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Acclimation to various temperature and pCO_2 levels does not impact the competitive ability of two strains of *Skeletonema marinoi* in natural communities

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Understanding the long-term response of key marine phytoplankton species to ongoing global changes is pivotal in determining how oceanic community composition will respond over the coming decades. To better understand the impact of ocean acidification and warming, we acclimated two strains of *Skeletonema marinoi* isolated from natural communities to three pCO_2 (400 µatm, 600 µatm and 1000 µatm) for 8 months and five temperature conditions (7°C, 10°C, 13°C, 16°C and 19°C) for 11 months. These strains were then tested in natural microbial communities, exposed to three pCO_2 treatments (400 µatm, 600 µatm and 1000 µatm). DNA metabarcoding of the 16S and 18S gene for prokaryotes and eukaryotes respectively was used to show differences in abundance and diversity between the three CO_2 treatments. We found there were no significant differences in acclimated *S. marinoi* concentrations between the three pCO_2 treatments, most likely due to the high variability these strains experience in their natural environment. There were significant compositional differences between the pCO_2 treatments for prokaryotes suggesting that indirect changes to phytoplankton-bacteria interactions could be a possible driver of bacterial community composition. Yet, there were no differences for eukaryotic community composition, with all treatments dominated by diatoms (but not the acclimated *S. marinoi*) resulting in similar biodiversity. Furthermore, strain-specific differences in community composition suggests interactions between prokaryotic and eukaryotic taxa could play a role in determining future community composition.

KEYWORDS

acclimation, prokaryotes, eukaryotes, community composition, strain-specific, biodiversity

Introduction

Rapidly escalating global changes have led marine organisms to adapt to unprecedented rates of change (Collins et al., 2014), with global warming and ocean acidification being two of the most serious challenges facing phytoplankton. Northern oceans have warmed by on average 0.7°C (IPCC, 2022) and are projected to rise by an additional 3°C by the end of the century (Collins et al., 2014). In addition, the world's oceans have absorbed 26% of anthropogenic CO_2 emissions, leading to an average pH decline of 0.1 units since pre-industrial times (Friedlingstein et al., 2022), with implications for the chemical balance of surface waters (Caldeira and Wickett, 2003). If anthropogenic CO_2 emissions continue at a similar rate, future atmospheric CO_2 concentrations are projected to reach 1000 µatm, which will result in a further decline in the mean surface pH of 0.3-0.4 units by 2100 (Pörtner et al., 2019). Moreover, oceanic pH is also influenced by a variety of biochemical processes such as primary production, respiration, algal blooms and organic matter decay (AMAP, 2018), which leads to diurnal and seasonal fluctuations in pCO_2 (Schulz and Riebesell, 2013; Liu et al., 2017; Vargas et al., 2022). As pCO_2 of seawater increases, the decline in buffering capacity will lead to amplifications in pCO_2 fluctuations (Angeles Gallego et al., 2018). However, the biological effects of oceanic warming and acidification vary between species and locations (Vargas et al., 2022), therefore, understanding how organisms respond is critical in determining the impact on the marine food web.

The effects of temperature and pCO_2 have been extensively studied, highlighting a wide range of responses to primary productivity and community composition (Feng et al., 2009; Gao and Campbell, 2014; Hoppe et al., 2018; Kim and Kim, 2021). Ocean warming increases stratification, constraining nutrient supply and altering the thermal regime in the surface layer (Winder and Sommer, 2012), leading to a reduction in phytoplankton biomass and productivity (Lewandowska et al., 2014; Behrenfeld et al., 2016). Increased pCO_2 concentrations have been shown to facilitate inorganic carbon acquisition in some phytoplankton, enhancing primary production (Rost et al., 2008), leading to changes in phytoplankton-bacteria interactions. As bacteria are highly dependent on algae exudates for growth (and also regenerate ammonium for phytoplankton growth), any changes to pCO_2 concentrations will influence the interactions between these two groups (Van Den Meersche et al., 2004). Previous studies have shown that the response of bacteria to changing pCO_2 concentrations is linked to phytoplankton, rather than being a direct effect of pCO_2, therefore, any increases in phytoplankton (due to higher pCO_2) could lead to an enhancement of bacteria biomass (De Kluijver et al., 2010).Changes to these interactions can also influence nutrient fluxes and have implications for the marine food web (Seymour et al., 2017). Ocean acidification can also cause varying responses of phytoplankton, resulting in shifts in the competitive fitness between different phytoplankton groups, substantially altering the community composition (Dutkiewicz et al., 2015). Other studies found only subtle changes in community composition (Kim et al., 2006), whilst Hoppe et al. (2018) found that Arctic phytoplankton community composition and primary production were unresponsive to ocean acidification. As seawater pCO_2 concentrations increase, it has been shown that species which benefit from CO_2 fertilisation may become more dominant at the expense of those species which are unresponsive or negatively affected by an increase in CO_2 (Bach et al., 2017). Studies have shown increasing pCO_2 concentrations give a neutral or slight benefit to diatoms and is strongly beneficial for N_2 fixing cyanobacteria, with coccolithophores negatively affected (Rost et al., 2008; Sommer et al., 2015). Collins et al. (2014) found that diatoms possess RubisCOs with high CO_2 affinities which could partially explain why they have shown small responses to high pCO_2; however, species-specific responses must be considered. Nevertheless, the response of acclimated phytoplankton communities to future high pCO_2 conditions have yet to be investigated.

Skeletonema marinoi is one of the most important and abundant primary producers in coastal temperate regions and, is critical for the marine food web (Sefbom et al., 2022). As ocean warming and acidification have the potential to influence microbial community composition, they can drive physiological and evolutionary changes within taxa such as *Skeletonema* (Maugendre et al., 2015). Acclimation of a dominant species such as *S. marinoi* to the projected future conditions (temperature and pCO_2 concentrations), allows for an investigation into the impacts of environmental changes to the microbial community (Collins et al., 2014), whilst still considering the pCO_2 variability species experience in their natural environments. As this species inhabits a wide geographical area and displays population genetic differentiation (Sefbom et al., 2018), acclimating *S. marinoi* strains from different geographical regions, allows us to understand the physiological and adaptive capabilities of a key phytoplankton taxa and how these vary within species (Li et al., 2021). Previous studies have resulted in conflicting reports on how increased temperature and pCO_2 concentrations influences the growth rate of *Skeletonema* (Zheng et al., 2015; Gao et al., 2019; Chen et al., 2021). Inoculating *S. marinoi* strains acclimated to future environmental scenarios into mesocosm experiments permits the replication of more realistic conditions allowing us to test their competitive ability against natural microbial communities (Briddon et al., 2022).

The main aim of this study is to understand whether the long-term response of *S. marinoi* to global changes, specifically, ocean warming and acidification could be predicted by using strains that were previously acclimated to various conditions. To determine how different pCO_2 concentrations will influence eukaryotes and prokaryotes, we compared three pCO_2 treatments, 400 µatm, 600 µatm and 1000 µatm. We also assessed if acclimation to increased temperature or pCO_2 gave two strains of *S. marinoi*, a competitive advantage against natural microbial communities exposed to different pCO_2 treatments. Changes in biomass were measured using a PHYTO-PAM-II (Multiple Excitation Wavelength Phytoplankton and Photosynthesis Analyser) and DNA metabarcoding to show differences in abundance and diversity

between the three CO_2 treatments. Understanding the response to global stressors of dominant marine phytoplankton species can help to project more accurately their response to forecasted future conditions and the impact on the marine food web.

Materials and methods

Biological materials and growth conditions of the *Skeletonema* strains (S8 and S17)

The *Skeletonema* strains used in this study were isolated from two locations along the Norwegian Coast. Water samples were collected using the automated sampling system operated by NIVA (Norwegian Institute for water Research) on board the coastal steamer *MS Trollfjord*. Strain S8 originated from a water sample (temperature; 10.02°C and 33.86 psu) collected in the outer part of Tanafjorden, Northern Norway (70.8306° N, 28.4723° E; average summer surface water temperature of 11°C, Figure 1). Strain S17, originated from a water sample (temperature; 13.37°C and 25.54 psu; Table S1) collected in Sognesjøen (61.1554° N, 6.5806° E; average summer surface water temperature of 17°C; Figure 1) in the Sognesjøen region on the Norwegian West Coast. The strains were brought into unialgal culture using a combination of serial culturing technique and single cell isolation (Andersen and Kawachi, 2005). The strains were then deposited as non-axenic cultures in the Collection of Cyanobacteria and Algae (AICB) at the Institute of Biological Research in Cluj-Napoca, Romania (Dragoș, 1997). The phylogenetic identity of the strains was confirmed using the 18S rDNA gene amplified with specific primers (Hadziavdic et al., 2014). The PCR fragments were sequenced by a third-party company (Macrogen Europe, Amsterdam, The Netherlands), who confirmed species identification.

The S8 and S17 strains were exposed to five different temperature conditions (7°C, 10°C, 13°C, 16°C and 19°C) for 11 months (August 2021 to July 2022) and three pCO_2 conditions (400, 600 and 1000 µatm) for 8 months (November 2021 to July 2022; Table 1; Figure 2). The strains were grown under a range of different average temperature conditions to reflect the projected temperatures for 2050 and 2100 under scenarios SSP5-8.5 and SSP2-4.5 from the latest IPCC climate model output (CMIP6; IPCC, 2018) for both sample collection sites (Tanafjorden and Sognesjøen, Norway). To acclimate the strains to different pCO_2 concentrations, each sample was bubbled daily with tanks filled with artificial air containing either 400 ppm, 600 ppm or 1000 ppm CO_2 (Messer, Bad Soden, Germany) for 90 minutes (15 minutes aeration every 4 hours). Frequent bubbling was also needed to prevent cells clumping. The strains were acclimated in semi-batch conditions (corresponding to approx. 100 generations) in artificial seawater (Kester et al., 1967) with macronutrients (Guillard and

FIGURE 1
Map of Norway highlighting the two sample locations (Sognefjord and Tanafjord, red dots) and main urban areas (black dots). The green dot shows the location of the research institute in Sweden, Kristineberg, where the mesocosm experiment took place. The Geographical coordinate system GCS_WGS_198: Source Arc GIS Open Data, ESRI.

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TABLE 1 List of the two S. marinoi strains (S8 and S17), the acclimated conditions used in the mesocosm experiment (temperature and pCO_2) and the corresponding pCO_2 treatment each sample was tested in.

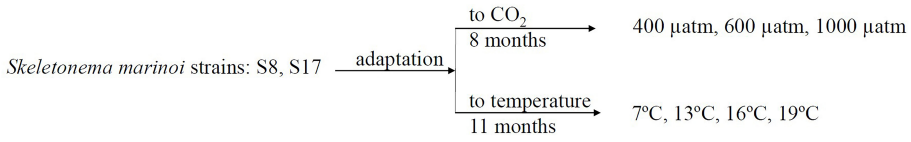
Strain	Acclimation conditions		Treatment CO_2 condition	Mesocosm Number
	Temperature (°C)	CO_2 (µatm)		
S8	7	400	400	1
			600	2 and 3
			1000	4
S8	16	400	400	1
			600	2 and 3
			1000	4
S8	19	400	400	1
			600	2 and 3
			1000	4
S8	7	600	600	2 and 3
S8	13	600	600	2 and 3
S8	19	600	600	2 and 3
S8	7	1000	600	2 and 3
			1000	4
S8	13	1000	600	2 and 3
			1000	4
S8	19	1000	600	2 and 3
			1000	4
S17	13	400	400	1
			600	2 and 3
			1000	4
S17	16	400	400	1
			600	2 and 3
			1000	4
S17	7	600	600	2 and 3
S17	13	600	600	2 and 3
S17	19	600	600	2 and 3
S17	7	1000	600	2 and 3
			1000	4
S17	13	1000	600	2 and 3
			1000	4
S17	19	1000	600	2 and 3
			1000	4

The mesocosm number in which each sample was placed, is also listed as: 1) Strains acclimated to 400 µatm and tested in 400 µatm condition, 2)and 3) contained a random placement of strains acclimated to all pCO_2 conditions tested in 600 µatm condition and 4) Strains acclimated to 400 and 1000 µatm pCO_2 conditions tested in 1000 µatm condition.

Ryther, 1962) and micronutrients/trace metals (Guillard, 1975) under controlled 16h:8h light:dark conditions provided by white LED lamps (100 µmol photon m^{-2} s^{-1}). Samples were grown in triplicates in 100 ml glass tubes.

Land-based mesocosm experiment

The impact of acclimated *S. marinoi* on natural microbial communities was tested at the Kristineberg Center for Marine



Adaptation							Test condition
to CO_2 (400 µatm, 600 µatm, 1000 µatm)	to temperature (7°C, 13°C, 16°C, 19°C)						CO_2
400 µatm		13	16	7	16	19	400 µatm
		13	16	7	16	19	600 µatm
		13	16	7	16	19	1000 µatm
600 µatm	7	13	19	7	13	19	600 µatm
1000 µatm	7	13	19	7	13	19	6000 µatm
	7	13	19	7	13	19	1000 µatm
	S17			**S8**			

FIGURE 2
Schematic representation of the experimental setup.

Research and Innovation, University of Gothenburg, Sweden during a mesocosm experiment (25^{th}-31^{st} July 2022). The experiment lasted 6 days as previous research has shown that by this time the *Skeletonema* strains had reached the stationary growth phase. Three 1500 L water tanks were used, in which four 200 L plastic containers, the mesocosms, were deployed. For the mesocosms themselves, surface (55%; collected from 0.5 m depth) and bottom (45%; collected from 5 m dep) fjord water (58.2497° N, 11.4448° E) was mixed to ensure the presence of sufficient phytoplankton (found in surface waters) and nutrients (from the bottom waters). The fjord water was filtered (using a 250 µm mesh) to remove macro- and mesozooplankton before being added to the mesocosms. We added nutrients to each mesocosm prior to the beginning of the experiment as analysis of nutrients in a preliminary experiment showed undetectable levels. Therefore, 2 L of synthetic artificial seawater with the associated macronutrients, micronutrients and trace metals (for the full list of added nutrients, see Table S2) was added to and mixed with the fjord water in each of the four large mesocosms (Guillard and Ryther, 1962; Kester et al., 1967; Guillard, 1975).

Three different treatments were used: 400 µatm, 600 µatm and 1000 µatm (Figure 2). Due to practical limitations, not all the acclimated strains were used in the 3 different pCO_2 treatments (See Table 1 for the list of strains, acclimation conditions and the tested pCO_2 conditions). For logistical reasons we only tested the samples in different pCO_2 treatments, as we were unable to control the temperature within the mesocosms. In order to distinguish between the different *S. marinoi* strains, acclimated conditions and pCO_2 treatments, the following labelling layout was used; strain_temperature_CO_2acclimisation_CO_2treatment. High pCO_2 levels were attained through bubbling pure CO_2 gas into the mesocosms and the pH was controlled using IKS Industrial Aquastar system (Karlsbad, Germany). Previous studies have found the bubbling of pure CO_2 gas to be a precise method for conducting ocean acidification experiments (Gattuso and Lavigne, 2009). Temperature was logged every 15 minutes using sensors linked to a HOBO MX2202 temperature/light data logger. The salinity of all mesocosms was 29 to ensure it is within the tolerable range for both strains (Table S1). The salinity of each mesocosm was measured twice a day using an Oxi 340i multiparameter (WTW, Weilheim, Germany). The four mesocosms were maintained for 6 days, to allow the planktonic resident communities to acclimate, prior to the addition of the dialysis bags containing the acclimated *S. marinoi* strains. Prior to the experiment, *S. marinoi* was also acclimated for 6 days with 50% synthetic seawater and 50% natural ocean water (55% surface and 45% bottom fjord water).

The use of small dialysis bags (10-20 k Dalton pore size; Nadir, Carl Roth, Karlsruhe, Germany), which are permeable for micro- and macro- nutrient (but not bacteria and phytoplankton), allowed for three replicates of each combination of strain and acclimation conditions (with an approximate volume of 520 mL each; Table 1) within the four mesocosm enclosures (Briddon et al., 2022; Drugă et al., 2022). Each dialysis bag was filled with the same water used in the mesocosms (55% surface and 45% bottom filtered -250 µm mesh- fjord water). All the dialysis bags were inoculated with *S. marinoi* (OD_{600} = 0.05; approximately $1.06x10^3$ cell L^{-1}, the corresponding chlorophyll *a* concentrations used in other phytoplankton-related experiments and in previous laboratory based experiments (unpublished); Briddon et al., 2022; Drugă

et al., 2022) except for the controls. The acclimated S8 and S17 were separately inoculated into all three pCO_2 treatments (Table 1). Nine additional dialysis bags were also used as controls (three for each pCO_2 treatment), which contained only mixed surface and bottom fjord water and no inoculated *S. marinoi*. In total, the experiment consisted of 108 dialysis bags distributed into four mesocosms (Table 1; Figure S1). After completion of the mesocosm experiment, the triplicates (99 dialysis bags plus an additional 9 dialysis bags used as a control for each pCO_2 treatment) were then combined, resulting in a total of 36 samples, which underwent further analysis.

Chlorophyll *a* and pigment measurements

Chlorophyll *a* concentrations (μg L^{-1}) were estimated *in vivo* every two days using a PHYTO-PAM-II Compact Version (Heinz Walz Gmbh, Germany), for a total of 6 days. An aliquot was taken from every dialysis bag, and this was used to determine the composition of the major algal groups. The PHYTO-PAM-II uses specific wavelengths to supply data on phytoplankton community composition using three defined functional groups of total chlorophyll *a* concentration. The three different major algal groups identified were: chlorophytes, cyanobacteria and a "brown" group (Chromophytes), which consists mainly of algae that have additional pigments that absorb in the yellow/orange wavelength range (e.g. diatoms, cryptophytes, dinoflagellates). Total chlorophyll a was determined using the sum of all three groups.

Nutrients

Samples for analysis of total nitrogen (TN), silica (Si), phosphate (PO_4^{3-}), iron (Fe), total phosphorus (TP) concentrations and total alkalinity were collected at the beginning and at the end of the 6-day experiment for all treatments. The analyses were standardised using calibration curves of samples with known concentrations and the use of blanks between samples. These analyses were completed using the HI83399 Multiparameter Photometer with COD for Water and Wastewater (HANNA Instruments, Germany). The nutrients were analysed from unfiltered water samples collected from each of the four mesocosms using the methods detailed in https://hannainst.ro/mwdownloads/download/link/id/939.

DNA analysis

The microbial community of each dialysis bag was analysed after the 6-day mesocosm experiment using DNA metabarcoding. This technique was chosen as it can determine both eukaryotic and prokaryotic diversity and abundance down to the genus level. Firstly, the water from each of the three replicates was combined (totalling approx. 1.5L), and then centrifuged to allow for the removal of the excess water. Total DNA was isolated using a E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions. The DNA concentration and quality were assessed using a NanoDrop™ 2000 Spectrophotometer (Waltham, MA, USA). The small ribosomal DNA (rDNA) subunit (16S for prokaryotes and 18S for eukaryotes) was then amplified using PCR (Herlemann et al., 2011; Hadziavdic et al., 2014). These primers targeted a 300 bp DNA fragment within the 16S gene in prokaryotes (341F 5'-CCTAYGGGRBGCASCAG-3' and 806R 5'-GGACTACNNGGGTATCTAAT-3'), and a 350 bp long fragment from the V4 region in the 18S gene in eukaryotes (528F 5'-GCGGTAATTCCAGCTCCAA-3' and 706R 5'-AATCCRAGAATTTCACCTCT-3').

DNA sequencing was performed by a third-party company (Novogen, United Kingdom). Following DNA sequencing, base calling and run demultiplexing were completed using the BaseSpace service (Illumina, San Diego, CA, USA) meters. The pair-end reads were joined in QIIME, and the quality filtration, dereplication and singleton removal was performed using Usearch v8. Both *de novo* and reference chimera checking were performed in Usearch v8, using the latest version of the Greengenes database ('13_8') as a reference (DeSantis et al., 2006). The taxonomy was assigned for the representative OTUs in QIIME using the SSU/LSU 138 SILVA database (Gurevich et al., 2013). The taxonomy was added to the OTU table with the biom-format package, and the mitochondrial and plastid sequences were filtered out of the final OTU table. Rarefaction was performed, followed by alpha- and beta-diversity estimation in QIIME (Lozupone and Knight, 2005; Caporaso et al., 2010). Multiple alpha diversity indexes were used to estimate the diversity of the communities at the end of the experiment.

Statistics (data analysis)

One-way, two-way and three-way ANOVAs (on chlorophyll *a* concentrations) were completed using the factors, time, temperature and level of pCO_2 acclimation and pCO_2 treatment for the two strains separately and both strains together. Prior to analysis, normality, the identification of outliers and assumption of sphericity were checked using a Shapiro Wilk's test, identify_outliers() function and Mauchly's test of sphericity respectively. For the ANOVAs, the controls were excluded as they contained no additional *S. marinoi* (and would distort the correlations). *Post-hoc* analysis included mean separation tests for the multiple comparisons (using Tukey-adjusted comparison) and least square means for the main effects. The analysis was completed using R (version 4.1.2). Furthermore, one-way ANOVAs and two sample *t-test* were also used to determine if the strain (S8 or S17) and/or the acclimation conditions resulted in any significant differences in OTU abundances of the main microbial groups.

Principal coordinate analysis (PCoA) was completed using the Weighted Unifrac of Bray-Curtis distances to determine any differences between the strains, temperature and pCO_2 acclimated and treatment. This analysis was completed using the calibrate package in R (version 4.1.2; Graffelman and van Eeuwijk, 2005; R Core Team, 2021). Indicator species analysis (Indval) was used to

assess the affinity of the different groups for the three pCO_2 treatments and was conducted using the Indval function in the indicspecies package in R (Version 3.4.2). Indval uses a species' relative abundance and occurrence to estimate the strength of their association to different groups. The test uses priori groups of interest and a simple randomisation test to evaluate the probability of a species' affinity to a certain group. Analysis of Similarities (ANOSIM) was also used to assess differences in bacterial and eukaryotic assemblages between the three pCO_2 treatments (Clarke, 1993; Clarke and Warwick, 1994). The three techniques complement each other as PCoA helps to determine what could be driving the differences between treatments, whilst Indval determines the affinity of each taxon to a specific pCO_2 treatment, and ANOSIM quantifies the similarities between the treatments.

Results

Land-based mesocosm experiments - temperature and light intensity

The temperature was similar in all pCO_2 treatments throughout the 6-day experiment (Figure S2A). The mean daytime and nighttime temperature averaged c.19-20°C and c.17°C respectively. Mesocosms 4 (1000 µatm pCO_2 treatment; Table 1) and 3 (600 µatm pCO_2 treatment) had a higher temperature compared to mesocosms 1 (400 µatm pCO_2 treatment) and 2 (600 µatm pCO_2 treatment) on the 26th July due to their location within the greenhouse and subsequent clouding over. Mesocosm 2 (one of the 600 µatm pCO_2 treatment) had a lower light intensity compared to the other three (Figure S2B). The remaining mesocosms had light intensity measurements that were similar across all treatments and followed near-identical patterns over the whole experiment. However, as mesocosms 2 and 3 contained a random placement of the triplicates being tested in the 600 µatm pCO_2 treatment, any influence of the reduced light intensity would have been removed once the triplicates were combined.

Nutrients

There were similar concentrations of TP, phosphate, iron, in all treatments at the start of the experiment (Table 2), whilst silica concentrations were lower in mesocosms 2 and 3. All the nutrients measured increased by the end of the experiment in all mesocosms except for iron which declined to undetectable levels. At the end of the experiment, there were again similar levels of silica, yet TP, phosphate and TN concentrations varied (Table 2). In mesocosms 1 and 4, there were substantially higher concentrations of TP and phosphate compared to the other mesocosms. Total alkalinity also showed a slight increase as pCO_2 concentration increased, most likely due to evaporation and/or nutrient consumption (Millero et al., 1998). Evaporation and the use of unfiltered water (which would contain microbes) for the nutrient analyses could explain the increase in nutrients (except for iron) observed at the end of the experiment (Zingel et al., 2023).

Phytoplankton group dynamics

There were no significant differences (ANOVA) between chlorophyll *a* concentration and any of the factors (temperature and level of pCO_2 acclimation, pCO_2 treatment and time) for either strain or both strains together (Table S3). There were also no significant differences between the different factors and their interactions. Chlorophyll *a* concentrations were not significantly different for both strains by the end of the experiment (Figure 3). Chromophytes (consisting mainly of diatoms) chlorophyll *a* concentrations were the highest of all the algal groups (15.3-43.7 µg L^{-1}) by Day 6, with no significant differences between S8 and S17 strains. Chlorophyte and cyanobacterial (consisting of only

TABLE 2 Total alkalinity and nutrient concentrations (total phosphorus, phosphate, iron, silica, silica dioxide and total nitrogen) of the samples collected on Day 1 (25th July 2022) and Day 6 (31^{h} July 2022) of the 6-day experiment.

	Mesocosm Number							
	Beginning of experiment – 25.07.2022				End of experiment – 31.07.2022			
	1	2	3	4	1	2	3	4
Total Alkalinity - Kg^{-1}	2440	2360	2300	2260	2640	2480	2520	2340
Total Phosphorus **(TP) - µg/L**	29	21	24	22	136	70	57	400
Phosphate **(PO_4^{3-}) - µg/L**	9	7	8	7	44	23	17	130
Iron **(Fe) - µg/L**	19	22	18	20	0	0	0	0
Silica **(Si) – µg/L**	340	60	60	160	350	260	330	300
Total Nitrogen **(TN) - mg/l**	*				5.1	0.8	7.2	1.2

* no data collected due to sample loss.

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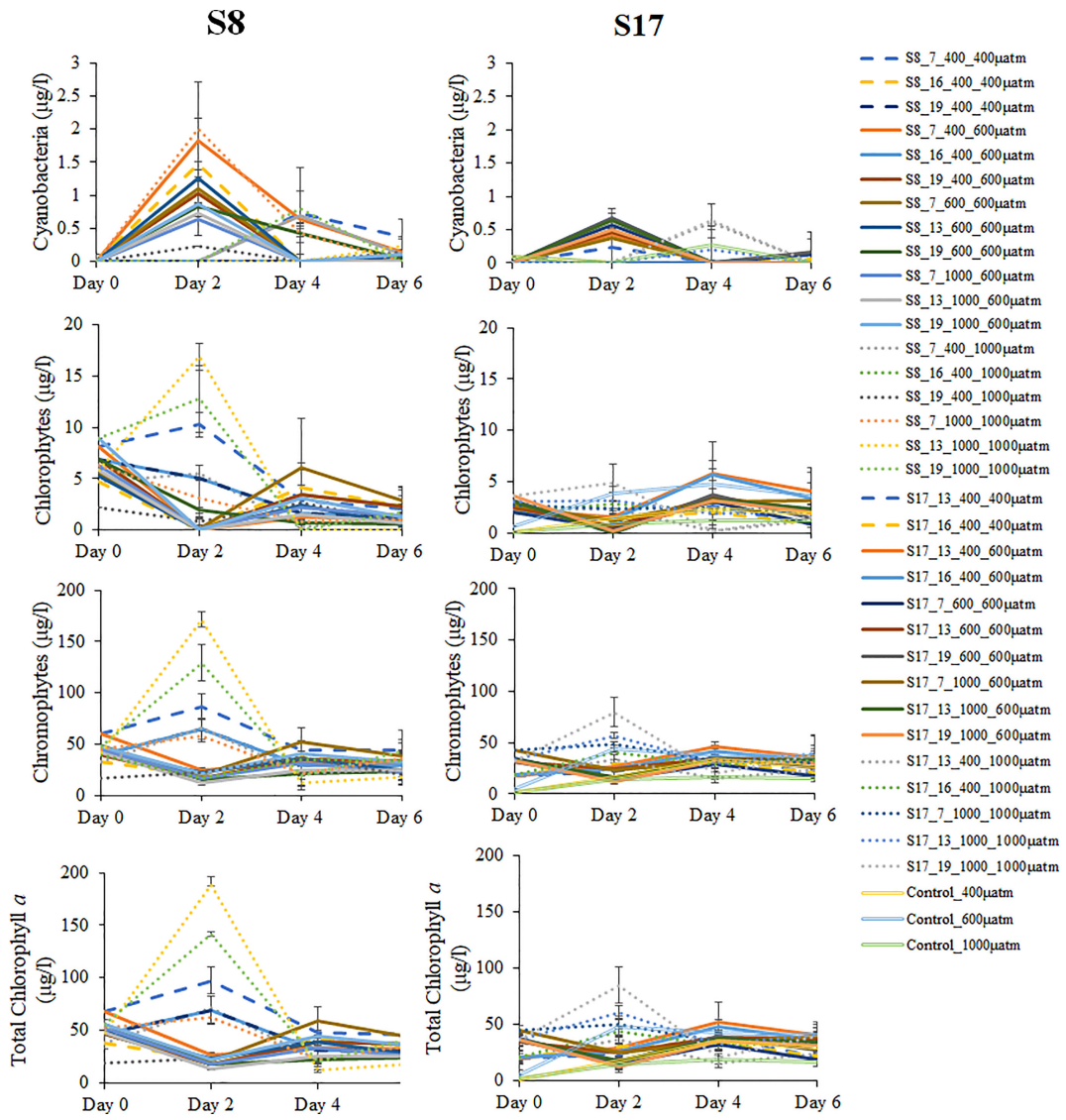

FIGURE 3
Mean total Chlorophytes, Chromophytes, Cyanobacteri and Chlorophyll a concentrations measured every two days of the experiment for all pCO_2 treatments (400 µatm, 600 µatm and 1000 µatm) for both S. marinoi strains (S8 and S17) and all the acclimation conditions. Each concentration was calculated using specific wavelengths of total chlorophyll a concentration for the three defined functional groups (Chlorophytes, Chromophytes and Cyanobacteria). The dashed, straight and dotted lines correspond to the samples tested in the 400 µatm, 600 µatm and 1000 µatm respectively. The controls sample are denoted by the unfilled lines. The error bars were determining using standard error. The following labelling was used to distinguish between the different S. marinoi strains, temperature and CO_2 acclimisation conditions and pCO_2 treatment; strain_temperature_CO_2acclimisation_CO_2treatment.

chloroplasts, endosymbiotic cyanobacteria, according to the DNA metabarcoding results) chlorophyll *a* concentrations were significantly higher in the samples inoculated with S8 compared to S17. Chlorophyll *a* concentrations fluctuated across the 6-day experiment for the three algal groups with no pattern between the temperature or pCO_2 concentration of acclimation or the pCO_2 treatment. Four samples consistently had the highest concentrations across all algal groups (S8_13°C_1000µatm _1000µatm, S8_19°C_1000µatm _1000µatm, S8_7°C _400µatm _400µatm and S17_19°C _1000µatm _1000µatm) but there was no relationship between temperature, pCO_2 acclimation or pCO_2 treatment.

16S and 18S metabarcoding data

A total of 3.83 million sequence reads were obtained following 16S rDNA amplicon sequencing and 3.87 million for 18S rDNA. 3.46 (16S) and 3.81 (18S) million combined reads passed the processing and filtering stages (sequencing quality and read length). After reassembly, alignment clean-up and mapping, the total abundance of OTUs from the 36 samples were 1306 prokaryotic and 1087 eukaryotic. The bacterial and eukaryotic composition at the class, order and family levels are shown in Figures S3–S8. The alpha diversity measurements (chao1, Simpson and Shannon diversity indexes) showed similar prokaryotic and

eukaryotic diversity in all pCO_2 treatments, yet less variability between the three triplicates in the 600 µatm treatment (Table S4).

All treatments had a similar bacterial community composition, yet the OTU abundances differed (Figure 4). All samples were dominated by cyanobacteria (all chloroplasts, endosymbiotic cyanobacteria), Proteobacteria and Bacteroidota. Cyanobacteria was the most abundant group (with a range of 49.1-78.4% for all samples) with significant differences between strain and the pCO_2 treatment. The highest cyanobacteria concentrations were found in the samples inoculated with strain S17 (mean of 37.9%) and those samples tested in the 600 µatm pCO_2 treatment (mean of 69.5%). The acclimation conditions (temperature and CO_2) did not result in any significant differences in cyanobacterial concentrations. The second most abundant group was proteobacteria (15.4-37.9%) with significantly higher concentrations for the samples inoculated with strain S8 (p=0.026) and those acclimated (p=0.005; F=6.31) and tested in the 400 µatm pCO_2 conditions (p=<0.001; F=20.42). The proteobacteria abundance mostly consisted of the orders Rhodobacterales, Caulobacterales, Rhizobiales and Thalassobaculales from the class Alphaproteobacteria (5.3-12.8%) and Alteromonadales, Oceanospirillales and Vibrionales from the class Gammaproteobacteria (9.1-28.1%). The third and final most abundant group was Bacteroidota consisting of the orders Flavobacteriales (1.1-10.9%), Cytophagales (0.1-1.8%) and Chitinophagales (0.2-1.9%). Within this group, the families Flavobacteriaceae and Alteromonadaceae had significantly higher OTU abundances in the 600 and 1000 µatm treatments. The abundances of Bacteroidota significantly increased along the pCO_2 treatments (p=0.005, F=6.31). There were no significant differences between strains or the acclimation conditions.

The eukaryotic populations in all samples were dominated by diatoms (78.6-96.1%; Figure 5). Most of the DNA reads were assigned to the pennate diatoms of the class Bacillariophyceae (77.5-94.5%) consisting of the orders Achnanthales, Bacillariales, Cymbellales, Dictyoneidales, Eunotiales, Lyrellales, Mastogloiales, Naviculales, Rhopalodiales, Surirellales and Thalassionematales. However, it is not possible to determine which families were dominating due to insufficient fragment lengths, needed for a more accurate identification. The samples inoculated with strain S8 had significantly higher diatom OTU abundances compared to those inoculated with S17 (p<0.001), with no discernable patterns between pCO_2 treatment and the acclimation conditions. *Skeletonema* OTU abundance was low in all the samples including the controls (0.1-27%) with no distinguishable trends between strains, pCO_2 treatment or the acclimation conditions, even though all samples (except the controls) were inoculated with acclimated *Skeletonema* strains. Most of the remaining eukaryotic OTU abundance consisted of Ciliphora (1.5-11.6%), Cercozoa (0.1-2.5%) and Dinoflagellates (0.3-2.3%). There were significant differences between the samples inoculated with S8 and S17 for Ciliphora (S17) and Cercozoa (S8) (p=<0.05), with no difference for Dinoflagellates (p=0.25). There were no significant differences between the pCO_2 treatments. There were low OTU abundances (0.1-0.5% average relative abundance) of the groups Ascomycota, Bicosoecida, Holozoa, Incertae Sedis and Ochrophyta in all samples.

The PCoA of the bacterial communities using the Weighted Unifrac of Bray-Curtis showed a clear grouping for the different pCO_2 treatments (Figure 6). The first group (southern quadrant)

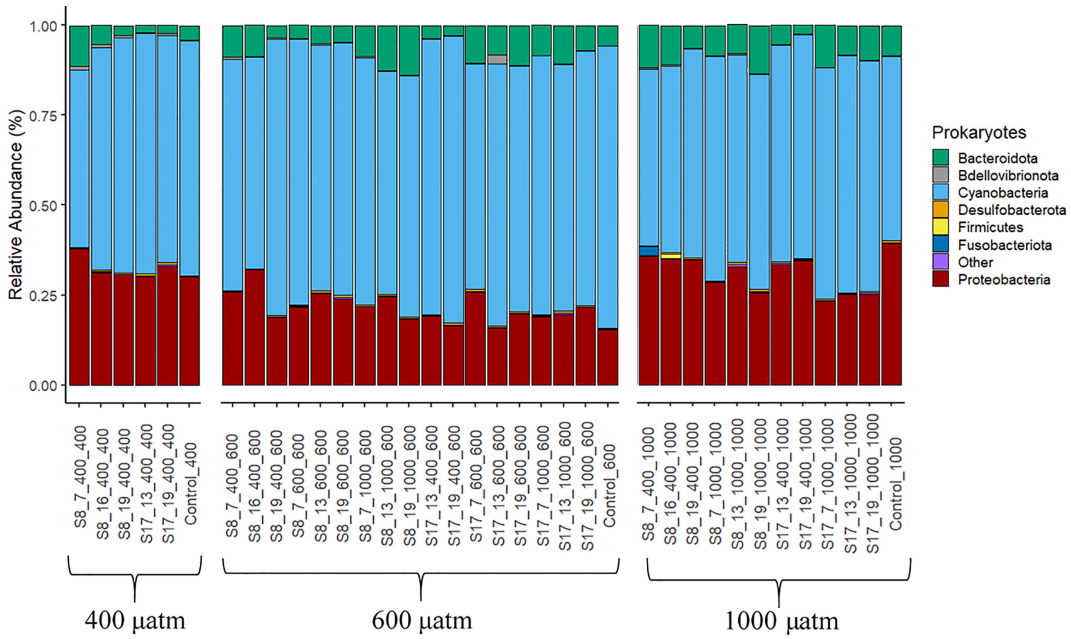

FIGURE 4
Prokaryote phyla according to the 16S gene DNA Metabarcoding analysis from the samples collected at the end of the 6 day experiment. The results are grouped by the three pCO_2 treatments (400 µatm, 600 µatm, 1000 µatm). The category "other" (in purple) is all remaining orders with <0.1% relative abundance. The following labelling was used to distinguish between the different S. marinoi strains, temperature and pCO_2 acclimisation conditions and pCO_2 treatment; strain_temperature_CO_2acclimisation_CO_2treatment.

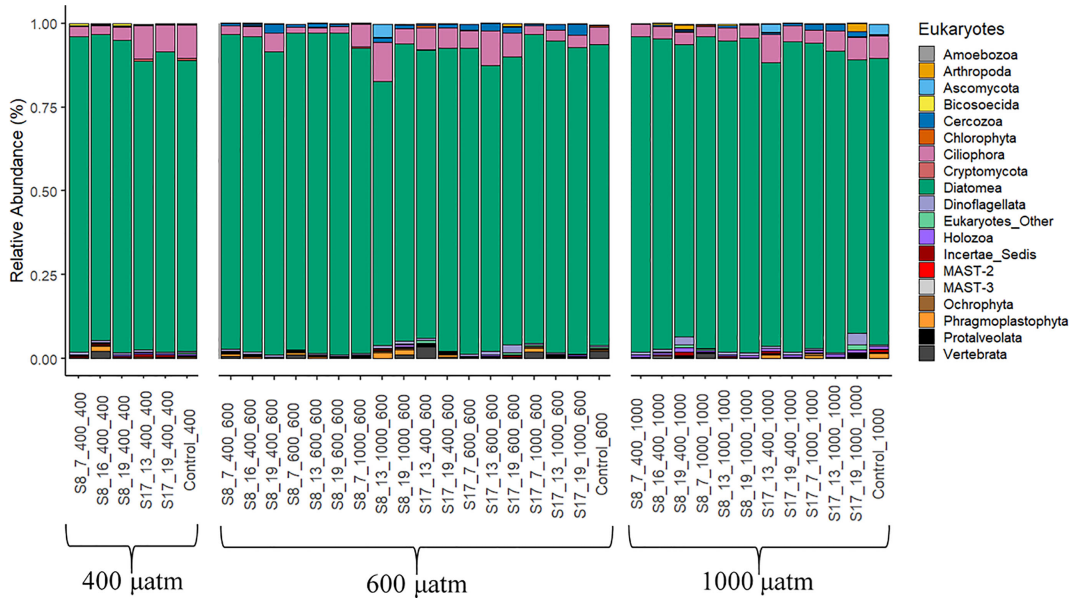

FIGURE 5
Eukaryote phyla according to the 18S gene DNA Metabarcoding analysis from the samples collected at the end of the 6 day experiment. The results are grouped by the three pCO_2 treatments (400 µatm, 600 µatm, 1000 µatm). The following labelling was used to distinguish between the different S. marinoi strains, temperature and pCO_2 acclimisation conditions and pCO_2 treatment; strain_temperature_CO_2acclimisation_CO_2treatment.

consisted of most samples from the 400 µatm treatment, the second (eastern quadrant) from the 600 µatm treatment and the third group (western quadrant) from the 1000 µatm treatment. In agreement, with the 16S PCoA, ANOSIM analysis demonstrated significant differences between the 600 µatm treatment and the other two pCO_2 treatments (r=0.51 and 0.43, p=0.001; Table S5). However, indicator species analysis did not highlight any taxa which had an affinity to the 600 µatm treatment. The only treatment with a significant indicator species, Bdellovibrio (IndVal value=0.707, p =0.006; Table S6), was the 400 µatm.

Compared to the 16S PCoA, the PCoA of the eukaryotic communities consisted of no groupings as well as no clear patterns or trends (Figure 6). This is supported by the 18S ANOSIM results which showed no significant differences between the three pCO_2 treatments (Table S5). Only the 400 µatm treatment had an indicator species (Cryptomycota; Indval value=0.537, p=0.033; Table S6). Some groups were more strongly associated with a combination of treatments, such as Chlorophyta (400 and 600 µatm treatments; Indval value=0.809, p=0.024) and Amoebozoa (600 and 1000 µatm treatments; Indval value=0.753, p=0.019).

Discussion

The incorporation of long-term acclimated *S. marinoi* into short-term mesocosms can help determine if previous acclimation to elevated temperature and pCO_2 concentration could give them a competitive advantage in natural microbial communities. Although, in this case, the lack of significant differences after acclimation to different temperature and pCO_2 concentrations suggests that *S. marinoi* did not gain any competitive advantage over other microbial groups. This is supported by Low-Décarie et al. (2013) who found that adaptation to high CO_2 concentrations (380 ppm vs. 1000 ppm) did not result in evolutionary change or increased growth rates at elevated CO_2 for two diatom genera (*Nitzschia* spp. and *Navicula* spp.). This could be due to the pCO_2 variability phytoplankton communities experience in their natural environment, both diurnally and seasonally (Schulz and Riebesell, 2013; Yasunaka et al., 2016). Diurnal fluctuations in seawater can range from 0.1 pH units in the Bay of Calvi in the Mediterranean sea to 0.5 in the Kerguelen Archipelago in the Southern Ocean (Delille et al., 2009; Schulz and Riebesell, 2013). Whilst in eutrophic systems, pH can vary between 0.7 and 3.2 units (Thomsen et al., 2010). So, the lack of variations between the different acclimation conditions could be because they are within the *p*CO2 variability the two strains experience in their natural environments (Thor and Dupont, 2015; Vargas et al., 2022). Whereas, for temperature, Briddon et al., (in review) determined that previous acclimation to two different temperatures conditions, 22°C (mean lake summer temperature) and 26°C (the predicted temperature by 2100), could be a possible driver of genetic change due to enhanced mutation rate and lower secondary metabolites production in heat-adapted cyanobacteria. However, as diatoms are diploids, any mutation or changes in one allele could be masked by the functional allele, making any genetic changes harder to detect (Low-Décarie et al., 2013).

The pCO_2 treatment could be a possible indirect driver of bacterial community composition through changes to phytoplankton-bacteria interactions (Krause et al., 2012; Héry et al., 2014). There were significant differences for the 600 µatm treatment (in comparison to the 400 and 1000 µatm treatments) for

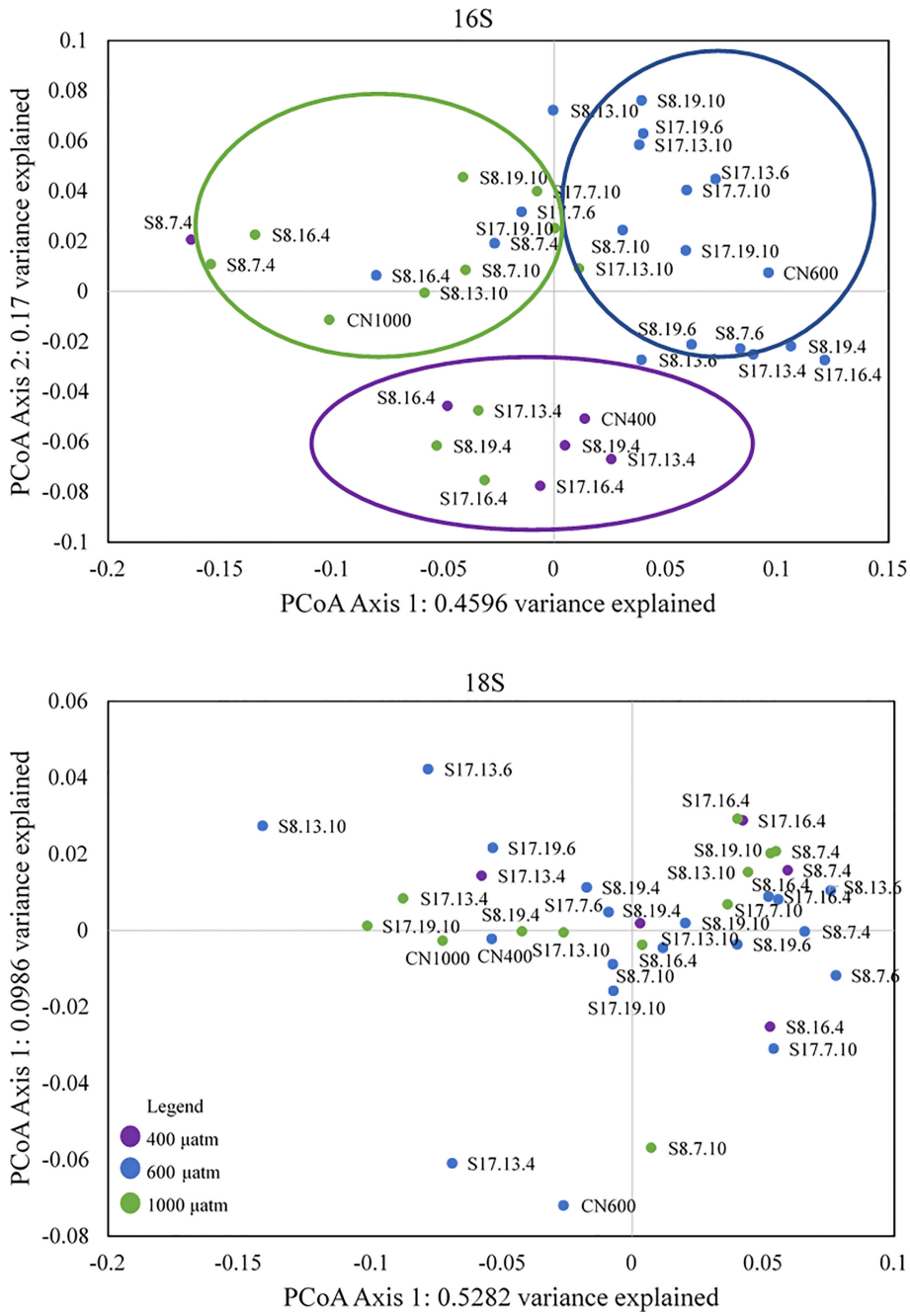

FIGURE 6
PCoA biplot of the 16S and 18S Bray-Curtis distance matrix for all samples. The three circles in the 16S PCoA plot correspond to the different groupings according to pCO_2 treatment, with the colours matching those in the legend, with purple= 400 µatm, blue= 600 µatm and green =1000 µatm CO_2 treatment. There were no associated groupings for 18S (eukaryotes).

the bacterial but not for the eukaryotic communities. It is unlikely to be due to differences in nutrient concentrations due to similar abundances between the 600 µatm treatment (mesocosms 2 and 3) and the mesocosm used for the 400 µatm treatment (number 1; Table 2). Krause et al. (2012), using a highly replicated microcosm experiment over the four seasons, found that even moderated changes in pH resulted in microbial community shifts, indicating that slight reductions in pH could be crucial for determining their composition. Yet, this depended on the community assembly and environmental factors such as light. Furthermore, Davidson et al. (2016) found there were little differences in Antarctic microbial abundance (including bacteria) between 84 and 643 µatm pCO_2. However, once concentrations exceeded 1281 µatm, the community structure changed favouring small protists (<5 µm) over larger taxa. This suggests that bacteria communities can acclimate to pCO_2 changes to a certain level until their rate of acclimation plateaus and they are no longer able to keep up with the continuous decrease in pH (Maas et al., 2013; Aguayo et al., 2020). An increase in

atmospheric CO_2 whilst not leading to significant changes in algal community composition, can lead to an increase in algal abundance (Bach et al., 2017) and, therefore, an enhancement of organic matter (Huang et al., 2021) and changes to phytoplankton-bacteria interactions (De Kluijver et al., 2010; Hornick et al., 2017). The families Flavobacteriaceae and Alteromonadaceae are highly abundant in marine environments and can become dominant in response to an increase in organic matter (Waśkiewicz and Irzykowska, 2014; Bunse et al., 2016). However, as the 600 and 1000 µatm treatments did not have significantly higher chlorophyll *a* concentrations, it suggests that other factors must also be influencing bacterial communities. Crummett (2020) found that both families (families Flavobacteriaceae and Alteromonadaceae) can have sub-OTU habitat specialisation to different pH environments, which can also vary seasonally. The season and the naturally occurring bacterial communities (present in the fjord water used in the experiment) could explain the significantly higher OTU abundances of Flavobacteriaceae and Alteromonadaceae in the 600 and 1000 µatm treatments. It would be interesting to replicate this mesocosm experiment in different seasons to determine if bacterial habitation specialisation plays a role in their response to ocean acidification

Even though there were a lack of differences for the eukaryotic communities between the different pCO_2 treatments, some groups preferred certain pCO_2 conditions. Cryptomycota preferred the 400 µatm treatment, Chlorophyta had an affinity for the 400 and 600 µatm treatment whilst Amoebozoa had an affinity to the 600 and 1000 µatm treatments. The lack of any significant affinity for the 1000 µatm treatment, which had the highest TP and phosphate concentrations, suggests that the high P concentrations are unlikely to be causing the differences between treatments. Cryptomycota is a fungus which consisted of *Incertae sedis*, meaning taxonomic groups with unknown relationships (Comeau et al., 2016). Rojas-Jimenez et al. (2017) suggests composition of this clade is influenced by site specific effects such as water-column stratification, anoxia and nutrient inputs, suggesting either the present-day pCO_2 concentration or an unknown factor led to higher OTU abundances of Cryptomycota in the 400 µatm treatment. Chlorophyta (consisting mostly of the genus *Tetraselmis*) is commonly found in marine environments. Figueroa et al. (2021) and Kang and Kim (2016) found that Chlorophyta growth increased with CO_2 concentrations when nutrients were not limiting. The opposite pattern observed here, could be due to the low or undetectable (iron) nutrient concentrations in all mesocosms. Amoebozoa's affinity to the higher pCO_2 treatment could be due to its ability to survive in a wide range of environments and the limited understanding on the ecological preferences of this taxonomic group (Kudryavtsev et al., 2021). The OTU abundances of Ciliphora (consisting mostly of the subclass Haptoria) was similar across all treatments. The subclass Haptoria is widely distributed in coastal waters (Earland and Montagnes, 2002) and can dominate planktonic communities in a wide variety of nutrient conditions (Barouillet et al., 2022). As micropredators they are exposed to both top-down and bottom-up controls, the removal of zooplankton prior to the beginning of the experiment could have resulted in high abundances in all treatments due to reduced predation.

Overall, elevated pCO_2 would increase the growth of photosynthetic organisms, it would also enhance the demand for nutrients (Low-Décarie et al., 2013). Therefore, the lack of iron in all treatments (Table 2) could have prevented elevated growth rates and explain the lack of differences for eukaryotes between the different treatments. This could be why the high pCO_2 (1000 µatm) treatment was not significantly different from the other treatment, even though the opposite would be expected. Moreover, the high tolerance of eukaryotes could be due to the highly variable pCO_2 conditions they experience in their natural environment. This variability suggests that local phytoplankton communities can acclimate to fluctuating pH conditions at least in the short term (Hinga, 2002; Rathbone et al., 2022). This could explain why all the major algal groups showed limited differences between treatments, supported by the DNA evidence, which showed similar bacterial and eukaryotic species richness among all CO_2 treatments (according to the Shannon Diversity Index; Table S4).

Cyanobacteria were the dominant prokaryotes in all treatments; however, all cyanobacterial reads had been assigned as chloroplasts (endosymbiotic cyanobacteria) suggesting that the reads could be fragments of organelles from living and/or dead photoautotrophic eukaryotes. It is now widely accepted that chloroplasts originate from cyanobacteria (Falcón et al., 2010; Sato, 2021). Therefore, it could be suggested that the reads assigned to chloroplasts would most likely belong to the diatoms as they are the most abundant eukaryotic group (>85%). However, it cannot be discounted that the reads also suggest the presence of cyanobacteria. An annual phytoplankton community study from Laholm Bay (south of Gothenburg, Sweden) found July to have the highest monthly relative abundance of cyanobacteria (Eberlein et al., 2017). This combined with stratified water column observed during the summer season, are ideal conditions for cyanobacterial blooms (Paerl and Huisman, 2008). Furthermore, Valencia et al. (2022) found that diatoms and cyanobacteria (specifically *Synechococcus*) were overrepresented in sinking particles in oligotrophic oceanic regions relative to other eukaryotes. As the water enclosed in the mesocosms used a mixture of surface and bottom water, this could include sinking particles. However, as metagenomic sequencing was not completed it is not possible to determine the origin of the chloroplasts.

Interestingly, even though all samples (except the controls) were inoculated with acclimated *Skeletonema*, it was not the dominant diatom species in terms of OTUs. Multiple studies have shown that diatoms dominate the phytoplankton community at the time of year the experiment was run (July; Höglander et al., 2013; Eberlein et al., 2017). This is supported by the chlorophyll *a* concentrations of the major algal groups, which showed that the chromophyte algae group (consisting of cryptophytes, dinoflagellates and diatoms) had the highest concentration in all treatments. All samples also had a high relative OTU abundance of diatoms (unclassified Bacillariophyceae; 77.5-94.5%). However, it is unlikely to be the acclimated *Skeletonema* strains (S8 and S17),

due to similar OTU abundances in the control samples which were not inoculated with these strains. As the diatom relative OTU abundance was similar in all treatments, the high OTU abundances are most likely due to the diatoms dominating the phytoplankton community at the time.

Strain-specific differences (between S8 and S17) suggests phenotypic and genotypic variants of the same species can result in different responses in natural microbial communities (Langer et al., 2009). This could explain the significantly higher relative abundances of diatoms and proteobacteria in mesocosms containing the seawater inoculated with the strain S8 and the high relative abundances of Ciliphora, Cercozoa and endosymbiotic cyanobacteria in mesocosms containing the seawater inoculated with the strain S17. Interspecies interactions can play a significant role in determining community composition and preclude or facilitate the coexistence of specific community members (Leventhal et al., 2018). Goyal et al. (2022) found that strains belonging to the same species were the key element of long-term community dynamics due to pre-existing genetics. It is plausible that strain interactions between the acclimated *S. marinoi* and the other major eukaryotic and bacterial groups could explain some of the significant differences between the microbial communities (Vuong et al., 2017). However, without metagenomic analyses it is not possible to determine if genetic variation or differentiated genotypes played a role.

Conclusion

In summary, this study showed that increases in pCO_2 could result in significant changes in OTU diversity of prokaryotic communities but have a limited impact on eukaryotes. Even though all dialysis bags (except the controls) were inoculated with acclimated *S. marinoi*, there were insignificant differences between the pCO_2 treatments showing the species plasticity to variations within its natural environment. This suggests that previous acclimation to increased temperature and pCO_2 did not give them a competitive advantage in natural microbial communities. Furthermore, strain specific differences indicated that interspecies interactions between different eukaryotic and bacterial groups could have played a role in determining community composition. In the future, the use of metagenomic sequencing of the resulting biomass from mesocosm experiments could improve our understanding of strain-specific interactions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: European Nucleotide Archive, PRJEB61711.

Author contributions

RB, WE, and BC collected and described the *Skeletonema* strains. CB, BD, MN, and AH performed the long-term acclimation experiment. BD, RB, and SD designed the experimental setup. CB, MN, AN, and BD completed the mesocosm experiment. MN and AN completed the DNA extraction and purification process. CB analysed the DNA metabarcoding results and wrote the first version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1197570/full#supplementary-material

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