (Jeong et al. 1999; Adolf et al. 2006) increase significantly in the presence of suitable prey. Supporting the case for potential reliance on mixotrophy by *Alexandrium* sp.in our experiments, growth rates were very slow in unialgal culture but increased dramatically in coculture, and throughout the 12-month experiment, conditioning and competition pCO$_2$ levels were not a significant influence on its dominance or growth rates. *P. micans* also responded with increased growth rates in the 12-month artificial community experiment, even though both conditioning and competition pCO$_2$ were also significant influences on its dominance and growth. In contrast, *L. polyedrum* and *Gonyaulax* sp. exhibited much less stimulation or even a reduction of growth rates in some mixed culture treatments, perhaps indicating mortality losses to grazing. If mixotrophy was a significant influence on community structure, as seems likely, it appears that *Alexandrium* sp. and *P. micans* may have taken on the role of grazers at the expense of the other two species.

Other, less understood interspecies interactions such as allelopathy (Granéli and Hansen 2006) may have also influenced growth in our mixed cultures. For instance, *Alexandrium fundyense* can inhibit the growth of competing phytoplankton species through the production of allelochemicals (Hattenrath-Lehmann and Gobler 2011). Other variables, such as growth factors in the form of vitamins (Tang et al. 2010) and co-occurring bacteria may also have played a role in the algal population dynamics in our experiments. Whether the competition between the dinoflagellates was influenced by these types of interactions is unknown, but the common occurrence of mixed species blooms of dinoflagellates and other phytoplankton groups such as raphidophytes and diatoms (Zhang et al. 2006; Schnetzer et al. 2007) suggest that interspecific facilitation or inhibition interactions could be a widespread phenomenon. Despite these necessary qualifications about factors other than pCO$_2$ that may have influenced our experimental results, outcomes of the four community trials consistently revealed a significant restructuring of our dinoflagellate communities based on pCO$_2$. This suggests the possibility that ocean acidification may play an influential role in bloom dynamics of these organisms in the future ocean, but in combination with biotic factors including mixotrophy, alleopathy, and facilitation.

We used final relative abundance as an indicator of competitive success, whereas growth rates were assumed to be indicative of any fitness changes under our long-term pCO$_2$ conditioning treatments. However, higher growth rates did not always correspond to competitive dominance at the end of the experiment.

This is partly because initial abundances of each species were different by design (reproducing their initial abundances in the original community), and partly because we focused on growth rates during the second week of each competition experiment. We avoided drawing conclusions based on initial (first week) growth rates because we assume that the cultures may not have been fully acclimated directly after being placed in the mixed culture communities. Because the final abundance of each species integrated the relative growth rates in both weeks, as well as initial abundances, the second week growth rates presented may not have been uniformly predictive of final relative abundance trends.

The responses of the four species in the switch experiments conducted at the 12-month timepoint did not provide unambiguous evidence that either adaptation or acclimation during the conditioning period at a particular pCO$_2$ subsequently provided a strong competitive advantage in a mixed community growing at that pCO$_2$. Neither *Alexandrium* sp. nor *Gonyaulax* sp. showed statistical evidence that the conditioning pCO$_2$ level affected their competitive success in any of the switch pCO$_2$ treatments. *L. polyedrum* showed a highly significant effect of conditioning pCO$_2$ on its competitive dominance, but only among the low pCO$_2$-conditioned clones. Furthermore, there was no evidence that these *L. polyedrum* clones had adapted in a manner that favored their competitive success at the particular conditioned pCO$_2$ (low→low treatments), as the net effect of long-term conditioning at low pCO$_2$ was to increase the competitive success of *L. polyedrum* at all pCO$_2$ levels tested (Fig. 6A–C).

For *P. micans* there was strong statistical support for an effect of all three conditioning CO$_2$ concentrations on its relative dominance in the switch experiments. Because all conditioning treatments increased its competitive abilities, though, we cannot rigorously attribute its success to selection by pCO$_2$ alone. *P. micans* could have also been favored by selection by one or more of other shared environmental conditions common to all the long-term cultures. However, low pCO$_2$-conditioned clones of *P. micans* did compete significantly better in the low→low treatments than in the low→medium and low→high treatments (Fig. 6A–C), suggesting that in this single case, conditioning at low pCO$_2$ may have conferred a competitive advantage in communities growing at this CO$_2$ level.

Dinoflagellates are a ubiquitous protistan functional group in marine ecosystems, and their multispecies blooms are excellent systems in which to experimentally test community competitive interaction dynamics in response to changing environmental factors such as pCO$_2$. However, these organisms are not typically amenable to cryopreservation, have extraordinarily large genomes (Hackett et al. 2004), and in general have slow growth rates compared to many microorganisms and thus present some inherent difficulties as experimental evolution model organisms. Our experiments were not intended to rigorously distinguish physiolog-

ical acclimation from genetic adaptation, and it is questionable to what degree selection could have led to significant adaptive responses within the limited number of generations encompassed by the relatively slow-growing clonal cultures. In contrast to studies with much faster growing organisms (Collins and Bell 2004; Lohbeck et al. 2012), our dinoflagellates only completed approximately 35 to 120 generations over the 1-year period. In addition to low intrinsic growth rates, and the reduced growth in unialgal cultures relative to mixed communities discussed above, slow growth in our cultures may have also been partially because of our attempts to keep our experiments environmentally relevant by using relatively modest nutrient concentrations.

Contemporary populations of algae are adapted to CO$_2$ concentrations roughly equivalent to our "medium" treatment. Nevertheless, they still may be exposed to large diurnal and seasonal ranges in dissolved inorganic carbon and pH levels. in habitats such as productive estuaries (Hinga 1992; Hansen et al. 1997) and coastal upwelling regimes (Feely et al. 2008). The seawater carbonate buffer system can vary dramatically during blooms, especially in semi-enclosed embayments. The low pCO$_2$ treatment in our study can be viewed as simulating a preindustrial value, but also serves as a good model for contemporary CO$_2$-depleted bloom water. Indeed, experiments suggest there are dinoflagellate taxa that thrive at low pCO$_2$ (Hinga 1992). Particularly notable in our study was the observation that the relative abundance of *L. polyedrum* always increased as pCO$_2$ decreased, suggesting that this organism may be better adapted to growth at lower CO$_2$/higher pH than many co-occurring species. Because CO$_2$ depletion commonly occurs in dense blooms, it may be no coincidence that *L. polyedrum* is usually the dominant dinoflagellate in recent recurring in the Southern California coastal region (Gregorio and Pieper 2000).

Shifts away from *L. polyedrum* at high CO$_2$ and toward greater dominance by species such as *Alexandrium* and *Gonyaulax* spp. that can produce virulent phycotoxins (Rhodes et al. 1996; Schantz et al. 1966) may have implications for the future environmental impacts of Southern California coastal blooms. In addition to increased abundance, some harmful bloom species such as the dinoflagellate *Karlodinium veneficum* and the diatom *Pseudo-nitzschia* spp. produce much more toxin when physiologically acclimated to high pCO$_2$ (Fu et al. 2010; Sun et al. 2011; Tatters et al. 2012). How toxin production rates will respond to selection by prolonged growth in acidified seawater is, however, presently unknown.

Our experiments examined only pCO$_2$, and so cannot predict the full spectrum of community responses to interactive effects from multiple global change variables (Boyd et al. 2010). Experiments incorporating not only higher pCO$_2$ but also projected concurrent warming, nutrient shifts, and irradiance changes need to be conducted with different assemblages (Rost et al. 2008;

Boyd et al. 2010). Our experiments suggest a need for such multivariate experiments to encompass longer time scales. Despite the escalating complexity such experiments entail, elucidation of how multiple interactive variables will modulate algal acclimatization and adaptation is crucial for a better understanding of their ecology in a future greenhouse ocean. Investigations into how the phenotypic plasticity and genetic diversity of algal populations are involved in long-term responses to a complex changing environment are a critical component of this effort. Our research adds to the very sparse data on long-term phytoplankton community structure responses under global change regimes. To our knowledge our study is the first to take an experimental approach; other efforts to examine long-term phytoplankton community responses have considered mainly warming, and present observational (Cloern et al. 2005; Paerl and Huisman 2009; Hinder et al. 2012) or modeling (Moore et al. 2008) datasets. Future experimental efforts distinguishing physiological acclimation from genetic adaptation are needed to provide a foundation for a more in-depth understanding of these processes in the future.

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