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# Effects of increased $pCO_2$ and temperature on the North Atlantic spring bloom. II. Microzooplankton abundance and grazing

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ABSTRACT: The North Atlantic spring bloom is one of the largest annually occurring phytoplankton blooms in the world ocean. The present study investigated the potential effects of climate change variables (temperature and $pCO_2$) on trophic dynamics during the bloom using a shipboard continuous culture system. The treatments examined were (1) 12°C and 390 ppm $CO_2$ (ambient), (2) 12°C and 690 ppm $CO_2$ (high $pCO_2$), (3) 16°C and 390 ppm $CO_2$ (high temperature), and (4) 16 °C and 690 ppm $CO_2$ (greenhouse). Individually, increasing temperature and $pCO_2$ initially resulted in significantly higher total microzooplankton abundance and grazing rates over the ambient treatment mid-experiment, with significantly greater increases still in the greenhouse treatment. By the end of the experiment, microzooplankton abundance was highest in the 2 low temperature treatments, which were dominated by small taxa, while the larger ciliate *Strombidium* sp. numerically dominated the high-temperature treatment. Microzooplankton community composition was dominated by small taxa in the greenhouse treatment, but total abundance declined significantly by the end after peaking mid-experiment. This decrease occurred concurrently with the growth of a potentially unpalatable phytoplankton assemblage dominated by coccolithophores. Our results suggest that indirect effects on microzooplankton community structure from changes in phytoplankton community composition as a result of changing temperature or $pCO_2$ were likely more important than direct effects on microzooplankton physiology. Similar changes in trophic dynamics and whole plankton community composition may also be important for future climate-driven changes in the North Atlantic spring bloom assemblage.

KEY WORDS: Microzooplankton · Herbivory · Temperature · $pCO_2$ · North Atlantic spring bloom · Climate change

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## INTRODUCTION

Protistan heterotrophs are key grazers of phytoplankton, heterotrophic nanoplankton, and bacteria (Sanders et al. 1992, Sherr & Sherr 1994). Microzooplankton (between 20 and 200 µm in size) can consume significant amounts of primary production in the marine environment, and their contribution to phytoplankton mortality can exceed that of mesozooplankton such as copepods (Gifford et al. 1995, Landry et al. 1997, Strom et al. 2001). Grazing by microzooplankton can result in significant remineralization of nutrients in surface waters, reducing potential export flux (Caron & Goldman 1990, Dolan 1997). Microzooplankton can

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also be an important food source for mesozooplankton and, as such, can contribute to carbon transfer to higher trophic levels (Sherr et al. 1986, Atkinson 1996, Schnetzer & Caron 2005).

The annually occurring and spatially extensive spring bloom of phytoplankton in the North Atlantic Ocean has been extensively studied since the early days of oceanography (e.g. Sverdrup 1953). The US Joint Global Ocean Flux Study (JGOFS; Ducklow & Harris 1993), US Marine Light Mixed Layer research initiative (MLML; Plueddemann et al. 1995), UK Biogeochemical Ocean Flux Study (BOFS; Savidge et al. 1992), UK Plankton Reactivity in the Marine Environment program (PRIME; Savidge & Williams 2001), and French 'Programme Océan Multidisciplinaire Méso Echelle' (POMME; Memery et al. 2005) have all been large-scale efforts to combine physical, chemical, biological, and modeling approaches to understand ecosystem dynamics throughout the stages of the bloom. This phytoplankton bloom is initiated by a reduction in mixed layer depth and is typified by an initial community of large diatoms shifting to dominance by smaller prymnesiophytes such as *Emiliania huxleyi* (Robinson 1965, Colebrook 1982, Sieracki et al. 1993). However, considerable interannual variability has also been reported (Henson et al. 2006).

Virtually all studies of microbial dynamics during the North Atlantic spring bloom have identified microzooplankton as active and important grazers (Burkill et al. 1993, Verity et al. 1993b, Gifford et al. 1995), consuming much of the daily primary production (Stelfox-Widdicombe et al. 2000, Fileman & Leakey 2005, Karayanni et al. 2005). The microzooplankton community is typically dominated by aloricate ciliates (Verity et al. 1993b, Stoecker et al. 1994, Fileman & Leakey 2005) and, less frequently, by heterotrophic dinoflagellates and tintinnids (Verity et al. 1993a, Stelfox-Widdicombe et al. 2000). Microzooplankton have been hypothesized to be particularly important in the late to post-bloom season, when average phytoplankton size is smaller and chain- or colony-forming phytoplankton abundance is reduced (Gifford et al. 1995). The importance and efficiency of grazing by microzooplankton during the spring bloom was linked to low rates of carbon export during the JGOFS North Atlantic Bloom Experiment (Buesseler et al. 1992, Verity et al. 1993b).

While the North Atlantic spring bloom itself has been the subject of considerable study, it is not known how future predicted climate changes will affect this complex oceanic system. Recent models predict sea surface temperature increases of 1 to 4°C by the end of this century (Bopp et al. 2001, Alley et al. 2007). Atmospheric carbon dioxide partial pressure ($pCO_2$) has increased from 280 to 380 ppm since the pre-industrial era and is predicted to exceed 700 ppm by the end of this century (Alley et al. 2007). Increased sea surface temperature should increase potential phytoplankton growth rates (Eppley 1972) and may alter dynamics between phytoplankton and their microzooplankton grazers (Rose & Caron 2007). Increased atmospheric $pCO_2$ has been predicted to affect photosynthesis, calcification, and elemental composition of individual phytoplankton (Riebesell 2004, Feng et al. 2008), as well as alter the species composition of phytoplankton communities (Tortell et al. 2002, Rost et al. 2003, Hare et al. 2007). Interactive effects of increased temperature and $pCO_2$ on growth rates, photosynthetic parameters, and elemental cell quotas have been reported in laboratory studies of phytoplankton cultures (Fu et al. 2007, Feng et al. 2008). Recent work has suggested that winter warming may favor microzooplankton communities and, as a result, potentially suppress phytoplankton blooms in temperate waters (Aberle et al. 2006). Another recent study reported little to no effect of increased $pCO_2$ alone on microzooplankton grazing of an incubated natural phytoplankton assemblage (Suffrian et al. 2008). Virtually nothing is known, though, about interactive effects of increased temperature and $pCO_2$ on microzooplankton herbivory in marine systems.

The goals of this experiment were to use a new shipboard continuous culture system (Ecostat) to study the effect of increased temperature and $pCO_2$ on phytoplankton physiology, community composition, biogeochemistry, and herbivory by microzooplankton within the North Atlantic spring bloom setting. The Ecostat system has been developed to more realistically simulate natural environmental changes, by allowing extended manipulative experiments using whole seawater with intact plankton communities. The Ecostat system modifies traditional shipboard bottle incubation methods and applies continuous culturing methodology (Hutchins et al. 2003, Hare et al. 2005, 2007). Responses of the phytoplankton community and effects on biogeochemical parameters are reported in Feng et al. (2009, this volume). The observed responses in terms of microzooplankton abundance, community composition, and grazing rates are reported here.

## MATERIALS AND METHODS

**Experimental design.** The experiment was conducted onboard the RV 'Seward Johnson II', from June 20 to July 4, 2005, with water collected at 57° 58′ N, 15° 32′ W. Four treatments were used with 6 replicates each: (1) 12°C and 390 ppm $CO_2$ (ambient), (2) 12°C and 690 ppm $CO_2$ (high $pCO_2$), (3) 16°C and 390 ppm $CO_2$ (high temperature), and (4) 16°C and 690 ppm $CO_2$ (greenhouse). Sea surface temperature at this location was 12°C at the time of water collection,

and the increase of 4°C in the high temperature treatments is consistent with projections for the end of this century (Sarmiento et al. 1998, Bopp et al. 2001, Alley et al. 2007). The 2 $CO_2$ concentrations chosen also represent present day and projected year 2100 atmospheric carbon dioxide partial pressures, respectively (Alley et al. 2007). Experiments were run using a seawater continuous culture system, termed an 'Ecostat' (Hutchins et al. 2003, Hare et al. 2005, 2007). Briefly, whole seawater was collected from 5 to 10 m depth using a trace-metal-clean, towed-intake Teflon pump system (Hutchins et al. 2003), prefiltered through 200 µm Nitex mesh to remove mesozooplankton and incubated in twenty-four 2.7 l trace-metal-clean, clear polycarbonate bottles. Bottles were placed in racks in a temperature-controlled deck incubator with recirculating water and shaded to 30% of surface irradiance ($I_0$) using a neutral-density shade screen. Temperatures in the 16°C incubator were gradually increased over a period of 24 h to avoid heat-shocking the plankton. Bottles were bubbled with either air or a commercially prepared air/$CO_2$ mixture with 750 ppm $CO_2$ using an inflow tube through the cap and an airstone to maximize gas transfer to the liquid phase. The gases used for bubbling were filtered through a 0.2 µm HEPA filter to avoid contamination of experimental bottles by trace metals (Hare et al. 2005). The system was run in batch mode for 3 d prior to turning on the pumps, in order to stimulate phytoplankton growth and prevent wash-out of slower growing species.

After this batch growth period, whole seawater in each incubation bottle was slowly diluted at a continuous rate using seawater collected at the initial site. This seawater medium was filtered through a 0.2 µm inline capsule filter initially, then re-filtered through a second 0.2 µm inline capsule filter immediately prior to use as a diluent. The medium was stored in trace-metal-clean, 50 l carboys in the dark. Initial *in situ* nutrient concentrations were low (0.32 µmol nitrate $l^{-1}$, 0.12 µmol phosphate $l^{-1}$, 0.7 µmol silicate $l^{-1}$), so the medium and the whole water in the incubation bottles were amended with 5 and 0.31 µmol $l^{-1}$ (final concentration) of nitrate and phosphate. The dilution rate of 0.5 $d^{-1}$ was controlled in each incubation bottle using a peristaltic pump and calibrated daily to ensure constant flow rate. This flow rate constituted a 50% dilution of the experimental bottle volume daily. Incubation bottles were mixed by inverting the rack 120° every 5 to 15 min using a compressed-air-driven system. Diluted seawater flowed out of the incubation bottles at a continuous rate and into 2.7 l polycarbonate bottles stored in the dark, which were used as outflow collection vessels. Incubation bottles were sampled daily for chlorophyll *a* (chl *a*), algal community composition (determined by flow cytometry and microscopy), dissolved nutrients (N, P, Si), particulate dimethylsulfoniopropionate ($DMSP_p$), dissolved inorganic carbon (DIC), and pH. Daily samples were taken directly from the bottles with a sampling syringe. Samples for total particulate carbon (TPC), particulate organic carbon (POC), particulate organic nitrogen (PON), biogenic silica (BSi), and particulate phosphorus (POP) were taken from outflow bottles every few days, along with samples for pigments. Particulate inorganic carbon was calculated as the difference between TPC and POC concentrations. For the final day (T14) sampling, all the samples were taken directly from the incubation bottles. A previous examination and comparison of outflow and experimental bottles showed that within 24 h of outflow collection there were no differences between them in terms of both algal biomass and community composition (data not shown). Detailed methods and results of biogeochemical analyses, as well as more detailed information about the phytoplankton assemblage are reported in the companion papers (Feng et al. 2009; Lee et al. 2009, this volume).

**Phytoplankton.** Total chl *a* was measured daily from samples taken directly from experimental bottles. Samples were filtered onto GF/F filters at low vacuum pressure and extracted in 90% acetone for 24 h in the dark at –20°C. Samples were read on a Turner 10-AU fluorometer. Nano- and picophytoplankton were enumerated using standard flow cytometric techniques (Olson et al. 1983, Yentsch et al. 1983). Samples were removed daily directly from experimental bottles, and 2 ml were preserved with 10% formalin buffered with seawater (1% final concentration), then frozen at –80°C until analysis. Samples were analyzed in the laboratory on a FACSCalibur benchtop flow cytometer. Phytoplankton were identified in cytograms based on forward angle light scatter (size) and red fluorescence (chlorophyll). Phytoplankton were classified within cytograms as picoplankton (0.2 to 2 µm) or nanoplankton (2 to 20 µm) using 2 µm green fluorescent beads (Invitrogen) added to each sample.

**Dilution experiment.** Microzooplankton grazing was measured using the modified dilution technique of Landry et al. (1995), without the addition of fluorescently labeled prey. This technique involves the successive dilution of whole seawater to reduce encounter rate between microzooplankton and phytoplankton and thus release grazing pressure on phytoplankton. Phytoplankton growth and mortality rates can then be estimated using the slope and intercept of graphs of total chl *a* versus percentage dilution of whole seawater.

Experiments were conducted on the initial phytoplankton community from the same water used for the experiment and on Day 8 of the Ecostat experiment using outflow water from experimental bottles. Out-

flow water was collected for approximately 24 h to obtain enough volume to conduct the dilution experiments on T8. Replicate bottles from treatments within the continuous culture system were combined to provide adequate volume for the grazing treatments, so the dilution experiment itself was not replicated. The dilution series was run in 1.2 l polycarbonate bottles that had been soaked in 10% HCl and rinsed thoroughly with Milli-Q water. Each dilution treatment was run in triplicate from the combined experimental treatments. Four dilution treatments were used, including approximately 100, 75, 50, and 25% whole seawater. Bottles were incubated in the deck incubators housing the seawater continuous culture system for 24 h. Bottles were maintained at the appropriate experimental water temperatures, but were not bubbled during the 24 h incubation. The dilution experiment conducted on the initial phytoplankton community was incubated at ambient water temperatures to avoid the potential for mortality due to thermal stress at the beginning of the experiment. Water for the dilutions also came from the outflow bottles and was filtered through 0.2 µm inline capsule filters then used immediately in the experiment. Nutrients were added at the beginning of the dilution experiments to ensure phytoplankton growth in the dilution series was nutrient replete. Nutrient additions consisted of 10 µmol nitrate $l^{-1}$, 10 µmol silicate $l^{-1}$, and 0.63 µmol orthophosphate $l^{-1}$ (all final concentrations). Triplicate bottles of unamended, 100% unfiltered seawater were used as controls to determine phytoplankton growth in unenriched conditions.

Samples for total chl *a* were removed initially and after 24 h and measured as described above (Strickland & Parsons 1972). Phytoplankton mortality rates ($m$) were calculated based on the slopes of the regressions of total chl *a* versus percentage dilution. Phytoplankton growth rates in unamended ($\mu_0$) and nutrient-enriched ($\mu_n$) dilution treatments were calculated from the 100% unfiltered seawater bottles. Net phytoplankton growth rates were calculated as the changes in chl *a* concentration over 24 h in the unamended, whole-seawater control treatment.

**Microscopy.** Samples for enumeration of microzooplankton were removed from outflow bottles on Days 4, 9, and 14 of the experiment. Triplicate samples were counted on the initial day. Duplicate samples on Days 4 and 9 were mixed to obtain enough volume for accurate counts, yielding 3 replicates per treatment on these days. All 6 replicates were counted on the final day of the experiment. All samples were preserved with 10% acid Lugols solution and stored at room temperature in the dark until enumeration of microzooplankton in the laboratory (Throndsen 1978). Then, 100 ml of sample was settled for at least 18 h into Utermöhl chambers before counting using an inverted Zeiss Axiovert S100 microscope at 200× magnification (Utermöhl 1958). Microzooplankton were identified to genus level when possible. The use of Lugol fixative obscured chl *a* fluorescence and rendered the distinction between phototrophic and heterotrophic dinoflagellates based on autofluorescence impossible. However, certain heterotrophic dinoflagellates such as *Protoperidinium* and *Gyrodinium* were identified based on morphology, and were thus included in the counts.

**Statistics.** Microzooplankton abundances were compared on Days 4, 9, and 14 separately using a percentile bootstrap method to perform multiple comparisons, as described by Wilcox (2003). This test was chosen over the more commonly used ANOVA *F*-test since the percentile bootstrap test does not have the standard assumptions of normality and homoscedasticity and generally has a higher power than the ANOVA *F*-test. Tests were performed using the freeware statistical program R Version 2.3 (www.r-project.org). The same percentile bootstrap test was performed to test multiple comparisons of trimmed means for the unenriched net growth rates of the total phytoplankton community in the dilution experiments (Wilcox 2003). This percentile bootstrap test was also used to compare abundances of nanophytoplankton and picophytoplankton on the final day of the experiment. Before analyzing the flow cytometry data, outliers were removed using the Hampel identifier, as modified by Rousseeuw & van Zomeren (1990). A percentile bootstrap method and Theil-Sen estimator were used to test for significant differences among mortality rates. This method only compares the slopes of 2 groups at a time, so a full pairwise comparison was performed for all treatments, as described by Wilcox (2003). All tests were done at the $\alpha = 0.05$ level.

Microzooplankton community composition was compared using the multivariate statistical software package PRIMER Version 6 (Clarke & Warwick 2001). A square-root transformation was applied to data to slightly upweight the contribution of rarer species in the measure of similarity among samples. The similarity matrix was generated with a Bray-Curtis coefficient, and non-metric multidimensional scaling (MDS) was used to visualize sample relationships. The use of MDS allowed whole communities within individual samples to be compared to each other, illustrating high-level sample relationships. Each symbol on an MDS plot represents the whole microzooplankton community from a single sample. Two samples with very similar microzooplankton communities (e.g. replicates from a single treatment) have symbols located relatively close to each other within the 2-dimensional MDS plot. Two samples with very different microzooplankton communities (e.g. samples from different

treatments) have symbols located relatively far away from each other within the plot. The MDS plots illustrate relative differences between samples within a group. Since the distances plotted are relative and not absolute, the axes of an MDS plot are without absolute scale and thus unlabeled.

We used the ANOSIM test for differences between groups of samples to determine the significance of similarity between microzooplankton community composition and treatment factor on each day that microzooplankton samples were analyzed from the Ecostat experiment (Clarke & Green 1988). This is a nonparametric (rank-based) procedure that uses Bray-Curtis similarities and a permutation test to compare the overall similarity of samples within an individual treatment to the overall similarity of samples between treatments. In this way, the test is able to determine the significance level of community composition differences between treatments. The ANOSIM test is computed using distances from the similarity matrix, not distances on the 2-dimensional MDS plot.

## RESULTS

### Phytoplankton measurements

Total chl *a* increased in all 4 treatments during the first 3 d of the experiment, while the Ecostat was run in batch mode (Feng et al. 2009). Between Days 4 and 8, total chl *a* declined in all treatments. During the last 4 d of the experiment, total chl *a* was stable in the ambient, high $pCO_2$, and high temperature treatments. On the final day of the experiment, total chl *a* was highest in the greenhouse treatment. Total chl *a* on the final day was significantly higher in the greenhouse treatment when compared to the other 3 treatments (all $p < 0.05$). Total chl *a* on the final day of the experiment was not significantly different in the control versus high $pCO_2$ treatments ($p = 0.13$), control versus high temperature treatments ($p = 0.22$), or high temperature versus high $pCO_2$ treatments ($p = 0.67$).

Picophytoplankton (0.2 to 2 µm) abundance in the ambient, high temperature, and greenhouse treatments doubled in the first 4 d of the experiment (Fig. 1A). Abundances in the greenhouse treatment fluctuated between 3000 and 5000 cells $ml^{-1}$ until the last 4 d of the experiment, when they increased to 3-fold the initial values. Abundances in the high temperature treatment ended at twice the initial values. In both low temperature treatments, abundances declined between Days 9 and 14 to less than initial levels. Due to high variability of picophytoplankton abundances in the greenhouse and ambient treatments, only high temperature was significantly greater than high $pCO_2$ ($p < 0.05$). All other pairwise comparisons were not statistically different ($p > 0.05$).

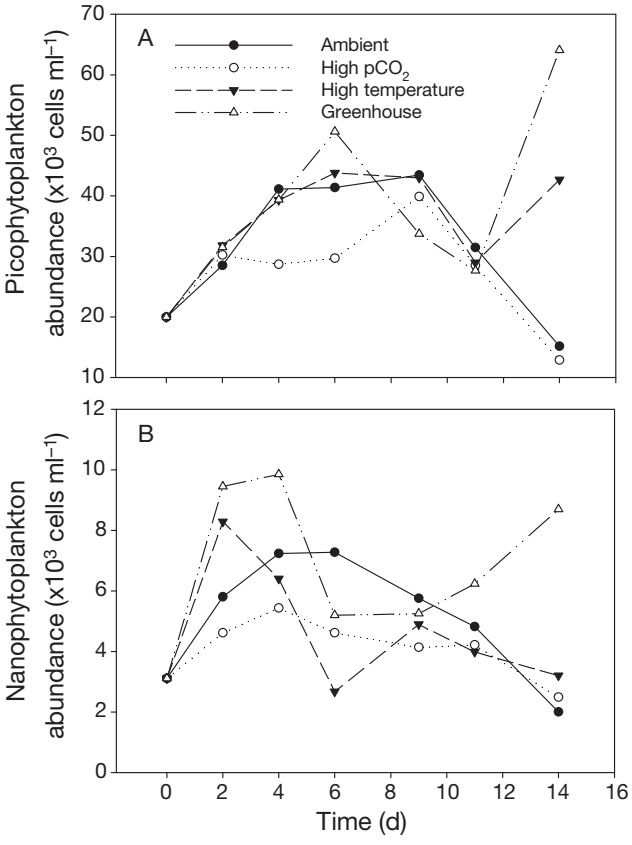


Fig. 1. Phytoplankton dynamics over the course of the Ecostat experiment. Abundance of (A) picophytoplankton and (B) nanophytoplankton in 4 treatments (see 'Materials and methods: Experimental design' for details)

Nanophytoplankton (2 to 20 µm) abundance increased earlier and more rapidly in all treatments than did picophytoplankton abundance (Fig. 1B). The ambient and high $pCO_2$ treatments showed a similar initial increase to approximately twice the initial abundance by Day 4, then a steady decline throughout the rest of the experiment, ending at abundances similar to initial values. The high temperature and greenhouse treatments increased 3-fold in abundance by Day 4 and then rapidly declined to twice the initial values in the greenhouse treatment and to initial values in the high temperature treatment by Day 6. Nanophytoplankton abundance in the high temperature treatment then remained relatively stable at initial values until the end of the experiment. In contrast, abundances in the greenhouse treatment increased back to 3-fold initial values by Day 14. Final day nanophytoplankton abundances were significantly higher in the greenhouse treatment than in all other treatments ($p < 0.05$), as were the abundances in the high temperature compared to the high

$pCO_2$ treatment. There were no significant differences between the final day nanophytoplankton abundances in the ambient compared to either the high $pCO_2$ or high temperature treatments ($p > 0.05$).

### Dilution experiments

Results from the dilution experiments are shown in Table 1. There was little difference in phytoplankton growth rate in the enriched and unamended whole-water treatments for the initial phytoplankton community, suggesting no nutrient effect on the initial community. Average mortality rates for the initial phytoplankton community were also relatively high at 0.65 $d^{-1}$. Phytoplankton net growth rates were slightly positive at 0.17 $d^{-1}$.

Mortality rates in the second set of dilution experiments ranged from the lowest in the ambient treatment (0.49 $d^{-1}$) and increased to similar mortality rates in the high $pCO_2$ and high temperature treatments (0.86 and 0.84 $d^{-1}$, respectively), and the greatest mortality in the greenhouse treatment (1.1 $d^{-1}$). Significant differences in mortality rate were obtained in pairwise comparisons of all treatments ($p < 0.05$), except when comparing the high $pCO_2$ versus high temperature treatments ($p > 0.05$). Phytoplankton net growth rates were all negative, and all pairwise comparisons were significantly different ($p < 0.05$). The phytoplankton net growth rate in the ambient treatment was virtually zero (–0.005 $d^{-1}$), was lower in the high $pCO_2$ treatment (–0.512 $d^{-1}$), lower still in the high temperature treatment (–0.88 $d^{-1}$), and lowest in the greenhouse treatment (–1.4 $d^{-1}$).

### Microzooplankton abundance

Total microzooplankton abundance increased in all treatments over the course of the experiment (Fig. 2). The 2 low temperature treatments (ambient and high $pCO_2$) showed a similar pattern to each other, with modest increases in total microzooplankton abundance between Days 0 and 9 and an order of magnitude increase between Days 9 and 14. Total microzooplankton abundance was highest in the 2 low temperature treatments on the final day of the experiment. Total microzooplankton abundance in the high temperature treatment followed a similar but less pronounced pattern, increasing between Days 0 and 9 to 4-fold the initial abundance, then peaking and declining between Days 9 and 14 to 3-fold the initial abundance. Total microzooplankton abundance in the greenhouse treatment increased over an order of magnitude from Days 0 to 9, then peaked and declined between Days 9 and 14, ending at 5-fold the initial abundance. There was no significant effect of $CO_2$ at either temperature at the end of the experiment ($p < 0.05$). There were significant differences between the ambient versus high temperature, ambient versus greenhouse, and high $pCO_2$ versus greenhouse treatments on the final day of the experiment ($p < 0.05$).

Table 1. Phytoplankton growth and mortality rates for the initial phytoplankton community (T0) and the 4 experimental treatments (see 'Materials and methods: Experimental design' for details) on Day 8 of the 14 d experiment. $\mu_n$: phytoplankton growth rate in bottles amended with nutrients; $\mu_0$: phytoplankton growth rate in bottles not amended with nutrients; $m$: mortality rate; $r^2$: regression coefficient

| Treatment | $\mu_n$ ($d^{-1}$) | $\mu_0$ ($d^{-1}$) | $m$ ($d^{-1}$) | $r^2$ |
|---|---|---|---|---|
| T0 | 0.17 | 0.17 | 0.65 | 0.50 |
| Ambient | 0.33 | –0.005 | 0.49 | 0.80 |
| High $pCO_2$ | –0.31 | –0.51 | 0.86 | 0.96 |
| High temperature | –0.16 | –0.88 | 0.84 | 0.97 |
| Greenhouse | –0.56 | –1.38 | 1.1 | 0.97 |

### Microzooplankton community composition

Microzooplankton community composition was described using a combination of multivariate statistical techniques (Fig. 3) and by genera (Figs. 4 to 6). Fourteen taxa of microzooplankton were identified to the genus level when possible, and enumerated over the course of the experiment. A MDS plot was used to compare assemblages on the 4 different days sampled (Fig. 3A). MDS plots of samples were also used to visualize differences in microzooplankton community composition among treatments on individual days of the experiment (Fig. 3B to D).

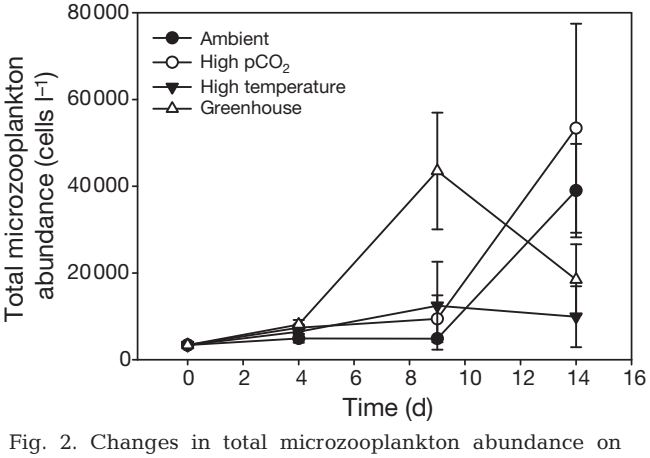


Fig. 2. Changes in total microzooplankton abundance on Days 0, 4, 9 and 14 of the Ecostat experiment. Error bars represent 1 SD from the mean. See 'Materials and methods: Experimental design' for treatment details

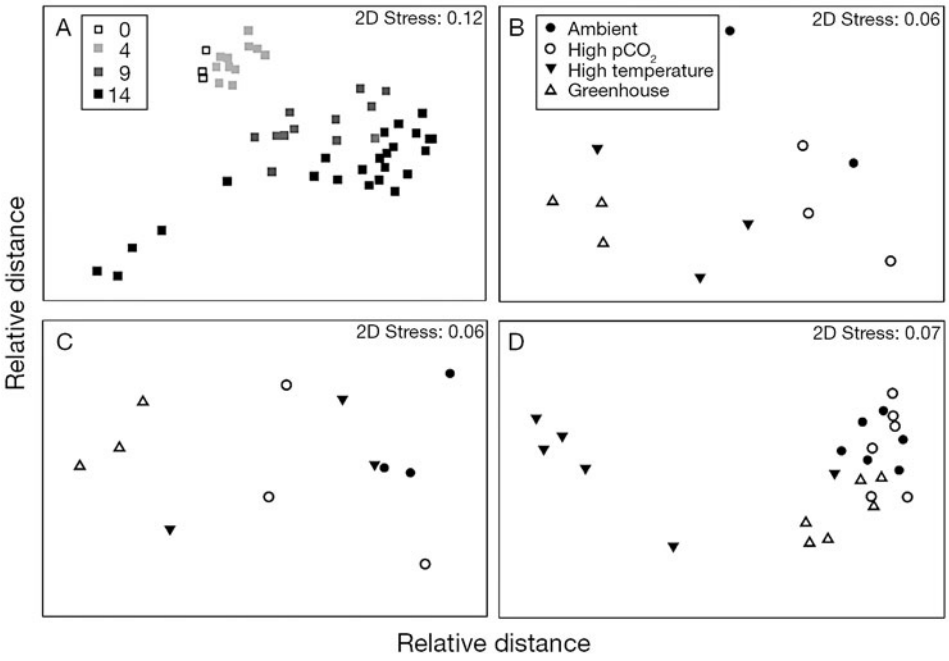


Fig. 3. Similarities among microzooplankton assemblages plotted using non-metric multidimensional scaling (MDS) with square-root transformation and a Bray-Curtis resemblance matrix. Relative distances between samples are illustrated and, thus, the axes are without absolute scales. (A) MDS plot showing relative differences between assemblages on all 4 sampling days. MDS plots for (B) Day 4, (C) Day 9, and (D) Day 14 (of the 14 d Ecostat experiment) are also shown for the 4 treatments

We observed a statistically significant degree of similarity between microzooplankton communities grouped according to incubation temperature on all sampling days, while significant similarity of microzooplankton communities grouped according to $pCO_2$ concentration was only observed on the final day (Fig. 3D). Significant grouping of microzooplankton communities according to incubation temperature was observed on Days 4 and 9, with no significant grouping of microzooplankton community structure based on $pCO_2$ concentration on these days ($p = 0.001$ and 0.003 for temperature and $p = 0.14$ and 0.06 for $pCO_2$ concentration on Days 4 and 9, respectively; Fig. 3B,C). On Day 14, the microzooplankton communities were significantly grouped based on temperature at both $pCO_2$ concentrations; they could also be grouped based on $pCO_2$ concentration, but only at high temperature (Fig. 3D). The ANOSIM test yielded significant differences between all pairs of treatments on Day 14, except for the ambient and high $pCO_2$ treatments (ambient vs. high $pCO_2$, $p = 0.83$; all other treatments, $p < 0.02$).

The initial microzooplankton community was relatively diverse, with 13 taxa represented and no single genus dominating. One day after the pumps were turned on (T4), the microzooplankton assemblages in all 4 treatments were similar to the initial community (Figs. 3A & 4). The assemblages were numerically dominated by *Gyrodinium* sp., a heterotrophic dinoflagellate, with substantial numbers of *Lohmaniella* sp., a small oligotrichous ciliate, also present. The assemblages were still relatively diverse at this time point. By contrast, the T9 and final day microzooplankton communities were less diverse, being dominated by small taxa, with differences in the microzooplankton community being largely driven by changes in the relative abundance of 4 genera (Figs. 5 & 6). Both low temperature treatments (ambient and high $pCO_2$) were dominated on T9 and T14 by *Lohmanniella* sp., and also had relatively high numbers of a small species of the dinoflagellate genus *Protoperidinium*. In contrast, the high temperature treatment was numerically dominated by a large species of the ciliate genus *Strombidium* that grew from relatively low abundance on Day 9 to relatively high abundance on Day 14. *Lohmanniella* sp. and the same small species of *Protoperidinium* sp. observed in the 2 low temperature treatments were still present in this treatment by the end of the experiment, but at relatively low abundance. On Day 9, the community in the high temperature treatment was dominated by the same *Lohmanniella* sp. and *Protoperidinium* sp. that were observed in the other treatments (Fig. 5), but these numbers declined sharply (along with most of the phytoplankton numbers) as the large *Strombidium* sp. came to numerical dominance

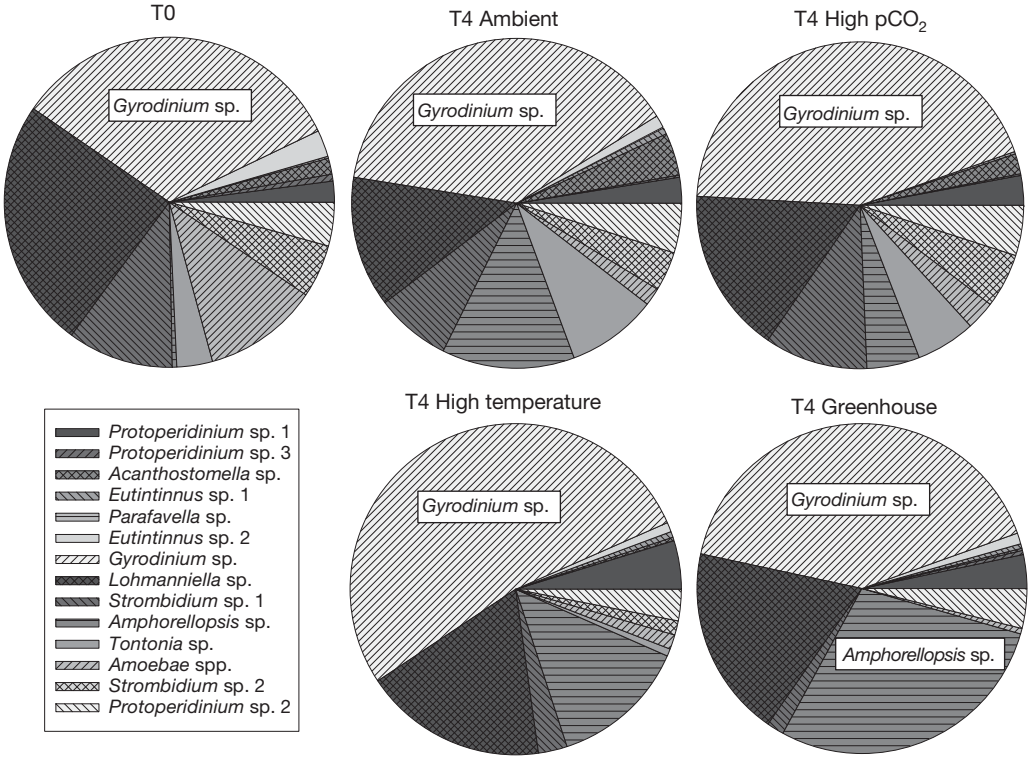


Fig. 4. Microzooplankton assemblage composition (at the genus level) on Days 0 (T0) and 4 (T4) of the Ecostat experiment, according to treatment (see 'Materials and methods: Experiment design' for details). Each genus is plotted as a fraction of the entire assemblage at that time point

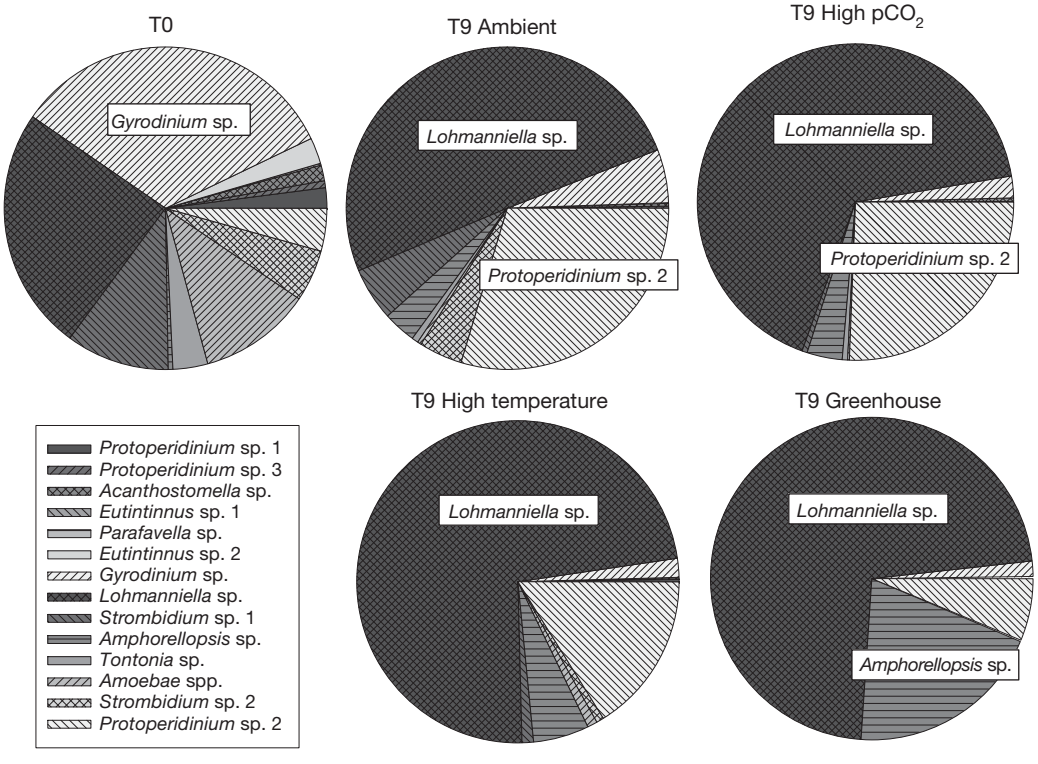


Fig. 5. Microzooplankton assemblage composition on Days 0 (T0) and 9 (T9) of the experiment. Other details as in Fig. 4

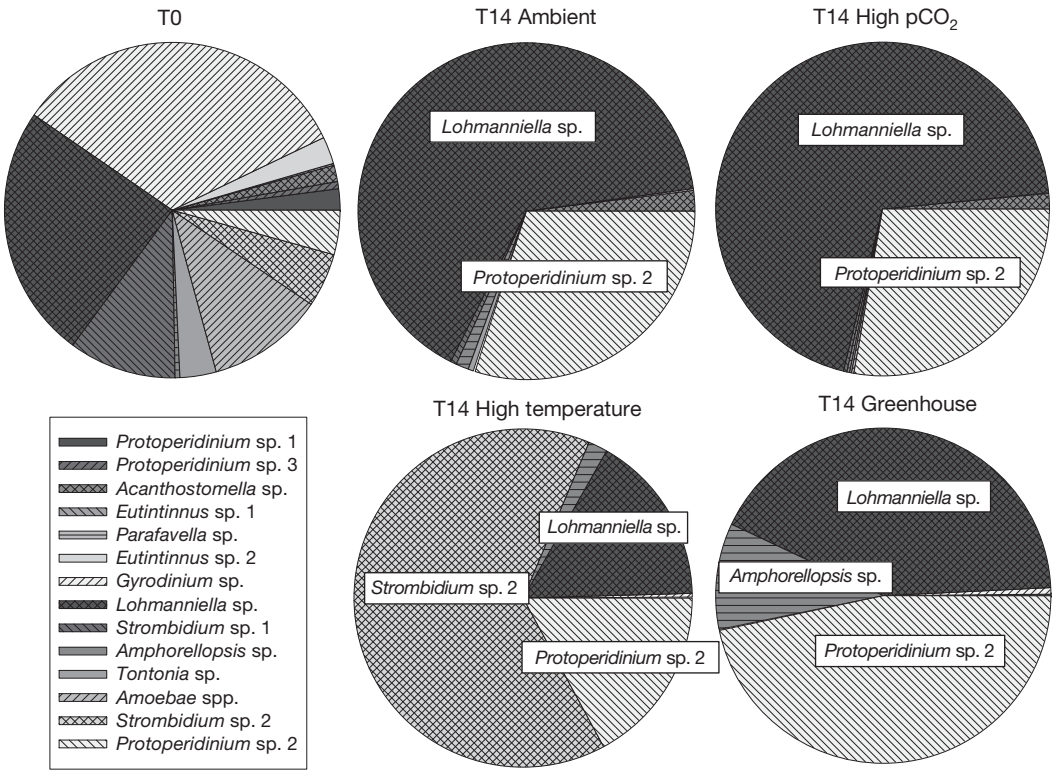


Fig. 6. Microzooplankton assemblage composition on Day 0 (T0) and Day 14 (T14), the final day for each treatment of the experiment. Others details as in Fig. 4

at the end of the experiment. This *Strombidium* sp. did not increase in numbers in the greenhouse treatment. The small *Protoperidinium* sp. became numerically dominant in the greenhouse treatment between Days 9 and 14, and the *Lohmanniella* sp. that dominated on Day 9 was the second most abundant genus by Day 14, along with substantial numbers of the tintinnid ciliate *Amphorellopsis*, which was not observed in great abundance in any of the other 3 treatments.

## DISCUSSION

Increasing temperature and $pCO_2$ alone and in concert led to significant changes in phytoplankton and microzooplankton abundance and community composition in the Ecostat experiment. These results were due to inter-related effects on multiple trophic levels within the experiment and included a combination of direct effects on the phytoplankton community, direct and indirect effects on the microzooplankton community, and changes in top-down controls on the phytoplankton community. When temperature and $pCO_2$ were increased together in the treatment simulating projected year 2100 climate conditions, the results were significantly different than those in the single-variable treatments. The plankton assemblage was dynamic in both abundance as well as community composition throughout the entire experiment, and shorter, more traditional batch-style experiments would likely not have run long enough to observe the large changes displayed during the last 5 d of the experiment.

Phytoplankton mortality rates were relatively high at the onset and during the Ecostat experiment. The significant grazing pressure that we observed is consistent with the results of previous work within the JGOFS and BOFS studies of prymnesiophyte-dominated phytoplankton communities in the North Atlantic spring bloom (Burkill et al. 1993, Verity et al. 1993b, Fileman & Leakey 2005). These studies and others include reports of microzooplankton dominance of total phytoplankton herbivory and microzooplankton consuming significant portions of phytoplankton daily production in the North Atlantic (Gifford et al. 1995, Stelfox-Widdicombe et al. 2000).

Increases in microzooplankton abundance and grazing mortality by Day 8 of the experiment in the 2 high temperature treatments were consistent with the known effects of increased temperature on protistan

physiological rate processes (Eppley 1972, Rose & Caron 2007). We also observed increases in both the abundance and grazing rates of microzooplankton within the 2 high $pCO_2$ treatments relative to ambient $pCO_2$ treatments. If the dominant microzooplankton were mixotrophs and able to fix carbon, they may have directly benefited from increased $pCO_2$ availability. Unfortunately, we do not know the extent of mixotrophy in the microzooplankton assemblage. If the microzooplankton present were obligate heterotrophs, they could not have directly benefited from increased $CO_2$ availability. While changes in pH also accompany increased $pCO_2$ concentration, we deliberately chose to increase $pCO_2$ concentrations through bubbling rather than the addition of acid to more realistically simulate effects of increased atmospheric $CO_2$ on pH and avoid artificial perturbations of total alkalinity. The minor changes in pH associated with the experimental treatments should not have been enough to depress growth rates or inhibit physiological functioning of the several common taxa, based on previous results of pH changes on laboratory cultures of microzooplankton and mixed plankton assemblages (Pedersen & Hansen 2003a, b, Weisse & Stadler 2006). We suggest that changes in pH in the high $pCO_2$ treatments were likely not enough to explain the differences observed in microzooplankton abundance and grazing rates.

Increases in microzooplankton growth and grazing rates in the high $pCO_2$ treatments could have also been related to increased prey availability and/or changes in prey species composition. Prey availability (estimated daily, based on chl *a* concentration and nano- and picophytoplankton abundance) was significantly higher in the greenhouse than high temperature treatment by the end of batch phase. The increased microzooplankton physiological rates at the higher temperature (as discussed in the previous paragraph) may have allowed microzooplankton grazers to take advantage of an increased prey encounter rate. Microzooplankton grazing rates are often well-correlated with standing stocks of algal prey (Calbet & Landry 2004). Shifts in phytoplankton community composition resulting from higher $pCO_2$ effects towards prey of better quality may have favored microzooplankton growth as well. This explanation is also consistent with the results of a recent mesocosm study of $pCO_2$ effects on microzooplankton grazing off the coast of Norway (Suffrian et al. 2008). These authors reported no effect of increased $pCO_2$ on rates of microzooplankton herbivory, yet also reported low grazing rates in general and no major differences in either phytoplankton growth or microzooplankton community composition among $pCO_2$ treatments. Their results suggest $pCO_2$ did not have a significant direct effect on the microzooplankton community and indirectly support our hypothesis that interactions with the phytoplankton community may have been more important in dictating microzooplankton dynamics than the direct effects of increased $pCO_2$.

Initial total microzooplankton abundances were relatively low when compared to reports for early summer in this region (Verity et al. 1993b, Fileman & Leakey 2005), although the peak abundances observed in our 4 treatments were similar to the maximal levels previously reported in the North Atlantic (Burkill et al. 1993, Stelfox-Widdicombe et al. 2000). The initial microzooplankton community composition was diverse. The taxa observed in the present study were similar to the mix of aloricate ciliates, tintinnids, and heterotrophic dinoflagellates described in several previous studies, and the ciliates and dinoflagellates that grew to high abundance (i.e. *Lohmanniella* sp., *Strombidium* sp., *Protoperidinium* sp.) have been reported to occur at high abundance in this region (e.g. Verity et al. 1993a, Stoecker et al. 1994, Fileman & Leakey 2005).

The microzooplankton community composition at the end of the experiment was significantly related to temperature and $pCO_2$ concentration. In general, significant differences in community composition between temperature treatments were more frequently observed. Our results for temperature effects on community composition were consistent with a recent mesocosm study of winter warming in the Baltic Sea by Aberle et al. (2006). We observed a similar shift at high temperature in the microzooplankton community to large oligotrichous ciliates (*Strobilidium caudatum* in their study, *Strombidium* sp. observed here) and a numerical dominance by small ciliates at low temperature (*Lohmanniella* sp. in both studies) (Fig. 6). We observed no relationship between community composition and $pCO_2$ at low temperature, but a significant grouping of microzooplankton communities based on $pCO_2$ at high temperature on the final day (greenhouse treatment; Fig. 3D), with dinoflagellate growth favored more in the greenhouse treatment than in the other 3 treatments (Fig. 6). Both temperature and $pCO_2$ had significant effects on phytoplankton community composition and abundance (Feng et al. 2009). Temperature was more likely to have a significant direct effect on the microzooplankton community than $pCO_2$, as discussed above; however, there were likely also significant indirect effects of phytoplankton community composition changes on the microzooplankton community composition. It was also clear that interactions between $pCO_2$ concentration and temperature had significant direct or indirect effects on microzooplankton community composition. These findings thus highlight the importance of examining interactions among trophic levels in experiments with climate vari-

ables and also the need for multivariate experiments to identify interactions between climate change variables that would not be apparent from single-variable studies.

While total microzooplankton abundance was highest in the greenhouse treatment mid-experiment, on the final day of the experiment, it was the 2 low temperature treatments that had the highest total microzooplankton abundance (Fig. 2). The microzooplankton communities in these treatments were numerically dominated by a small ciliate (*Lohmanniella* sp. 15 to 20 µm in diameter). A decrease in the numbers of picophytoplankton in the same treatments to one-quarter of their peak abundance by the end of the experiment was also observed. Ciliates at the smallest end of the microzooplankton size range have been reported to preferentially graze on picophytoplankton over nanophytoplankton (Rassoulzadegan et al. 1988, Kivi & Setala 1995, Christaki et al. 1998). We speculate that the peak in total microzooplankton abundance in the 2 low temperature treatments in the last few days of the experiment was fueled by the consumption of the smallest phytoplankton size fraction. Total chl *a* was dominated by phytoplankton in the nano- and microplankton size classes (Feng et al. 2009), and the grazing pressure applied by these micrograzers to the picophytoplankton likely did not strongly affect production by the larger phytoplankton.

Total microzooplankton abundance in the greenhouse treatment declined after peaking on Day 9 (Fig. 2). Low final microzooplankton numbers in this treatment were not due to large species composition shifts. The 200 µm pre-screening of the initial community effectively removed mesozooplankton predators. Mesozooplankton were not observed in any of the microzooplankton counts on the final day of the experiment, suggesting the decline in microzooplankton was not caused by increased top-down pressure (data not shown). In addition, the decease in total microzooplankton abundance coincided with an increase in total chl *a*, nano- and picophytoplankton abundance (Feng et al. 2009; Fig. 1). This inverse relationship between the abundance of phytoplankton and grazers suggests 1 of 2 possibilities. The phytoplankton may have grown in response to reduced grazing pressure from the declining microzooplankton assemblage, without directly causing the decline in microzooplankton abundance. Alternatively, the phytoplankton that grew in spite of the relatively strong grazing pressure observed mid-experiment may have been toxic, noxious, or unpalatable to the dominant microzooplankton taxa, a phenomenon discussed in detail by Irigoien et al. (2005). Some combination of these 2 scenarios is also possible. Unfortunately, we were not able to measure microzooplankton grazing rates on the final day of the Ecostat due to limitations in sample volume. Thus, we were not able to directly determine if grazing was reduced in the greenhouse treatment at the end of the experiment. However, indirect evidence suggests that this may have been the case. The phytoplankton group that dominated the microphytoplankton community at the end of the experiment was a coccolithophore, based on microscopic counts (Feng et al. 2009). Additionally, the pigment data and $DMSP_p$ concentrations from the total phytoplankton community indicate that the phytoplankton community as a whole was also dominated by coccolithophores, suggesting that there was also a strong coccolithophore component to the nanoplankton size class (Feng et al. 2009, Lee et al. 2009). Coccolithophores such as *Emiliania huxleyi* have been hypothesized to produce chemicals reducing microzooplankton grazing rates according to previous field- and laboratory-based studies (Wolfe et al. 1997, Olson & Strom 2002, Strom et al. 2003), which would be consistent with our results. A significant decrease in dissolved dimethylsulfoniopropionate ($DMSP_d$) was noted in the greenhouse treatment relative to controls on the final day of the experiment (Lee et al. 2009). Microzooplankton grazing has been shown to contribute significantly to $DMSP_d$; thus, a decline in $DMSP_d$ in the greenhouse treatment would also be consistent with reduced microzooplankton grazing in this treatment (Lee et al. 2009). Our data suggest that the initial increased grazing pressure observed in the greenhouse treatment may have opened the door for growth of unpalatable phytoplankton taxa, resulting in overall net gains for the phytoplankton community in this treatment.

## CONCLUSIONS

It has been demonstrated in the present study and others that microzooplankton are able to respond rapidly to changes in phytoplankton abundance and community composition in the environment. Microzooplankton in our experiment quickly consumed initial gains made by phytoplankton due to increased temperature and $CO_2$ concentration. While the outcome of bottle incubation experiments is always dependent on the composition of the initial plankton communities, our results were consistent with those of Aberle et al. (2006). These authors demonstrated a stimulation of microzooplankton growth (and a shift in the microzooplankton community towards larger ciliates of the genus *Strombidium*) by increased temperature alone in a set of mesocosm experiments. This suggests that our observations may reflect a more general effect of temperature on microzooplankton abundance and community composition.

Aberle et al. (2006) reported no evidence of a bloom of noxious phytoplankton in their experiments. However, the combination of increasing both temperature and $CO_2$ concentration in our Ecostat experiment may have resulted in the unexpected growth of potentially unpalatable phytoplankton at the end of the experiment. If so, this result suggests that the interactive effect of these variables may have caused changes in phytoplankton bloom composition, and the strong grazing pressure observed mid-experiment acted as a positive feedback favoring growth of specific phytoplankton. Temperature and $pCO_2$ directly affected phytoplankton physiology and community composition, while we hypothesize that effects on the microzooplankton community were more likely a combination of direct (through temperature increases) and indirect (due to 'bottom-up' changes in the phytoplankton abundance and community composition) influences. At the same time, our results also suggest the possibility of a 'top-down' feedback loop in the greenhouse treatment, where high grazing pressure mid-experiment could itself have shaped the phytoplankton community composition and final day phytoplankton abundance.

Similar changes in trophic dynamics and whole plankton community composition may also turn out to be a feature of future climate-driven changes in the North Atlantic spring bloom assemblage. Our results suggest that future increases in temperature and $CO_2$ concentration may favor the formation of coccolithophore blooms. If the phytoplankton groups that are favored by these conditions are toxic, noxious, or unpalatable to microzooplankton grazers, reduced microzooplankton grazing could result and carbon export from this annual bloom event could potentially increase. If our results are predictive of future potential changes in the North Atlantic spring bloom, the changes in trophic dynamics we observed could act as an overall negative feedback to atmospheric $CO_2$ concentration.

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