Physiological characterization of the mixotrophic dinoflagellate *Amphidinium carterae*.

by
Emma Tornberg

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 23, 2019
Commencement June 2019

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Abstract approved:_____

Kimberly Halsey

Marine primary production can be modeled and estimated using remotely-observable physiological signatures such as chlorophyll and carbon. Current models are based on strict physiological relationships based on photoautotrophic phytoplankton, and discrepancies between modeled and in situ data may stem from unaccounted-for physiological deviations from photoautotrophy. Mixotrophic phytoplankton can obtain energy from photosynthesis and phagocytosis of prey, and so their expressions of chlorophyll and carbon may deviate significantly from these parameters previously described in photoautotrophs. The physiology of *Amphidinium carterae*, a well-studied mixotroph, was characterized under a range of different growth irradiances and availabilities of dissolved organic carbon and prey. We found that *A. carterae* upregulated its photosynthetic machinery (i.e., chlorophyll *a* and carbon fixation) when growing with dissolved organic carbon and bacterial prey compared to growth on only CO_2. Additionally, the relationship between the chlorophyll *a* to carbon ratio (Chl:C) vs. growth irradiance differed substantially from the modeled Chl:C vs. growth irradiance relationship (Westberry et al. 2008). These results provide evidence that the current approach to modeling primary production fails to capture the heterogeneity of phytoplankton physiology.

Key Words: phytoplankton, mixotrophy, primary production, chlorophyll, carbon

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Emma Tornberg, Author

Characterization of physiological signatures observable from space in mixotrophic dinoflagellate *Amphidinium carterae.*

Emma Tornberg

May 23, 2019

Introduction

Photosynthetic organisms perform the initial introduction of inorganic carbon into organic forms that are useable by other organisms. In doing so, they help mediate global carbon cycles. This conversion of inorganic forms (e.g., CO_2) into organic forms (e.g., glucose) is known as photosynthesis. The amount of CO_2 fixed through photosynthesis is known as primary production and is most often reported as the carbon fixed per area or volume over time. Primary production can be estimated globally using remotely-observable physiological signatures (chlorophyll and carbon), however substantial discrepancies still exist between modeled and in situ data (Behrenfeld et al. 2016). Primary production is affected by a multitude of environmental factors, ranging from light intensity to nutrient status. When studied in culture, many organisms display significant physiological flexibility, as demonstrated by C:N ratios and chlorophyll content that vary depending on nutrient and light availability (Halsey and Jones 2015). It is therefore plausible that discrepancies in modeled and in-situ data are rooted in organism physiology and the complex ways phytoplankton regulate their photosynthetic machinery.

Approximately half of global primary production occurs in the oceans, emphasizing the importance of understanding physiological responses of oceanic photosynthetic organisms (Field et al. 1998). The majority of marine primary production is done by microscopic single celled organisms called phytoplankton. Unlike plants, which have limited flexibility in their chlorophyll levels once developed, phytoplankton can up- or downregulate chlorophyll in response to factors like light nutrient availability (Halsey and Jones 2015). This physiological flexibility means that relationships between remotely-sensed parameters like chlorophyll and carbon and primary production need to be corroborated by laboratory experiments in order to develop accurate models of global primary production, such as the Carbon-Based Production Model (CbPM) in Behrenfeld et al. (2005). Subsequent ecosystem model development has linked some discrepancies between modeled and in situ observations to nutrient deficiency (Westberry et al. 2008), but further exploration of phytoplankton physiology remains necessary. The presence of mixotrophs further complicates studies of physiology. We hypothesize that mixotrophs, or single-celled organisms that exhibit both photoautotrophy and heterotrophy, are responsible for some of the observed discrepancies between production models and direct measurements.

Until recently, it was thought that nearly all marine plankton were either strict photoautotrophs, like diatoms, incapable of phagotrophy, or strict heterotrophs, incapable of photosynthesis (Flynn et al. 2012). Recent work has shown that almost all phytoplankton (and

many heterotrophs) exhibit some degree of mixotrophy—the ability to both photosynthesize and phagocytize (Stoecker et al. 2017). Mixotrophs can be further broken down into groups based on whether or not cells are capable of producing their own photosynthetic machinery (Mitra et al. 2016). Constitutive mixotrophs (CMs), commonly described as "plants that eat," encode and produce their own plastids. Plastids are organelles that contain photosynthetic pigments such as chlorophyll *a* and are the main site of photosynthesis in eukaryotic cells. The group of CMs contains many species that were originally thought of as strict photoautotrophs and includes many organisms responsible for harmful algal blooms (Leles et al 2017). There are also non-constitutive mixotrophs (NCMs) which are cells that acquire their photosynthetic machinery from their prey. This group is further broken down into generalist non-constitutive mixotrophs (GNCMs), which are cells that can acquire their chloroplasts from any prey, and specialist non-constitutive mixotrophs (SNCMs), which are cells that acquire their plastids from specific prey, including through symbiotic relationships with smaller phytoplankton.

Each of these strategies for obtaining photosynthetic energy, or mixotypes, have different environmental implications. For example, evidence from modeling studies show that CMs could more readily dominate an ecosystem as they are able to photosynthesize without the need for prey (Mitra et al. 2016), while NCMs rely on plastid acquisition from prey so are likely less abundant. CMs have also been found to dominate in eutrophic and shallow habitats, and some oligotrophic gyres, and they have been shown to thrive in ecosystems where conditions are poor for both strict phototrophs or strict heterotrophs (Faure et al. 2019). Furthermore, while NCMs are ubiquitous in the open oceans, NCM subgroups have wildly diverging global distributions. (Leles et al. 2017).

With mixotrophs making up about 50% of pigmented biomass in coastal waters and responsible for between 40 and 95% of bacterivory in the euphotic zone, their impact on global marine carbon cycling is substantial (Ghyoot et al. 2017). So far, models of global primary production have been based on photosynthetic physiology of strict photoautotrophs. (Stoecker 2017). Results from ocean system models that include mixotrophs as the key primary producers demonstrate significantly different implications for global ecosystems and the biological carbon pump, which describes movement of carbon into the deep ocean, compared to models that rely on strict autotrophy. For example, primary production was generally enhanced through the process of mixotroph grazing because it increased the availability of inorganic nutrients for the entire mixotrophic community (Mitra et al. 2014). Controlled culturing studies are needed to characterize mixotrophic physiology and provide "ground-truthing" of modeled behaviors. Combining lab-based and modeling approaches is important to determine the impact of mixotrophs on primary production and ecosystem function.

This study investigated physiological parameters of a model mixotroph, *Amphidinium carterae*, in response to light and organic carbon availability. I hypothesized that when grown

under a range of light levels and when given different carbon sources, *A. carterae* would express significantly different chlorophyll to carbon ratios than the ratios expressed by strict photoautotrophs. Mixotrophs can acquire energy by photosynthesizing, taking up dissolved organic carbon from the environment, or preying on cells. Thus, prey availability may influence how much chlorophyll mixotrophs need to produce for growth. Because strict photoautotrophs have less metabolic flexibility than mixotrophs, I also predicted that photoautotrophs require more pigment to achieve the same growth rate as mixotrophs. These differences would mean that current models based on photoautotrophic physiology may need to be amended to account for mixotrophs.

Materials and Methods

Culture Conditions

The dinoflagellate *A. carterae* (CCMP1314) was grown in semi-continuous culture in polycarbonate flasks using f/2 + Si medium with 1mM $NaNO_3$ (Guillard 1975) at continuous light levels of 30 μE (low light, LL), 80 μE (medium light, ML), and 130 μE (high light, HL) at 18°C. Stock cultures were maintained at these light levels for several weeks to allow for acclimation to both irradiance and carbon source. Carbon treatments were: no added organic carbon (CO_2 treatment), dissolved organic carbon in the form of 50 μM glucose as in Carini et al. 2012 (DOC treatment), and the DOC media used in the DOC treatment inoculated with the heterotrophic marine bacteria SAR 92 (HTCC 2207) (BAC treatment). For the BAC treatment, SAR 92 was maintained in semi-continuous culture and fed to *A. carterae* in amounts between 10^2 and 10^4 cells ml^{-1}. This three by three experimental design yielded nine treatments. Each treatment was grown in triplicate flasks at minimum. All treatments were harvested during exponential phase growth to ensure consistent physiological activity that best parallels balanced growth and avoid physiological changed associated with nutrient starvation at high densities.

Cell Properties

To track growth, *A. carterae* concentration was determined using a Multisizer 3 Coulter Counter with a 70 μm aperture. Specific growth rate was used to determine if cultures were still in exponential growth prior to harvesting for other cell properties. Specific growth rate is reported in units of per day (d^{-1}), calculated using the equation below where the subscripted 'f' and 'i' refer to the final and initial cell concentrations, respectively:

$$\frac{\ln concentration_f - \ln concentration_i}{\Delta time}$$

To measure the chlorophyll *a* content per cell, 5 mL of culture was filtered onto Whatman glass fiber filters (GF/F) and then the chlorophyll was extracted using 5mL of 90% acetone. After extraction for 24 hours at -20°C, the absorbance of each sample at 630 nm, 647

nm, 664 nm and 750 nm was measured using a Shimadzu UV-Vis Spectrophotometer. The absorbance at 664 nm corresponds to chlorophyll *a,* 630 nm and 647 nm correct for accessory pigments, and the absorbance at 750 nm corrects for solvent absorption. Measurements were done in triplicate for technical replication as well as biological replicates. Using the absorbance values at each wavelength, the chlorophyll *a* per cell was calculated in accordance with the Ritchie (2006) equation for dinoflagellates.

Cellular carbon was measured by filtering 4, 3, and 2 mL samples of each culture as well as 5mL of flow-through filtrate onto pre-combusted GF/F filters. The flow-through filtrate provides a baseline for the carbon content measurements to account for non-*A. carterae* carbon. Filters were stored at -20°C. For analysis, filters were dried for 24 h at 40°C and analyzed for carbon content using an Exeter Analytical EA1 Elemental Analyzer. Carbon per cell was then determined by dividing the resulting carbon values in µg/mL by cellular concentration.

Primary production

Primary production was measured via radiolabeled carbon uptake. 7 mL of culture was spiked with 0.7 µCi of $NaH^{14}CO_3$. 4 mL of the radiolabeled culture was then incubated at the growth irradiance of the culture, and 3 mL was in the dark (wrapped in foil) to arrest any photosynthetic activity and serve as a negative control. Two 50 µL subsamples of the spiked sample were combined with 50 µL phenethylamine and 900 µL F/2 media to determine the exact radioactive activity in each sample. After 24 hours of incubation at the growth irradiance, each sample was aliquoted in 1 mL amounts, treated with 10% HCL, and allowed to off-gas for 24 hours. Eco-Scint A Scintillation Cocktail was then added to the samples and measured using a Beckman Liquid Scintillation Counter to determine the total uptake of radiolabeled carbon. An additional measure of carbon production (in units of pg $cell^{-1}$ d^{-1}) was calculated by multiplying light-driven growth rate (µ, day^{-1}) by cellular carbon.

All statistical tests and figures were made using R. Two-way ANOVA testing was completed in order to determine the effect of growth irradiance and carbon availability on the measured parameters. Following a significant result from the ANOVA testing ($p < 0.05$), pair-wise t-tests were performed between all growth irradiances and all carbon availability levels.

Results

A. carterae was grown at three different light intensities and in the presence of CO_2 only, DOC, or bacteria. All nine treatments of *A. carterae* showed steady growth, but there were significant differences in cell properties between treatments that are reported below.

Cellular Properties

Cellular chlorophyll *a* content decreased significantly with increasing light availability ($p < 0.001$, Figure 1). Chlorophyll content in cells grown at the two highest growth irradiances significantly differed from chlorophyll content in cells grown at the lowest growth irradiance ($p < 0.05$), but did not differ from each other ($p > 0.1$). Chlorophyll *a* also differed significantly with carbon availability, increasing with greater organic carbon availability ($p < 0.001$). Following pair-wise testing, the only significant difference was between the BAC treatment and the CO_2 treatment ($p < 0.05$), the treatments with the greatest difference in carbon availability. There was approximately a four-fold range of chlorophyll *a* content across treatments, with the lowest observations below 1 pg cell^{-1} and the highest observations close to 4 pg cell^{-1}. The carbon per cell values were relatively consistent across treatments at approximately 100 pg C cell^{-1}. No significant effects from growth irradiance or organic carbon availability were observed ($p > 0.05$, Figure 2).

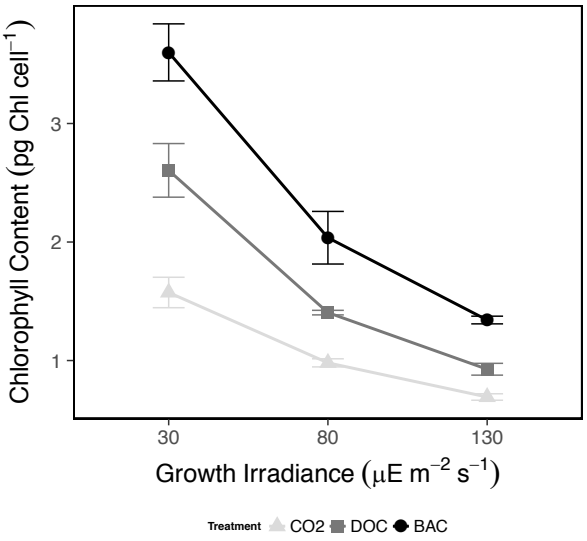

Figure 1: Chlorophyll *a* in pg cell^{-1}. Chlorophyll *a* increased significantly with growth irradiance and carbon availability increases ($p < 0.001$).

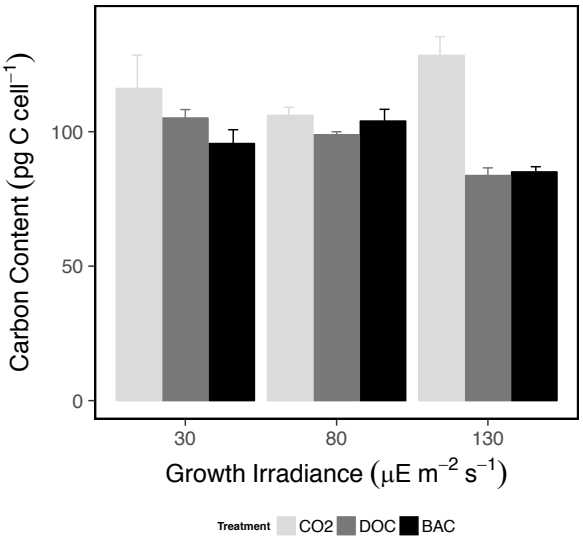

Figure 2: : Carbon content in *A. carterae* determined by filtering culture onto pre-combusted GF/F filters and reported in pg cell^{-1}.

Growth rates were calculated from the linear portion of each growth curve (see Materials and Methods). Growth irradiance significantly affected the growth rate of the cultures ($p < 0.001$), with the two higher growth irradiances differing from the lowest ($p < 0.05$), but not from each other ($p > 0.1$). Growth rate increased significantly with more forms of organic carbon available ($p < 0.001$). The BAC and DOC treatments had consistently faster growth rates than the CO_2 treatment ($p < 0.05$), but the BAC and DOC treatments did not differ significantly from each other ($p > 0.1$). The growth rates ranged from 0.07 ± 0.02 to 0.41 ± 0.02 d^{-1}, close to a ten-fold difference.

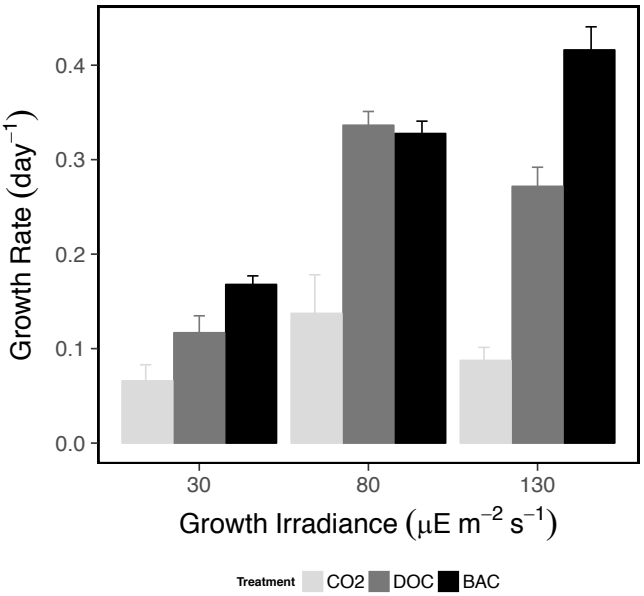

Figure 3: Growth rate differences in *A. carterae* across light and carbon treatments. Both growth irradiance and carbon availability significantly influenced growth rate ($p < 0.001$).

The chlorophyll *a* to carbon ratios across all carbon sources were higher at 30 µE and decreased as the light level increased ($p < 0.001$). Only the highest and lowest growth irradiances were significantly different from one another ($p < 0.05$), and the 80 µE treatment was not significantly different from the either two treatments ($p > 0.1$). Additional access to organic carbon increased the Chl:C ratio ($p < 0.001$), and in paired t-tests only the BAC and CO_2 treatments were significantly different from one another ($p < 0.05$). In all treatments the Chl:C ratios were well below the relationship for Chl:C ratios for the equivalent irradiances and nutrient replete conditions, reported in Westberry et al. (2008) and used to model global primary production (Figure 4).

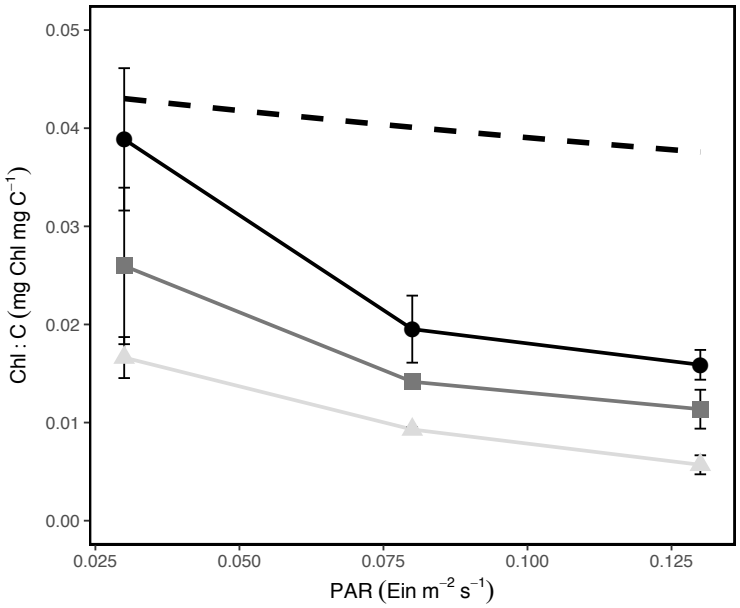

Figure 4: Chlorophyll to Carbon Ratios Across Treatments. Ratio was determined from the Chlorophyll *a* and carbon data shown in previous figures. Lightest grey is CO2 treatment, medium grey is DOC treatment, darkest grey is BAC treatment. Dashed line is modeled chlorophyll *a* to carbon ratio as described in Westberry et al. (2008).

Primary Production

Total production as calculated from growth rate and cellular carbon increased with increasing growth irradiance ($p < 0.001$). Total production was significantly higher at growth irradiances of 80 and 130 µE compared to total production at the lowest light level of 30 µE ($p < 0.005$ and $p < 0.05$, respectively). Additional organic carbon significantly increased the NPP ($p < 0.01$) with both DOC and BAC treatments being significantly higher than the CO2 treatment ($p < 0.05$). Total production varied between 6.19 ± 1.61 and 35.30 ± 2.15 pg C cell^{-1} day $^{-1}$(Figure 5).

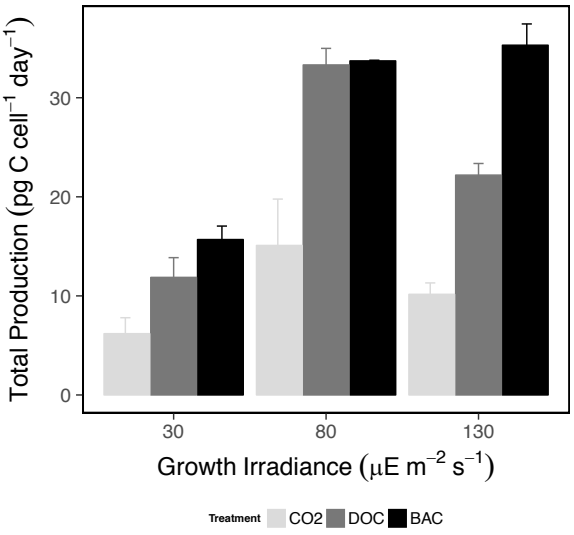

Figure 5: Total production from growth rate and cellular carbon. Both growth irradiance and carbon availability generally increase primary production ($p < 0.001$).

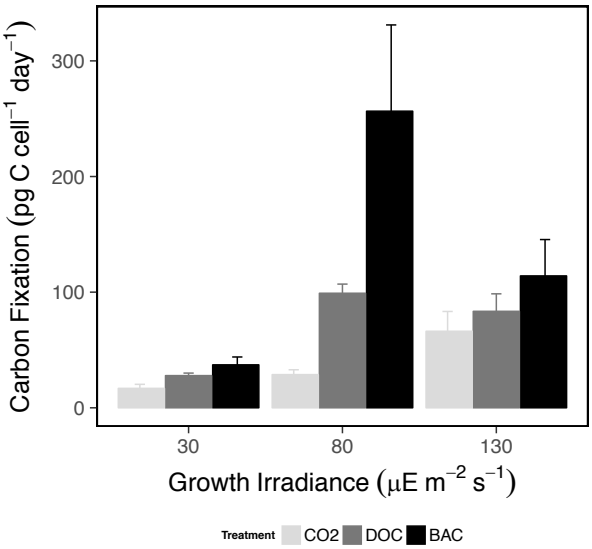

Figure 6: Carbon Fixation as determined by 24 hour ^{14}C Uptake Assays.

Carbon fixation as calculated by ^{14}C uptake assays incubated over 24 h was not significantly impacted by growth irradiance or organic carbon availability ($p > 0.05$). There were, however, visible trends in the data, with carbon fixation rates increasing with carbon availability, and both higher levels of growth irradiance fixing more carbon than the lowest growth irradiance (Figure 6).

Discussion

This research investigated the physiology of a model mixotrophic phytoplankton species *Amphidinium carterae*, in response to light and carbon availability. The collection of cell properties reported here give a new view of mixotrophic behavior. The amount of light and chemical energy available to mixotrophs influences a range of cell properties from light-harvesting to growth. Collectively, these properties also influence their productivity. Importantly, mixotrophs alter their physiology in ways that are different from strict photoautotrophs. These differences have important consequences on estimates of primary production in situ.

Photoautotrophic organisms downregulate chlorophyll in response to higher light availability (Laws and Bannister, 1980). This physiological adjustment balances the light energy captured in the chloroplast reaction centers and the rate at which carbon can be fixed while protecting the cell from taking in more energy than it can utilize (Falkowski and Owens, 1980).

A. carterae also displays this expected trend. Yet, *A. carterae* significantly upregulated chlorophyll content in response to available organic carbon, increasing its production capacity even further. This may be an adaptive response for the organisms to maintain chlorophyll while nutrients are replete, and this chlorophyll pool may act as a safeguard during periods of nutrient or prey limitation. Nitrogen from chlorophyll could be redirected to other cellular functions during nutrient limitation, and while prey is scarce this additional chlorophyll could increase photoautotrophic capability.

Similar to the chlorophyll content, growth rate varied in response to changes in growth irradiance and access to organic carbon. The DOC and BAC cultures had access to more chemical and light energy via increased chlorophyll which likely supplemented their faster growth rates. Cultures with no added carbon were not able to increase chlorophyll levels or carbon fixation rates to a level that would make their productivity equal to the added carbon cultures. At roughly 100 pg cell $^{-1}$, the carbon content was similar to previously established values (Menden-Deuer and Lessard, 2000). Despite differing growth rates, the carbon per cell was fairly consistent across treatments. This is unexpected, as generally slower growing algae are larger (Halsey et al. 2010), but this behavior was not seen in diatoms (Fisher and Halsey 2016) and so may be limited to certain taxonomic groups.

The two measures of production, μ•C and radiolabeled ^{14}C uptake assays, yielded substantially different numerical results for primary production. The value calculated by μ•C takes into account both primary production from photosynthesis and secondary production from phagocytosis and DOM incorporation. The product of growth rate and cellular carbon does not account for non-biomass organic carbon that could have been fixed and then excreted by *A. carterae*.

The radiolabeled ^{14}C uptake assays showed a high degree of variability, and therefore no clear conclusions could be made based on those data. The variability may have been due to experimental error. If however, the generally higher values for primary production determined by ^{14}C uptake compared to μ•C are correct even with this imprecision, then about 50% to 85% more carbon is being fixed than is being incorporated into the cells. This excess carbon is then excreted and/or egested via fecal pellets. These results have implications for the fate of carbon fixed through mixotrophy and that becomes available for carbon export. It would be useful to re-assess NPP as measured by ^{14}C uptake to reduce the possible error in these experiments.

A. carterae is relatively cosmopolitan but mainly resides in tropical and temperate waters (Chen et al. 2015). In coastal waters where *A. carterae* is known to bloom, organic matter is relatively plentiful and in all ocean environments (even in oligotrophic gyres) the concentration of bacteria is at least 10^5 cells m^{-1} (Calbert et al. 2001). In situ, *A. carterae* would rarely encounter a complete lack of organic carbon as was controlled in the CO_2 treatment. The

availability of organic carbon thus suggests that *A. carterae* did not evolve to be completely autotrophic. The complete lack of dissolved organic matter or prey could therefore be suboptimal for *A. carterae* and would explain the consistently lower growth rate, NPP and chlorophyll *a* across growth irradiances in cells grown on CO_2 alone compared to when grown in the presence of organic carbon sources. It is possible the DOC and BAC treatments would be more representative of the physiology of *A. carterae* in the environment. *A. carterae* and other constitutive mixotrophs are thought to be facultative mixotrophs (Stoecker et al. 2017), and thus they are considered to be more dependent on photoautotrophy than on heterotrophy. In this study however, *A. carterae* grew significantly better with access to diverse forms of organic carbon. Future experiments that test whether mixotrophs can grow under strictly heterotrophic conditions could help determine preferred energy acquisition strategies.

Given the patterns of significant differences between treatments, it appears that a more nuanced picture of carbon, chlorophyll, and primary production as driven by growth irradiance could be found by studying levels between 30 µE and 80 µE, as the difference between 80 µE and 130 µE was often not significant. Previous work had shown photoinhibtion in *A. carterae* at levels lower than those of other phytoplankton, (Samuelsson and Richardson 1982), so exploration of physiology below 30 µE could be warranted as well.

Likewise, the differences in chlorophyll *a,* growth rate, and NPP between the DOC and BAC treatments were often not significant. Cultures were grown in the presence of bacteria at concentrations of 10^2 or 10^3 per ml. Given that the concentration of bacteria in seawater is around 10^5 or 10^6 cells ml^{-1} (Li 1998) It is possible that higher bacterial concentrations would promote larger differences in pigment and growth. On the other hand, the DOC concentration (50 µM Glucose) may have been sufficient to make additional phagocytosis either unnecessary or comparably more costly than pinocytosis. *A. carterae* has been shown to both phagocytize and pinocytize regularly (Bronk et al. 2007), so preferential pinocytosis may have decreased the effect of bacterial phagocytosis on *A. carterae* physiology.

Chlorophyll was upregulated with more organic carbon availability and lower light, however all treatments fell below the modeled Chl:C relationship with growth irradiance (Westberry et al. 2008). Previously, values that fell below the modeled maximum Chl:C were hypothesized to be due to nutrient limitation (Westberry et al. 2008, Halsey et al. 2010). Since nutrient limitation was not an experimental factor, this departure from the model is likely caused by the unique physiology of the mixotroph, *A. carterae*. With this being said, aspects of our experimental design such as constant light and low bacterial concentration do not perfectly replicate in situ conditions, so further exploration of physiology remains necessary. If the physiology of *A. carterae* is representative of other constitutive mixotrophs, it is possible high concentrations of mixotrophs in natural communities could cause errors in estimates of NPP based on Chl:C ratios. Further study of the mechanisms underlying these differences is warranted

given the scope and impact of mixotrophs on global carbon cycling (Ghyoot et al. 2017). Additionally, the Chl:C ratios of mixotrophs in relation to their light-driven growth rate must be experimentally determined in order to be incorporated into models. The physiological differences between *A. carterae* and the expected physiology for strict photoautotrophs merits further exploration into mixotrophs physiology.

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