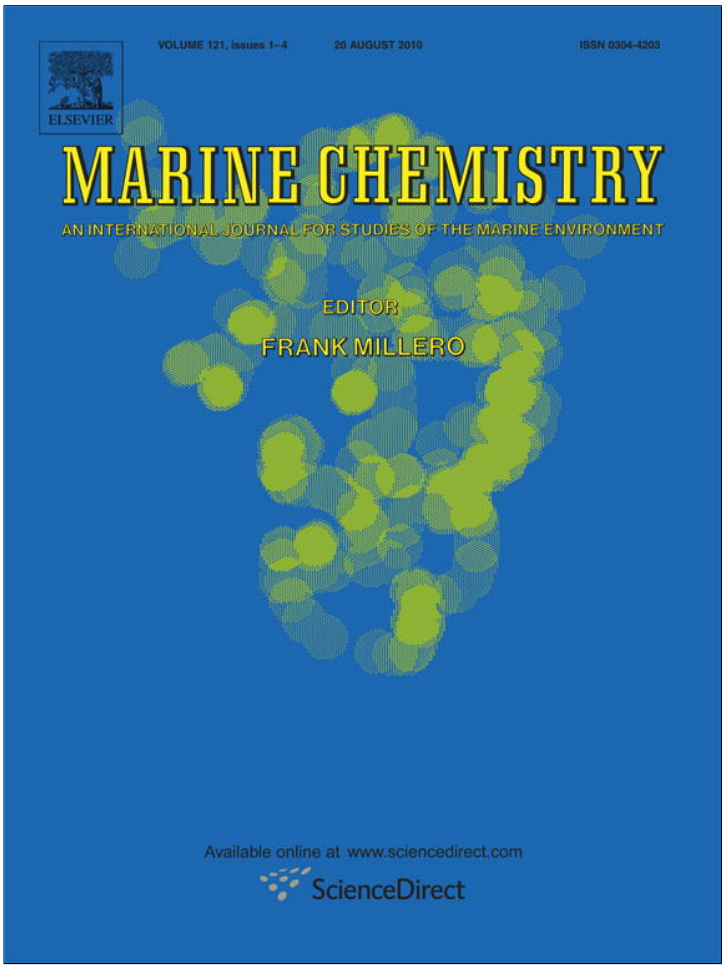

Marine Chemistry 121 (2010) 27–36

Contents lists available at ScienceDirect

Marine Chemistry

journal homepage: www.elsevier.com/locate/marchem

Nutrient temperature and light stress alter phosphorus and carbon forms in culture-grown algae

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ARTICLE INFO

Article history:
Received 8 May 2009
Received in revised form 1 December 2009
Accepted 2 March 2010
Available online 17 March 2010

Keywords:
Phosphorus forms
P NMR
C NMR

ABSTRACT

Particulate organic matter (POM) in seawater contains a wide range of chemical compounds, many of which come directly from phytoplankton. Phytoplankton may alter their biochemical characteristics in response to stress, which in turn alters the relative abundance of carbon (C) and phosphorus (P) compounds in the POM. The objective of this research was to examine changes in the P and C forms of algae cultured under light intensity, temperature and P nutrition stress, using ^{13}C CPMAS and ^{31}P NMR spectroscopy. Of the C forms, lipids and protein were significantly related to light intensity, while lipids were significantly increased at high temperatures. Low-P nutrition significantly altered ^{13}C NMR spectra, but did not alter modeled C forms. There were few changes in P forms with light and temperature stress. Low- and high-P nutrition altered C:P and N:P ratios. Low-P nutrition did not alter P forms. High-P nutrition increased pyrophosphate, indicating luxury P consumption. Polyphosphates did not appear to be related to light and temperature stress, because no significant changes in polyphosphates were observed in cultured algae under light and temperature stress.

1. Introduction

Particulate organic matter (POM) in surface seawater, primarily a direct derivative of living and dead organisms, contains a wide range of chemical compounds. Relatively little is known about the chemical composition of POM pools, or the changes in composition of POM when species assemblage or environmental conditions are altered (Hedges et al., 2001). There is considerable evidence that phytoplankton species differ substantially in their biogeochemical characteristics (e.g. Healy and Hendzel, 1979; Shifrin and Chisholm, 1981; Arrigo et al., 1999; Xu et al., 2006). Additionally, phytoplankton will alter their cell physiology in response to environmental stress, including changes in light, temperature and nutrients. Factors affecting photosynthesis have long been known to alter C fixation and the allocation of C into macromolecules. As such, light intensity has been shown to alter proteins (Morris et al., 1974; Cuhel and Lean, 1987; Mock and Kroon, 2002; Khotimchenko and Yakovleva, 2005) and lipids (Mock and Gradinger, 2000; Mock and Kroon, 2002; Fábregas et al., 2004; Khotimchenko and Yakovleva, 2005). Temperature stress will also alter C allocation, particularly lipids (Al-Hasan et al., 1991; Kakinuma et al., 2001; Khotimchenko and Yakovleva, 2005; Ventura et al., 2008). Nutrient stress has been reported to affect C forms: N starvation may enhance lipid storage (Shifrin and Chisholm, 1981) and may decrease protein content (Kilham et al., 1997; Lynn et al., 2000; Heraud et al., 2005), while increased carbohydrate:protein ratios are thought to be indicative of P deficiency (Healy and Hendzel, 1979; Kilham et al., 1997; Dean et al., 2008). Phosphorus stress can also alter the response of algae to light and temperature stress (e.g. Gauthier and Turpin, 1997; Sterner et al., 1997).

Little is known about changes in plankton P forms with environmental stress. Many of these forms are important metabolically and structurally, such as DNA, sugar phosphates and phospholipids, and are organic compounds chemically linked to C. Thus, if C fixation is altered by environmental stress, then organic P forms could also be altered. The C:P ratio has been shown to remain close to the Redfield ratio under low-light conditions, but to increase under high light conditions (Sterner et al., 1997), but it is not known if this also produces changes in the distribution of organic P forms. Inorganic P forms, such as orthophosphate and polyphosphate, may also be altered by environmental stress. Indeed, in bacteria, polyphosphates and associate enzymes such as polyphosphate kinase are involved in a wide range of aspects of metabolism, and play an important role in responding to a variety of environmental stresses (Jahid et al., 2006; Manganelli, 2007; Brown and Kornberg, 2008). For algae, luxury accumulation of polyphosphate is well known (e.g. Droop, 1973; Stevenson and Stoermer, 1982; Sterner and Elser, 2002). With respect to stress, it appears that polyphosphate accumulation in *Dunaliella salina* can be altered by alkaline stress (Pick et al., 1990) and osmotic stress (Bental et al., 1991). However, there are few reports to indicate that changes in

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0304-4203/$ – see front matter.
doi:10.1016/j.marchem.2010.03.002

polyphosphate synthesis represent an environmental stress response in algae to the same extent as has been observed for bacteria.

The objective of this research was to examine changes in plankton allocation of C and P in response to light, temperature or P nutrition stress, using solid-state ^{13}C CPMAS and solution ^{31}P NMR spectroscopy to characterize C and P forms or compound classes in culture-grown samples. Different algal species were grouped together based on the applied stress for statistical analyses, to determine if broad trends in C and P allocations could be detected. This is an important first step in understanding the factors controlling the production of organic compounds, and ultimately the factors controlling the oceanic biological pump.

2. Materials and methods

2.1. Algal cultures

A number of algal species were grown in culture to elucidate the role of various environmental stressors on allocation and synthesis of various C and P forms. Unless otherwise noted, species were cultured in F/2 marine phytoplankton culture medium (Sigma) added to standard artificial ocean water (Goldman and McCarthy, 1978) at 25 μmol photons $m^{-2} s^{-1}$, and 20 °C. These species included: *Thalassiosira pseudonana* (CCMP 1335), *T. weissflogii* (CCMP 1051), *Symbiodinium* sp. (CCMP 832), *Emiliania huxleyi* (CCMP 2090), *Dunaliella tertiolecta* (CCMP 1320), *Synechococcus* sp. (CCMP 2370), *Amphora salina, Imantonia rotunda, Fragilariopsis curta, F. cylindrus, Nitzschia subcurvata* and *Phaeocystus antarctica.* These species include diatoms (*T. pseudonana*, *T. weissflogii*, *A. salina*, *F. curta*, *F. cylindrus*, and *N. subcurvata*), a prymnesophyte (*P. antarctica*), a coccolithophore (*E. huxleyi*), a chlorophyte (*D. tertiolecta*), a haptophyte (*I. rotunda*), a dinoflagellate alga (*Symbiodinium* sp.) and a cyanobacterium (*Synechococcus* sp.). The species grown under low-temperature (10 and 15 °C) stress and control temperature (20 °C) were *A. salina* (10 and 20 °C) and *E. huxleyi* (10, 15 and 20 °C), constituting two species for the control and three comparison samples (two species, one grown at two different low temperatures) for the treatment. The species grown under high temperature (26 °C) stress and control temperature (20 °C) were *T. weissflogi*, *D. tertiolecta* and *Synechococcus* sp., with three comparison samples each used for the control and for the high-temperature treatment. The species grown under high-P (7.2 μM mol P) and control P (3.6 μM mol P) conditions were *A. salina*, *E. huxleyi*, *I. rotunda*, *T. pseudonana*, and *Synechococcus* sp., with five comparison samples each for control and treatment. The species grown under low-P (0.5 μM mol P) and control P (3.6 μM mol P) conditions were *E. huxleyi*, *Symbiodinium* sp., *T. pseudonana*, and *Synechococcus* sp., with five comparison samples (one species was cultured twice) each for control and treatment. Control light conditions were 25 or 125 μmol photons $m^{-2} s^{-1}$, depending on the species, high light conditions were 250 or 125 μmol photons $m^{-2} s^{-1}$, depending on the species, and low light conditions were 5 or 25 μmol photons $m^{-2} s^{-1}$, depending on the species. The species grown under low light and controlled light were *F. curta*, *F. cylindrus*, *N. subcurvata*, *P. antarctica*, *T. pseudonana*, *E. huxleyi* and *Symbiodmium* sp., with seven samples for control and eight comparison samples (one species cultured twice) grown under low light. The four species grown under high light and control conditions were *F. curta*, *F. cylindrus*, *N. subcurvata*, and *P. Antarctica* (four samples each for high light and control).

2.2. Chemical analyses and NMR spectroscopy

Total C and total N were analyzed on a Carlo Erba NA1500 Series II elemental analyzer, without acidification. Total P was determined by digestion (Parkinson and Allen, 1975; O'Halloran and Cade-Menun, 2008), followed by colorimetric analysis (Murphy and Riley, 1962).

2.3. ^{13}C NMR spectroscopy

Solid-state cross-polarizing magic angle spinning (CP/MAS) spectra were acquired at a ^{13}C frequency of 100.53 on a Varian VXR/Unity spectrometer with a wide-bore 9.4T magnet and a 7 mm Jakobsen probe. Oven-dried samples (0.052–0.249 g) were packed into a 7-mm silicon nitride rotor with a Kel-F end cap, and were spun at 6000 ± 10 Hz. For small samples, a spacer of hexagonal boron nitride was inserted into the rotor prior to packing the samples, to properly align them in the center of the rotor. A standard cross-polarization sequence was used, including a pulse length of 4.0 μs, a 0.5-s recycle delay, and a spectral width of 100,000 Hz. The number of scans acquired ranged from 25,860 to 483,424, depending on the sample size and C concentration. Chemical shifts were externally referenced to the methyl resonance of hexamethylbenzene at 17.36 ppm (Hedges et al., 2001).

Carbon NMR spectra were processed using NUTS software (Acorn NMR, Livermore CA), with a backward linear prediction of 2 points and a 200-Hz Gaussian multiplication Fourier transformation of the acquired FID. The spectra were baseline corrected between −40 and 300 ppm, and the rotor background signal was subtracted after processing. The spectra were divided into seven spectral regions (Hedges et al., 2002; Nelson and Baldock, 2005): I, 0–45 ppm (alkyl C); II, 45–60 ppm (N-alkyl and methoxyl C); III, 60–95 ppm (O-alkyl C); IV, 95–110 (di-O-alkyl C); V, 110–145 ppm (unsaturated C); VI, 145–165 ppm (O-Aromatic C); and VII, 165–220 ppm (carbonyl C). Example spectra are shown in Fig. 1. The distribution of ^{13}C NMR signal intensity was quantified by determining the relative contributions of total signal intensity associated with each spectral region (%). Spectral intensities areas were corrected for spinning side bands (SSB) by adding two times the signal intensity associated with the prominent SSB at 230 ppm to the signal intensity of region VII, and subtracting half the signal intensity of the 230-ppm SSB from each of

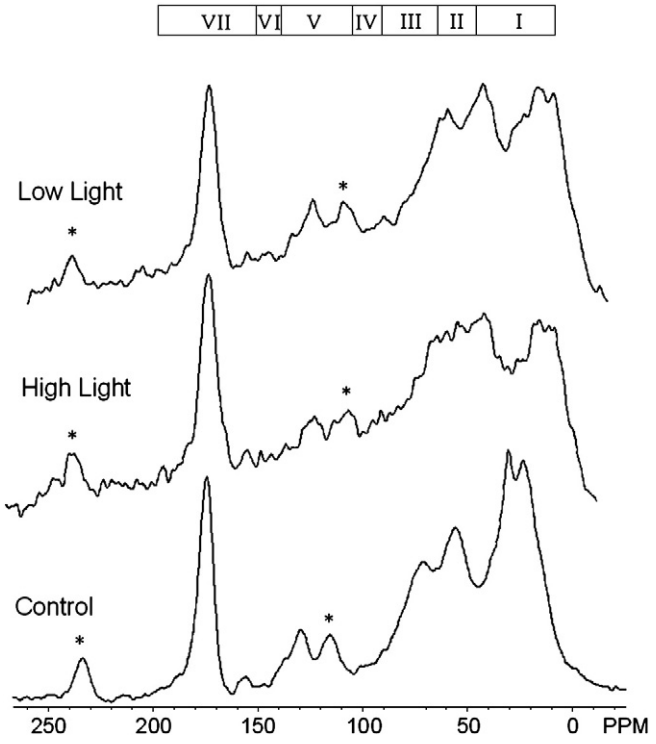

Fig. 1. Examples of ^{13}C CP/MAS NMR spectra for *Fragilariopsis cylindrus* grown under control (125 μmol photons $m^{-2} s^{-1}$), high (250 μmol photons $m^{-2} s^{-1}$) and low (5 μmol photons $m^{-2} s^{-1}$) light conditions. Roman numerals indicate C form classes I, 0–45 ppm (alkyl); II, 45–60 ppm (N-alkyl); III, 60–95 ppm (O-alkyl); IV, 95–110 (di-O-alkyl); V, 110–145 ppm (C C*–H (or –C)); VI, 145–165 ppm (C C*–O (or –N)); VII, 165–220 ppm (carbonyl). An * indicates a spinning side band.

regions IV and V. The molecular mixing model (MMM) was used to predict the molecular composition of samples, by defining the mixture of marine molecular structures that minimized the sum of squares of differences between measured and predicted ^{13}C NMR signal intensities (Baldock et al., 2004). The model was constrained by forcing the predicted molar N/C ratio to equal the corresponding measured value (Baldock et al., 2004). The MMM was fixed to describe five components: carbohydrate, protein, lipid, aromatics, and pure carbonyl.

2.4. ^{31}P NMR spectroscopy

Phosphorus was extracted for solution ^{31}P NMR spectroscopy with a modified version of the Cade-Menun and Preston (1996) procedure. A sample of variable size (0.033 to 0.35 g, depending on the availability of material) was placed in a 50-mL centrifuge tube with 25 mL of combined 0.25 M NaOH and 0.05 M Na_2EDTA, shaken gently for 16 h at 20 °C, and then centrifuged for 20 min at approx. 1500 *g*. A 1-mL aliquot was removed for total P analysis by ICP-OES, after which the remaining filtrates were frozen (24–48 h) and lyophilized (24–36 h).

The lyophilized extracts were re-dissolved in 1.0 mL D_2O, 0.6 mL 10 M NaOH and 0.6 mL of the NaOH–EDTA extracting solution, and were allowed to stand for 10 min with occasional vortexing. Samples were then centrifuged for 20 min at approx. 1500 *g*, transferred to 10-mm NMR tubes and stored at 4 °C prior to analysis within 12 h. Solution ^{31}P NMR spectra were obtained using a Varian Unity ^{INOVA} 600 MHz spectrometer equipped with a 10-mm broadband probe. The NMR parameters were: 90° pulse, 0.68 s acquisition time, 4.32 s pulse delay, 20 °C, and 5000–8500 scans (8–12 h).

Phosphorus compounds were identified by their chemical shifts (ppm) relative to an external orthophosphoric acid standard and the orthophosphate peak for each sample was standardized to 6 ppm. Peak areas were calculated by integration on spectra processed with 5 Hz line broadening, using NUTS software (Acorn NMR, Livermore CA, 2000 edition). Peaks were subsequently grouped into compounds, or compound classes if specific identifications could not be made, based on the literature (Cade-Menun, 2005). Example spectra are shown in Fig. 2. Detected inorganic P compounds include orthophosphate (6 ppm), pyrophosphate (−4.0 ppm), and polyphosphates (−17 to −20 ppm). Detected organic P compounds included orthophosphate monoesters between 5.9 and 3.7 ppm and orthophosphate diesters

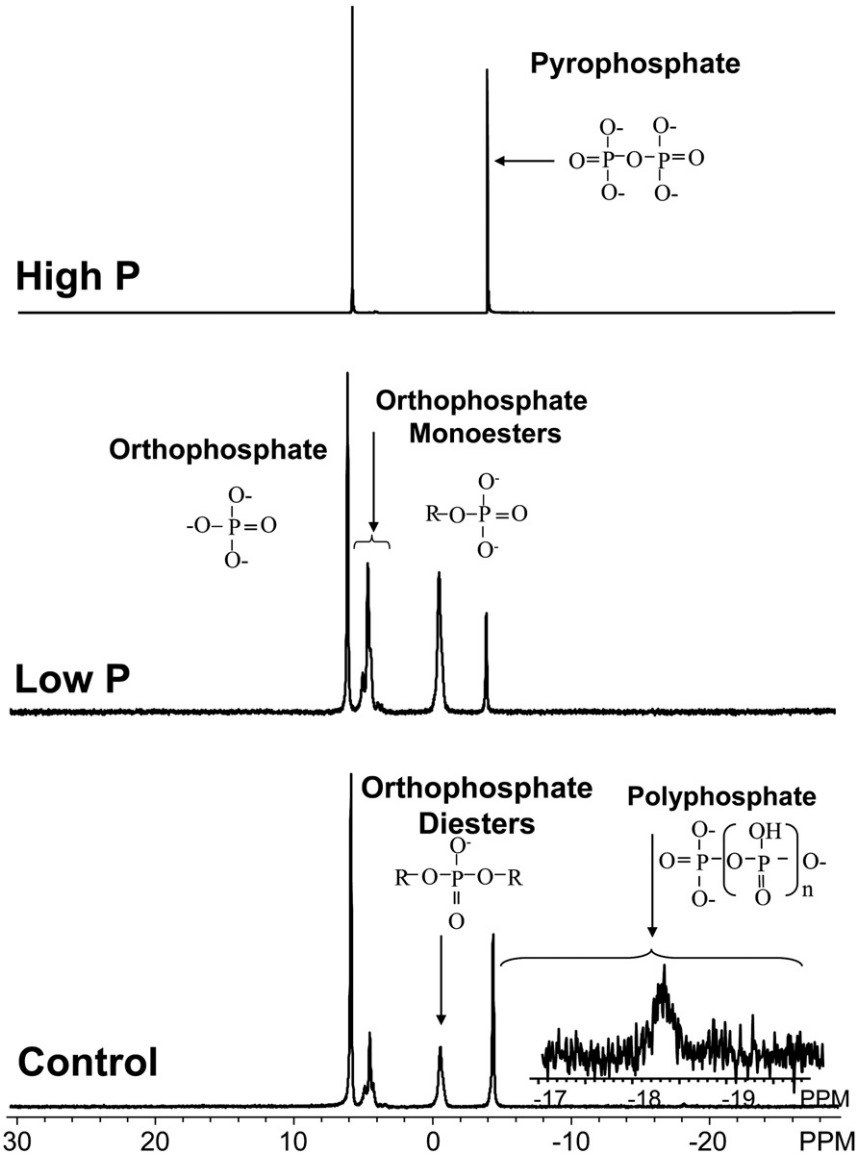

Fig. 2. Examples of ^{31}P NMR spectra for *Emiliania huxleyi* grown under high, low and control P conditions.

between 2.5 and −1 ppm (Turner et al., 2003; Paytan et al., 2003; Cade-Menun, 2005; Cade-Menun et al., 2005). The orthophosphate diesters were divided into DNA at −0.8 ppm, and other diesters from 2.5 to 0 ppm, which would include intact phospholipids. Orthophosphate monoesters, the dominant organic P compound class in these samples, were divided into two groups: monoester 1 included peaks from 5.9 to 4.8 ppm, while monoester 2 peaks ranged from 4.7 to 3.4 ppm. Both of these groups include sugar phosphates, inositol phosphates and orthophosphate diester degradation products if present (Turner et al., 2003). Degradation peaks for phospholipids, such as phosphatidic acid at 5.24 ppm, α-glycerophosphate at 5.2 ppm, and β-glycerophosphate at 4.91 ppm, would be found in the monoester 1 region (Turner et al., 2003; Turner and Newman, 2005; Bünemann et al., 2008; Doolette et al., 2009). Peaks in the monoester 2 region would include peaks for mononucleotides (4.3–4.7 ppm), some sugar phosphates such as α-glucose-1-phosphate at 3.4, and degradation peaks for RNA at 4.7, 4.4 and 4.2 ppm (Turner et al., 2003).

2.5. Statistical analysis

Statistical analyses (analysis of variance, Pearson pair-wise correlations and principal components analysis (PCA)) were performed with the statistical package JMP (version 4.04, SAS Institute, Inc.), with significance set at $P \leq 0.1$. The ^{31}P and ^{13}C NMR results were statistically analyzed both as relative percent and as concentration of forms or compound classes on a dry weight basis. Differences in statistical significance were found for both P concentrations and relative percents, but no differences in significance between relative percentages and concentrations were found for C, so only the C relative percent results are shown. The ^{31}P and ^{13}C NMR spectra were also compared with principal component analysis (unrotated).

3. Results

3.1. Light stress

There were no significant differences in total P, C, N or the C:N, C:P or N:P ratios between control cultures and those grown under low light conditions (Table 1). Cultures grown under high light conditions had significantly higher total P concentrations than control samples, but no differences for C or N, or the C:N, C:P or N:P ratios. Correlation analysis combining low light, high light and control samples shows that light intensity was positively correlated with total P ($r = 0.58$; $P = 0.01$), N ($r = 0.63$; $P = 0.004$), and C ($r = 0.47$; $P = 0.04$) and was negatively correlated with the C:N ratio ($r = -0.54$; $P = 0.02$).

No significant differences in any of the C spectral regions or modeled C forms were observed for samples grown under low light conditions, relative to controls (Table 2). Algae grown under high light conditions showed a significant increase in the O-Aromatic C (C VI) spectral region, but this did not produce any significant differences in modeled C forms. Correlation analysis combining low light, high light and control samples shows that light intensity was positively correlated with the C VI spectral region ($r = 0.43$; $P = 0.06$), and protein ($r = 0.46$; $P = 0.05$), and was negatively correlated with lipids ($r = -0.41$; $P = 0.08$). Principal components analysis for the ^{13}C CPMAS spectra (Fig. 3) did not show any significant grouping for samples grown under low or high light conditions, relative to controls.

There was a significant decrease in the relative percent of P in the monoester 1 region in the low light samples relative to controls (Table 3), but the concentration of P in the monoester 1 region was not significantly different (Table 4). There were no other differences in P forms, in relative percent or concentration, for the low light treatment. For the high light treatment, there were no significant differences in the relative percentages of P forms compared with controls (Table 3). However, the concentrations of orthophosphate, monoester 2 and organic P were significantly higher in the samples grown under high light conditions than the controls. Principal component analyses and correlation analyses combining low light, high light and control samples did not show any significant relationships for P forms or compound classes.

3.2. Temperature stress

Growth under low and high temperature did not produce any significant differences relative to control cultures for total P, C, and N, or the C:N, C:P and N:P ratios (Table 1). None of these parameters were significantly correlated with temperature in correlation analyses combining low temperature, high temperature and control samples.

There were no significant differences in C spectral regions or modeled C forms for low-temperature cultures relative to controls (Table 2). The high-temperature cultures, however, had a significant increase in the carbonyl C (C VII) spectral region, and a significant decrease in lipids relative to controls (Table 2). Correlation analyses combining low temperature, high temperature and control samples showed negative correlations to the C V ($r = -0.72$; $P = 0.02$) and C VI regions ($r = -0.62$; $P = 0.06$), and positive correlations to the C VII region ($r = 0.61$; $P = 0.6$) and to carbonyl ($r = 0.71$; $P = 0.02$). Principal components analysis also showed a significant difference in ^{13}C CPMAS spectra for high-temperature cultures relative to controls (Fig. 3), but no significant differences for low-temperature samples.

Low-temperature stress produced a significant decrease in the relative percent of orthophosphate (Table 3), but no changes in the relative percentages of other P forms (Table 4). There were no significant differences in concentrations of any P forms in the low-temperature samples compared with the controls. The high-temperature cultures were not significantly different from controls in any P

Table 1
Total P, total C, total N and the C:N, C:P and N:P ratios for algal cultures grown under control conditions and under light, temperature, and P nutrition stress. Results shown are averages and (standard deviation).

	Total P μmol g^{-1}	Total C mol g^{-1}	Total N mol g^{-1}	C:N	C:P	N:P
Low lt.	288.1 (102.6)	22.8 (6.71)	3.44 (1.01)	6.75 (1.26)	89.0 (47.8)	13.3 (5.77)
Control	278.3 (102.9)	23.0 (7.18)	3.44 (0.95)	6.83 (1.41)	90.6 (33.8)	14.0 (6.49)
High lt.	452.2 (111.3)*	37.0 (23.3)	8.56 (6.05)	4.82 (1.57)	77.7 (31.2)	17.9 (7.99)
Control	217.5 (96.2)*	18.3 (3.29)	3.26 (1.19)	6.03 (1.35)	97.9 (45.0)	16.8 (7.66)
Low tmp.	296.1 (253.8)	16.9 (3.31)	2.56 (0.83)	6.96 (2.41)	91.0 (26.6)	13.1 (9.23)
Control	203.6 (49.1)	11.4 (5.9)	1.44 (0.78)	8.01 (0.22)	61.4 (44.1)	7.74 (5.71)
High tmp.	490.8 (282.2)	21.4 (11.8)	3.50 (1.78)	6.04 (0.47)	45.7 (7.39)	7.55 (1.17)
Control	651.5 (336.0)	29.1 (3.33)	4.67 (0.38)	6.23 (0.51)	54.8 (29.7)	8.65 (4.38)
Low P	250.2 (263.4)	27.7 (4.46)	3.42 (1.53)	8.99 (2.71)	275.4 (203.2)*	22.0 (19.3)*
Control	556.1 (286.8)	26.9 (5.94)	3.80 (0.75)	7.11 (1.14)	59.5 (30.3)*	7.98 (3.14)*
High P	2837.5 (2062.6)	19.0 (10.0)	3.16 (1.96)	6.30 (0.65)	12.2 (12.1)*	2.03 (2.10)*
Control	881.0 (1328.0)	16.0 (8.00)	2.35 (1.19)	6.73 (1.51)	79.8 (64.3)*	11.3 (9.15)*

An * indicates a significant difference between a treatment and a control.

Table 2
^{13}C CPMAS spectral regions and modeled C forms for algal cultures grown under control conditions and under temperature (temp.), P nutrition and light stress. Carb, carbohydrate; Prot, protein; Arom, aromatics; Cbnl, carbonyl; Cr:Pr, carbohydrate:protein ratio. Results shown are averages and (standard deviation).

	C I	C II	CIII	CIV	C V	C VI	C VII	Carb	Prot	Arom	Lipid	Cbnl	Cr:Pr
Low light	32.9 (7.7)	12.9 (1.2)	23.2 (5.7)	3.2 (1.2)	9.0 (1.5)	2.8 (1.6)	16.1 (2.8)	32.3 (7.8)	54.9 (11.5)	4.3 (2.0)	8.3 (10.4)	0.3 (0.6)	0.63 (0.27)
Control	36.5 (7.7)	13.0 (1.3)	21.9 (6.2)	2.9 (1.8)	8.4 (1.0)	2.2 (0.9)	15.2 (2.0)	25.8 (14.0)	56.8 (20.8)	4.6 (4.1)	11.1 (9.6)	1.6 (2.2)	0.55 (0.35)
High light	27.1 (4.9)	13.8 (0.6)	24.9 (1.5)	4.2 (0.3)	9.6 (1.1)	3.9* (0.6)	16.7 (3.6)	20.9 (16.8)	76.0 (20.4)	2.0 (4.1)	0.0 (0.0)	1.1 (1.4)	0.34 (0.30)
Control	33.3 (8.7)	12.9 (1.8)	23.7 (2.2)	3.3 (1.2)	8.4 (0.8)	2.7* (0.8)	15.9 (17.8)	24.2 (24.9)	65.6 (5.5)	4.3 (7.2)	4.9 (2.2	1.1 (0.43	0.48 (6.1)
Low temp.	33.7 (13.1)	12.6 (1.4)	20.5 (2.2)	3.7 (3.4)	112.1 (1.8)	4.7 (7.6)	12.5 (7.6)	27.1 (2.7)	54.0 (14.3)	7.5 (3.8)	11.0 (13.4)	0.4 (0.8)	0.53 (0.14)
Control	37.4 (1.1)	12.7 (1.1)	19.6 (0.1)	3.1 (0.2)	10.9 (0.2)	2.6 (0.1)	14.0 (0.5)	26.7 (0.3)	44.5 (1.0)	12.7 (0.6)	15.9 (1.7)	0.3 (0.4)	0.60 (0.02)
High temp.	33.3 (4.1)	12.1 (0.3)	22.8 (1.3)	2.5 (1.7)	2.6 (2.3)	2.1 (0.4)	19.7* (0.6)	30.2 (2.1)	56.1 (5.1)	3.0 (2.8)	6.0* (3.1)	4.7 (2.5)	0.54 (05)
Control	39.7 (1.6)	12.1 (1.0)	21.1 (2.6)	2.1 (1.1)	7.9 (1.6)	1.8 (1.1)	15.5* (1.6)	29.0 (3.2)	54.3 (1.0)	2.4 (3.4)	13.8* (0.0)	0.6 (0.8)	0.53 (0.05)
Low P	33.6 (5.0)	11.9* (0.9)	27.6 (4.8)	3.9 (1.8)	7.8 (1.1)	1.5 (0.4)	13.9 (3.3)	38.6 (7.0)	40.8 (14.4)	5.6 (3.9)	13.1 (9.1)	2.0 (2.3)	1.04 (0.44)
Control	38.2 (6.3)	13.4* (0.6)	22.7 (8.3)	2.8 (1.3)	7.5 (1.9)	1.3 (0.5)	14.2 (2.4)	31.9 (11.4)	47.1 (5.0)	3.8 (3.0)	15.5 (8.6)	1.8 (2.4)	0.68 (0.24)
High P	31.4 (8.3)	12.9 (2.0)	22.6 (2.5)	4.1 (1.3)	9.5 (2.8)	3.6 (1.8)	15.9 (3.3)	30.2 (2.9)	54.8 (6.2)	6.4 (4.7)	6.4 (6.7)	2.2 (4.9)	0.55 (0.05)
Control	34.7 (2.8)	13.4 (0.6)	22.5 (5.5)	3.0 (0.6)	9.0 (2.4)	3.2 (1.6)	14.4 (1.4)	30.2 (7.9)	53.3 (11.6)	6.0 (6.8)	8.8 (5.7)	1.7 (3.5)	0.58 (0.16)

An * indicates a significant difference between a treatment and a control.

forms, in relative percent or concentration. Principal components analyses and correlation analyses combining low temperature, high temperature and control samples did not show any significant relationships for P forms or compound classes.

3.3. P nutrition stress

Growth under low-P conditions did not produce significant differences in total P, N or C, or the C:N ratio, relative to controls (Table 1). However, it did produce significantly higher C:P and N:P ratios. Growth under high-P conditions also did not produce significant differences in P, N or C, or the C:N ratio, relative to controls, but the C:P and N:P ratios were significantly lower than the controls. Correlation analysis combining high and low-P treatments with controls showed a positive correlation with total P ($r=0.67$; $P=0.001$) and negative correlations of P nutrition with the C:P ($r=-0.45$; $P=0.05$) and N:P ($r=-0.46$; $P=0.04$) ratios.

Low-P nutrition resulted in a significant decrease in the N-alkyl and methoxyl C (C II) spectral region, but no significant differences in modeled C forms or the carbohydrate:protein ratio (Table 2). High-P

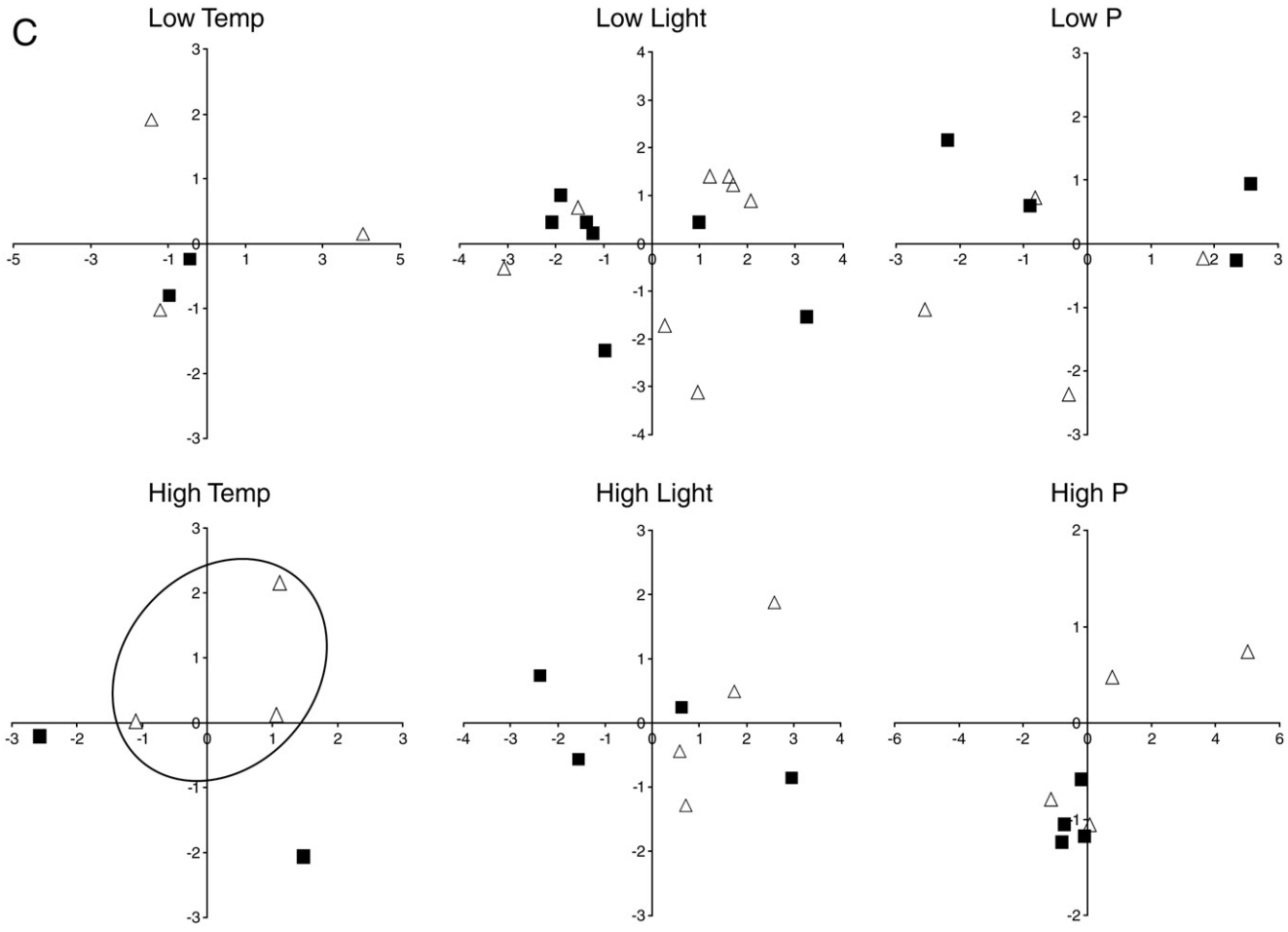

Fig. 3. Principal components analysis (unrotated) conducted using the relative percentages of C in the carbon classes defined by signal intensities in each region, from the ^{13}C CP/MAS NMR spectra. Solid squares represent control samples; open triangles represent stressed samples.

Table 3
Phosphorus forms, in relative percent (%), determined by ^{31}P NMR spectroscopy for algal cultures grown under control conditions and under temperature (temp.), P nutrition (low P, high P) and light stress. Orth, orthophosphate; pyro, pyrophosphate; poly, polyphosphate; mono1, orthophosphate monoester 1 region; mono2, orthophosphate monoester 2 region; OthDi, orthophosphate diesters other than DNA; Porg, organic P calculated from ^{31}P NMR spectra. See text for details on these regions. Results shown are averages and (standard deviation).

	Orth	Pyro	Poly	Mono1	Mono2	DNA	OthDi	Porg
Low light	41.3 (11.6)	13.5 (9.0)	0.7 (0.9)	5.5* (3.0)	32.3 (13.0)	5.8 (3.0)	1.0 (0.6)	44.5 (16.6)
Control	33.1 (11.8)	16.2 (13.2)	0.5 (0.7)	10.7* (6.3)	31.4 (17.3)	7.1 (7.8)	1.0 (1.3)	50.3 (19.4)
High light	46.0 (7.6)	9.4 (6.4)	0.0 (0.0)	6.5 (2.5)	33.9 (7.5)	3.7 (2.7)	0.4 (0.5)	44.6 (9.7)
Control	33.5 (12.3)	6.6 (3.1)	0.0 (0.0)	8.9 (3.1)	42.7 (5.7)	7.2 (8.1)	1.1 (1.2)	59.9 (14.9)
Low temp.	70.8* (3.9)	10.6 (11.0)	0.0 (0.0)	3.3 (1.1)	12.0 (6.4)	2.8 (1.8)	0.7 (0.6)	18.8 (8.7)
Control	79.3* (0.5)	1.5 (0.9)	0.4 (0.5)	3.1 (1.4)	13.2 (0.3)	1.9 (0.7)	0.8 (0.1)	19.0 (1.9)
High temp.	54.0 (6.5)	18.7 (6.3)	0.3 (0.5)	3.8 (1.2)	19.8 (7.2)	2.3 (1.4)	1.1 (0.6)	27.1 (7.4)
Control	59.4 (10.7)	11.9 (9.6)	0.3 (0.5)	3.8 (1.2)	21.0 (14.1)	1.8 (0.9)	0.3 (0.5)	28.5 (17.4)
Low P	37.8 (24.4)	9.0 (10.0)	0.3 (0.7)	9.1 (3.6)	27.2 (17.0)	13.1 (13.7)	3.4 (5.6)	52.9 (27.3)
Control	50.8 (27.5)	20.2 (13.8)	0.7 (0.7)	6.1 (6.8)	16.9 (9.7)	4.5 (7.4)	0.9 (1.1)	28.4 (18.7)
High P	54.1* (11.6)	37.2* (18.2)	0.0 (0.0)	2.1 (1.8)	5.2 (5.0)	1.2 (0.4)	0.2 (0.4)	8.7 (7.4)
Control	79.3* (10.1)	2.4* (0.6)	0.1 (0.3)	2.9 (1.6)	11.8 (6.5)	2.3 (1.8)	1.2 (1.3)	18.2 (10.5)

An * indicates significant difference between treatment and control.

nutrition did not produce any significant differences in C spectral regions or modeled C forms. Correlation analysis combining high and low-P treatments with controls showed P nutrition to be positively correlated with the O-aromatic C (C VI) spectral region ($r=0.47$; $P=0.05$). There were no significance differences in the ^{13}C CPMAS spectra from PCA (Fig. 3).

Low-P nutrition did not produce any significant differences in P forms, in relative percent or concentration (Tables 3 and 4), and did not show any differences with PCA of the ^{31}P NMR spectra (Fig. 4). High-P nutrition significantly decreased the relative percent of orthophosphate and increased the relative percent of pyrophosphate (Table 3). It also significantly increased the concentrations of pyrophosphate, monoester 1, monoester 2, DNA and organic P (Table 4), and produced significant differences in the ^{31}P NMR spectra, as shown by PCA (Fig. 4). Correlation analyses combining low-P, high-P and control samples showed that P nutrition was positively correlated with pyrophosphate ($r=0.67$; $P=0.001$) and negatively correlated with monoester 1 ($r=-0.41$; $P=0.07$), monoester 2 ($r=-0.50$; $P=0.02$) and organic P ($r=-0.51$; $P=0.02$).

4. Discussion

For all three stresses examined (light, temperature, and P nutrition), analysis combining low, high and "optimal" (control) levels for each stress produced significant positive correlations, although there may not have been any significant treatment effects. This is in part a function of our methods for testing significance: to study the effects of each stress, we compared the high and low treatments to controls, but not to each other. This was because we did not always use the same species in the high and low treatments. However, for the correlation analyses, all data for each stress were used. This may indicate some broader trends for the stresses, which will warrant further analyses.

4.1. Light stress

In natural aquatic systems, light has a complex distribution pattern, varying in intensity and spectral distribution with depth and time. Both low and high light intensities have the ability to limit algal growth, due to their effects on photosynthesis. At low light intensities, algae may not get enough energy to meet their physiological needs. At high light intensities, light can exceed the cellular capacity for energy utilization, resulting in photoinhibition. In nature, high light intensities from sunlight can also be accompanied by high ultraviolet radiation, which can damage cells (Balseiro et al, 2008; Garbayo et al., 2008.

Reduced photosynthesis at low light intensities and photoinhibition at high light intensities will limit the total C that can be fixed by algae. This in turn may alter the distribution of C into macromolecular compounds, as algae attempt to protect themselves from high light intensities or to maximize their energy-trapping capabilities at low light intensities. Photosystems and their subcomplexes (e.g. light harvesting complexes, reaction center proteins) are anchored in the thylakoid membrane through lipids exclusively present in the chloroplast (Mock and Kroon, 2002); thus, light intensity is expected to particularly affect the abundance of proteins and lipids.

In our samples, light intensity was positively correlated with total P, N and C, and negatively correlated with C:N. This suggests that the

Table 4
Phosphorus forms, in μmol g^{-1}, determined by ^{31}P NMR spectroscopy for algal cultures grown under control conditions and under temperature (temp.), P nutrition (low P, high P) and light stress. Orth, orthophosphate; pyro, pyrophosphate; poly, polyphosphate; mono1, orthophosphate monoester 1 region; mono2, orthophosphate monoester 2 region; OthDi, orthophosphate diesters other than DNA; Porg, organic P calculated from ^{31}P NMR spectra. See text for details on these regions. Results shown are averages and (standard deviation).

	Orth	Pyro	Poly	Mono1	Mono2	DNA	OthDi	Porg
Low light	124.2 (67.5)	44.8 (36.0)	2.6 (03.4)	14.8 (8.4)	84.2 (31.1)	15.2 (8.9)	2.3 (1.1)	116.5 (38.8)
Control	96.3 (49.7)	53.5 (49.4)	1.8 (2.3)	31.0 (26.7)	75.2 (43.0)	18.0 (22.9)	2.5 (3.5)	126.6 (47.6)
High light	212.0* (60.9)	38.3 (20.2)	0.0 (0.0)	27.9 (8.6)	156.1* (60.4)	16.1 (12.7)	1.8 (2.1)	201.8* (62.6)
Control	80.6* (48.8)	16.5 (12.7)	0.0 (0.0)	18.3 (8.5)	88.8* (31.6)	11.4 (6.8)	1.8 (1.3)	120.3* (35.7)
Low temp.	203.9 (165.2)	49.4 (74.6)	0.0 (0.0)	7.9 (3.4)	25.3 (5.4)	7.2 (4.8)	2.5 (3.1	42.9 (15.9)
Control	161.2 (37.9)	2.7 (1.2)	0.6 (0.6)	6.7 (4.4)	26.8 (5.9)	4.0 (2.4)	1.5 (0.5)	39.0 (13.2)
High temp.	149.4 (109.6)	62.5 (66.2)	0.4 (0.7)	12.4* (12.4)	58.4 (51.3)	8.7 (10.9)	4.3 (5.4)	83.8 (79.5)
Control	411.0 (269.3)	85.8 (68.8)	1.7 (3.0)	29.4* (12.4)	112.8 (41.8)	153.0 (39.7)	10.0 (4.3)	153.0 (39.7)
Low P	137.2 (231.1)	15.3* (12.2)	0.2* (0.4)	28.4 (38.9)	39.9 (43.6)	25.4 (29.5)	3.9 (3.4)	97.6 (71.6)
Control	332.1 (297.5)	87.5* (40.0)	2.6* (2.4)	27.0 (22.1)	84.4 (47.1)	18.8 (27.8)	3.7 (4.2)	134.0 (64.6)
High P	1366.1 (854.8)	1293.0* (1196.2)	0.0 (0.0)	38.1* (8.4)	89.0* (30.6)	29.9* (16.1)	1.4 (3.1)	158.3* (47.8)
Control	782.1 (1229.6)	22.8* 33.5)	0.2 (0.5)	16.9* (21.4)	46.3* (35.0)	10.0* (10.8)	2.7 (2.6)	75.8* (65.6)

An * indicates a significant difference between a treatment and a control.

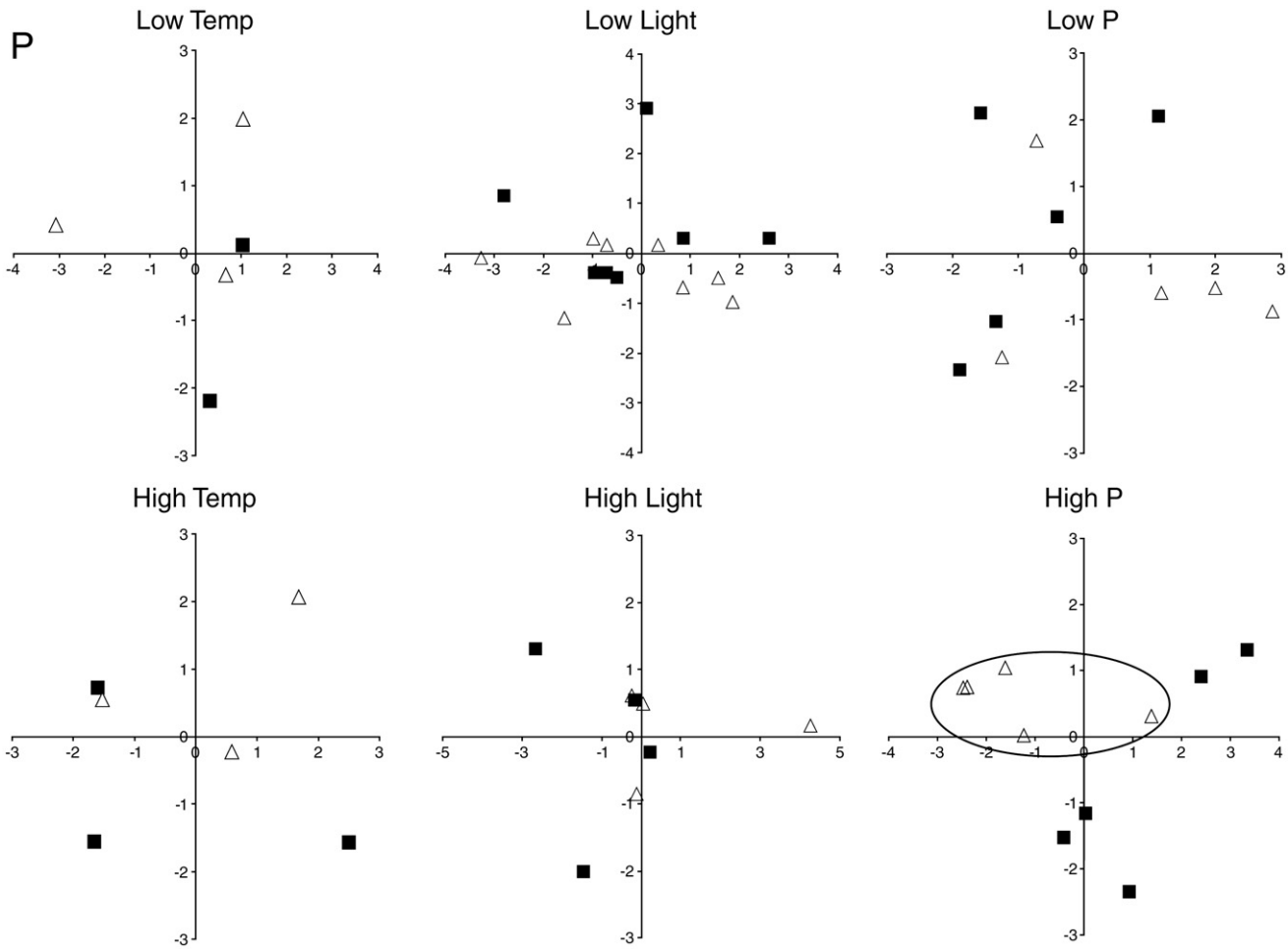

Fig. 4. Principal components analysis (unrotated) conducted using the relative percentages of P in the phosphorus form classes from the ^{31}P NMR spectra. Solid squares represent control samples; open triangles represent stressed samples.

cultures grown under high light conditions were better able to take up nutrients and fix C than those grown at low light. Algal species vary in their tolerances for light intensity. For example, photoinhibition for *E. huxleyi* (which we cultured under low light but not under high light intensities) was not observed until light intensities exceeded 1000–1500 μmol photons $m^{-2} s^{-1}$ (Nanninga and Tyrrell, 1996). The highest light intensity in our study was 250 μmol photons $m^{-2} s^{-1}$; thus, it is possible that our high light intensities were not high enough to fully stress all species and induce photoinhibition.

Light intensity was positively correlated with protein and negatively correlated with lipids in the cultures we analyzed. Under high light conditions, there was a significant increase in the O-aromatic C spectral region. Other studies have shown that under low-light conditions, algae will alter the composition of their light harvesting complex to maximize light uptake, reducing carbohydrates and increasing lipids (Mock and Kroon, 2002; Khotimchenko and Yakovleva, 2005). Under high light conditions, Cuhel and Lean (1987) reported an increase in both lipids and proteins at light intensities above 1600 μmol photons $m^{-2} s^{-1}$. High light intensity also appears to alter the composition of lipids, increasing storage lipids such as triacylglycerols and reducing structural lipids (Mock and Gradinger, 2000; Fábregas et al., 2004; Hotimchenko, 2002; Khotimchenko and Yakovleva, 2005). High light conditions also increased pigments such as carotenoids, which can assist with light absorption and protect against oxidative stress (Garbayo et al., 2008).

The cultures grown under high light conditions for our study had significantly higher concentrations of total P and significantly higher concentrations of orthophosphate, organic P, and P in the monoester 2 region. Low-light conditions did not significantly alter total P, but decreased the relative percent of P in the monoester 1 region. There are few reports of the effects of light intensity on algal P forms. Cuhel and Lean (1987) noted that light intensity did not alter nucleic acids, which is consistent with the lack of change in DNA in our results. Triacylglycerol synthesis requires large inputs of photosynthetically produced ATP and NAD(P)H (Khotimchenko and Yakovleva, 2005), which may account for the significant increase in total P under high light conditions. The reduction of P in the monoester 1 region under low-light conditions is consistent with a reduction in lipids. Although intact phospholipids would be detected in the "other diesters" region, phospholipids often degrade under the extraction conditions used for this study, and their degradation products are among the compounds found in the monoester 1 region (Turner et al., 2003; Doolette et al., 2009). Therefore, the significant decrease in this region could be due to a significant decrease in phospholipids. Further analysis would be required to confirm this, however, as other P forms besides phospholipid degradation products can be found in this region.

4.2. Temperature stress

In our algal cultures, high-temperature stress resulted in a significant decrease in lipids, and a significant increase in the C VII (carbonyl) spectral region. Temperature effects on algal C forms can vary, but changes in lipids are the most commonly observed responses. Lipids are modified to maintain membrane fluidity at low temperatures (Mock and Kroon, 2002) and to protect the thylakoid membrane, and photosynthesis, at high temperatures (Tchernov et al., 2004). The

specific response varies with algal species. Some species adjust total lipid concentrations, which are generally higher at high temperatures and lower at low temperatures (Al-Hasan et al., 1991). For other species, high and low-temperature stress alters the composition of the lipids, particularly the degree of fatty acid saturation (Al-Hasan et al., 1991; Khotimchenko and Yakovleva, 2005; Tchernov et al., 2004). Although we did not characterize the forms of fatty acids in our samples, temperature was inversely correlated with the unsaturated C (C V) spectral region, which might reflect an alteration in the degree of fatty acid saturation with temperature. Other thermally-induced changes reported in the literature include increased photosynthetic pigments (β-carotene, chlorophylls a and b, lutein), increased free amino acids, increased total C and C:N, and thickened cell walls from high-temperature stress (Kakinuma et al., 2001, 2006). There were no changes in total C or the C:N ratio in our cultures.

Low temperature significantly decreased the relative percent of orthophosphate, which may be due to differences in orthophosphate uptake from changes in membrane diffusivity (Mock and Kroon, 2002). Temperature stress did not produce any significant change in the relative percentage or concentration of compounds in the other diesters region, where intact phospholipids would be detected. However, there was a significant decrease in the P concentration in the monoester 1 region with high temperature. As noted above, phospholipid degradation products are among the compounds found in the monoester 1 region; thus, the significant decrease in this region could be due to a significant decrease in phospholipids. This is consistent with the decrease in lipids observed under high-temperature stress seen with the ^{13}C CPMAS NMR analysis.

4.3. P Nutrition

A number of algal species have been shown to alter the distribution of their C forms under P nutrition stress. Carbohydrates are often elevated in low-P environments, and it has been suggested that increased carbohydrate:protein ratios are indicative of P deficiency (Healy and Hendzel, 1979; Kilham et al., 1997; Beardall et al., 2001, 2005; Dean et al., 2008). The degree of change in the carbohydrate: protein ratio will vary with algal species (Stehfest et al., 2005). Elevated lipid levels have also been detected in P-limited algae (Lynn et al., 2000; Heraud et al., 2005; Sigee et al., 2007), but chlorophyll *a* and *b* concentrations were unchanged (Kozlowska-Szerenos et al., 2000, 2004) . In our cultures, C in the N-alkyl and methoxyl (C II) spectral region was significantly lower in low-P cultures than in controls, but no significant differences in modeled C forms were detected. It is important to note that our designation of "low-P" was based on external P concentrations rather than on the internal cell P concentration, also known as the P quota. Phytoplankton can adjust their cellular P requirements and relocate the cellular P pool to maintain a near-maximum growth rate when P availability is limiting, until their P quota drops below a threshold limitation level (Cembella et al., 1984; Ji and Sherrell, 2008), with the threshold limitation level varying among species (Litchman and Nguyen, 2008). Increased carbohydrate synthesis will only occur at very low P quotas (Sigee et al., 2007; Dean et al., 2008). It is possible that the P quotas in our low-P cultures had not fallen below threshold P deficiency levels. This is supported by the nutrient ratios: C:P and N:P were higher in our low-P cultures compared with controls, but total P was not significantly different. No significant changes in P forms were observed in our low-P cultures, although reduced polyphosphates and phospholipids in low-P cultures have been reported elsewhere (Eixler et al., 2006; Nishikawa et al., 2006).

Growth of algae in environments with higher P concentrations than are required for full growth is known to lead to luxury P consumption, which is the uptake of P in excess of growth requirements and subsequent intracellular storage of P, usually as polyphosphate (Droop, 1973; Stevenson and Stoermer, 1982). Pyrophosphate, a short-chain polyphosphate, was significantly higher both in relative percent and concentration in our high-P cultures than in controls. This apparent luxury consumption indicates that the P concentration of the high-P medium was in excess of growth requirements.

4.4. General discussion

For the purpose of this research, we chose to group different algal species together, rather than examining the stress response of individual species. Although different algal species are known to have different biochemical characteristics and different threshold levels for tolerance to stress (e.g. Healy and Hendzel, 1979; Shifrin and Chisholm, 1981; Arrigo et al., 1999; Xu et al., 2006), stress from light, temperature and P nutrition produced a number of significant changes in C and P forms and compound categories even when all species were grouped. The changes in C forms, in particular, in response to light and temperature stress correspond to trends observed previously using other methods (e.g. positive correlation of light intensity with proteins; negative correlations of light and temperature with lipids). This suggests that there may be some universal responses of algae to certain types of environmental stress. We did not, however, see all the stress responses that have been described in the literature. For example, increased carbohydrate: protein ratios and elevated lipids have frequently been reported in studies of low-P nutrition (Lynn et al., 2000; Heraud et al., 2005; Stehfest et al., 2005; Sigee et al., 2007) but we did not observe this in our cultures. This could be due to, among other things, the relatively small number of samples used in our study or to the natural range of tolerance of the algal species used, such that they were not fully stressed by the treatment. Further investigation is warranted, particularly studies assessing the effect of low intracellular P levels and quotas.

Although polyphosphates and their associate enzymes have been shown to be important stress mediators in bacteria (Jahid et al., 2006; Manganelli, 2007; Brown and Kornberg, 2008), they do not appear to play as large a role in algae, at least not in the species used for this study. The only significant change in polyphosphates was an increase in pyrophosphate in the high-P treatments, which is consistent with luxury uptake. Cultures grown at low and high temperatures had higher pyrophosphate concentrations than controls, but these differences were not significant. In *D. salina*, polyphosphate concentrations were reduced under low-P (Bental et al., 1991), osmotic and alkaline stress (Pick et al., 1990); therefore, it is possible that polyphosphate response to stress is species specific, and thus was masked by grouping different species in our study. In general, there are far fewer reports in the literature of changes in P forms than C forms in response to stress. This is in part due to fewer studies of P forms. It may also be because C forms respond more immediately to stresses that may impact photosynthesis and thus C fixation. Phosphorus forms may be less affected, because P can be reallocated and recycled within the cell to a greater extent. More research is needed to fully understand the effects of stress on algal P forms.

Although most of the P forms and compound classes identified by ^{31}P NMR were organic and thus contained C in some form, there were few discernable relationships of most P forms and compound classes to either the regions of the ^{13}C NMR spectra or to modeled C forms. When the entire data set (including data from all stresses) was analyzed, DNA was correlated to the alkyl C (C I) and di-O-alkyl C (C IV) regions ($r=0.25$; $P=0.06$ and $r=-0.28$; $P=0.03$, respectively) and to lipids ($r=0.23$; $P=0.09$), and the monoester 2 region was correlated with proteins ($r=0.23$; $P=0.09$). Interestingly, polyphosphate was also correlated with alkyl C ($r=0.29$, $P=0.03$), di-O-alkyl C ($r=-0.24$; $P=0.07$), protein ($r=-0.23$; $P=0.09$) and lipids ($r=0.42$; $P=0.001$), although polyphosphate does not contain C. Further research is warranted to clarify the relationships between P and C forms determined with these spectroscopic techniques.

5. Conclusions

Using ^{13}C CPMAS and ^{31}P NMR spectroscopy, this research demonstrated that light intensity, temperature and P nutrition stress could alter C and P forms in cultured algae. Of the C forms, protein was positively correlated to light intensity, while lipids were negatively correlated to light intensity and were significantly increased at high temperatures. Low-P nutrition significantly altered ^{13}C NMR spectra, decreasing the N-alkyl and methoxyl C spectral region, but did not alter modeled C forms. There were few changes in P forms with light and temperature stress. Low- and high-P nutrition altered C:P and N:P ratios. Low-P nutrition did not alter P forms. High-P nutrition increased pyrophosphate, indicating luxury P consumption. No significant changes in polyphosphates were observed in cultured algae under light and temperature stress. This suggests that, unlike what has been observed for bacteria, polyphosphate production is not a stress response for these algal species.

Acknowledgments

We are grateful to Gert van Dijken, Kate Mackey, Karen McLaughlin, Anton Post and Peter Bernhardt for supplying the algal samples. We are also grateful to Dave Mucciarone at Stanford University for total C and N analysis, Jonathan Stebbins, Geological and Environmental Sciences, Stanford University for instrument time and assistance with the ^{13}C CPMAS NMR spectroscopy, Corey Liu at the Stanford Magnetic Resonance Laboratory for assistance with ^{31}P NMR spectroscopy, and Jeff Baldock for the use of his molecular mixing model to interpret the ^{13}C CP/MAS NMR spectra. This project was funded by DOE grant number ER64164 to AP.

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