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LINKING PROTISTAN HERBIVORY
TO ENVIRONMENTAL AND BIOTIC CONTROLS

BY

FRANÇOISE MORISON

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

OCEANOGRAPHY

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2016

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

Phagotrophic protists have been established as the major consumers of ocean primary production and as such occupy a pivotal position in pelagic food webs, yet knowledge gaps remain regarding the seasonal and spatial variability of protistan grazing, and of its drivers, both environmental and biotic. The aim of the present research was to address such gaps. To do so, I gathered field measurements and observations and evaluated modifications and alternatives to current methods used to estimate grazing rates and characterize plankton communities. In a study conducted in the Western Antarctic Peninsula aimed at quantifying the seasonal variability of protistan herbivory (Chapter 2), the magnitude of grazing rates measured during austral fall 2013 and austral spring 2014 did not vary with prey biomass. Despite contrasting levels of phytoplankton biomass assessed by chlorophyll *a* measurements ($< 0.4 \mu\text{g L}^{-1}$ in the fall, up to $18.5 \mu\text{g L}^{-1}$ in the spring), grazing rates measured during austral fall ($0\text{-}0.26 \text{ d}^{-1}$) were as high or higher than rates measured during austral spring ($0\text{-}0.1 \text{ d}^{-1}$), and approximately half of the experiments in both seasons yielded no measurable grazing. Overall low grazing rates could not be explained by a lack of predators. Small cells dominated the austral fall phytoplankton community, and during austral spring, grazing was detected when the prey size-structure resembled fall conditions most, suggesting an association between detectable grazing and the dominance of small cells. These results indicate a lack of predators' functional response in the WAP, which is contrary to the assumption made when describing zooplankton grazing in models. Instead, plankton population dynamics and ultimately phytoplankton biomass accumulation rates in the WAP region may be best

predicted as a function of plankton community composition, emphasizing the importance of characterizing these communities concurrently while measuring rates of protistan herbivory. Results also underline the need to extend measurements for the global ocean to less productive seasons in order to verify whether the assumed enhancing effect of prey abundance on grazing rates is always observed in the field. Quantifying the variability of protistan grazing requires increasing the sampling resolution of grazing rate measurements, which is currently precluded by the sampling logistics associated with the standard multiple-dilution technique used to quantify grazing rates. In Chapter 3, I assessed an abbreviated version of the method that uses only two dilutions. I found that rate-estimates for either phytoplankton growth or grazer-induced mortality obtained using only two dilution levels did not substantially deviate from those obtained when using multiple dilutions, and that their accuracy was satisfactory and similar in magnitude to the inherent error associated with the dilution-series estimates ($\pm \sim 0.1 \text{ d}^{-1}$), supporting the usefulness of the abbreviated method. Routine characterization of phytoplankton communities in terms of their size structure and overall taxonomic composition is needed to decipher patterns of association between these characteristics and the level of grazing. In Chapter 4, I showed that a qualitative characterization of plankton populations could rapidly be achieved using the FlowCAM, an automated plankton imaging system. Expanding the spatial and temporal resolution of protistan grazing rate measurements and further investigating the factors influencing grazing magnitude, including plankton species composition, is essential to provide reliable parameters for plankton models, and to underpin the importance of phagotrophic protists in pelagic food webs.

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DEDICATION

For Sophie,

In hope that it will inspire her to be all she wants to be, and that my work will carve a special place in her heart for our great big ocean and all the wonderful living things in it.

PREFACE

This doctoral dissertation is presented in manuscript format, and is subdivided into 5 chapters. Chapter one is a general introduction describing the motivation for the research and its contribution to the understanding of the dynamics driving food web interactions among planktonic organisms. Chapter two is entitled “Seasonal similarity in rates of protistan herbivory in fjords along the Western Antarctic Peninsula”, which has been prepared for submission to the journal *Marine Ecology Progress Series*. Chapter three is entitled “Doing more with less? A cost-benefit analysis of sampling effort vs. data quality in protistan grazing-rate measurements”, which has been submitted for publication to the journal *Limnology and Oceanography Methods* and is in review. Chapter four is entitled “Evaluating FlowCAM to characterize phytoplankton communities, with examples from Narragansett Bay” and is being considered for submission to the *Journal of Plankton Research*. Chapter five serves as a reflection on the accomplished work and consideration of future work needed to address remaining knowledge gaps.

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CHAPTER ONE

GENERAL INTRODUCTION

Role of herbivorous protists in marine pelagic ecosystems

Marine phytoplankton generate half the organic matter that is produced on earth (Field et al. 1998), and in the process fuel marine food webs, regulate the ocean-atmosphere exchange of gases, and drive global climate cycles (Longhurst 1991; Falkowski et al. 1998). Predation is the major fate of primary production (Banse 2013) and modulates its dynamics. The most quantitatively significant phytoplankton mortality-losses are due to grazing by herbivorous protists (HP), a group of unicellular organisms often dominated by ubiquitous phagotrophic ciliates and flagellates (Smetacek 1981; Calbet and Landry 2004). HP are highly diverse, not only phylogenetically but also in size and feeding strategies (Sieburth and Smetacek 1978; Sherr and Sherr 2002; Caron et al. 2012), the latter allowing them access to a broad size-range of prey, from bacteria (Sherr and Sherr 2002; Worden and Binder 2003) to large diatom chains (Sherr et al. 2013). Many also function as mixotrophs (Flynn et al. 2013), often maintaining their algal prey or acquiring the prey's plastids (Stoecker et al. 2009). HP grow at rates similar to their prey, allowing their numbers to increase quickly after an increase in available prey (Sherr et al. 2003).

As active herbivores, protist grazers exert a significant influence on the species composition, size structure, and abundance of phytoplankton (Verity 1986; Strom et al. 2007; Mariani et al. 2013), thereby affecting primary production (Banse 2013) and the flow of carbon in the ocean (Legendre and Le Fevre 1995; Legendre and Rassoulzadegan

1996; Calbet and Landry 2004). Key agents of geochemical cycles (Buitenhuis 2010) and important constituents of the microbial loop (Azam 1983), HP also are prey to meso- and macro-zooplankton (Schmidt et al. 2006; Saiz and Calbet 2011) and thus can act as an important trophic link channeling otherwise unavailable primary production up the food web.

Thus knowledge of the grazing activity of these microbial predators and quantification of their feeding rates is essential to understanding trophic linkages and biogeochemical cycles. The need is made more pressing due to climate-driven changes in ocean conditions, with likely but uncertain impact on plankton communities and food webs, and on the export and sequestration of carbon and feedback on climate (Falkowski and Oliver 2007; Caron and Hutchins 2012; Winder and Sommer 2012).

Importance of protistan herbivory in Antarctic waters

Early models represented the Antarctic food chain as a simple system dominated by krill efficiently transferring diatom-dominated primary production to whales and other megafauna, and until the 1980's, the abundance, distribution, and trophic role of HP were largely unknown (Garrison 1991). The importance of diatoms in Antarctic waters was re-evaluated during an expedition that circumnavigated the Antarctic continent (Hewes et al. 1985). Extensive spatial sampling revealed that nano-phytoplankton ($<20\ \mu\text{m}$) often accounted for $> 50\%$ of total chlorophyll and provided preliminary empirical evidence that nano-phytoplankton biomass may be controlled by HP's grazing activity (Hewes et

al. 1985). As a result, the Antarctic food chain concept was revised to emphasize the importance of nano-plankton and the microbial loop (Azam et al. 1991).

Antarctic HP assemblages are often dominated by ciliates and athecate dinoflagellates (Burkill et al. 1995; Calbet et al. 2005; Garzio and Steinberg 2013). HP in Antarctic waters can be as abundant as at lower latitudes although their distribution can be patchy (Garrison 1991; Landry et al. 2002; Garzio and Steinberg 2013), and their biomass maxima sometimes occur at intermediate depths within the mixed layer (Burkill et al. 1995; Umani et al. 1998; Calbet et al. 2005).

HP grazing rates vary among studies and locations. Absence or low rates of grazing are often reported, in varying proportions of total experiments performed (14-75 %) within single studies (Tsuda & Kawaguchi 1997; Caron et al. 2000; Pearce et al. 2008), and have been attributed to the consistently cold Antarctic water temperatures (Caron et al. 2000). Due to a bias towards summer sampling (e.g. table 2 in Garzio et al. 2013), little is known about seasonal variability. Indeed few studies (Caron et al. 2000, Pearce et al. 2008) have provided the seasonal data necessary to assess the potential role of HP in the yearly cycles of primary production, and to build a year-round baseline of process-rates against which potential climate-driven changes may be detected.

Indeed, among all the regions of the world, perhaps the most vulnerable to changes in climate is Antarctica. In particular, during the latter half of the 20th century, the Western Antarctic Peninsula (WAP) underwent the most rapid warming on Earth (Turner et al. 2005). Despite evidence that the pace of warming has slowed, and that cooling has even occurred in the last two decades (Turner et al. 2016), data collected

from six coastal stations show that the Antarctic Peninsula is warmer now than it was at the start of temperature records (Fig. 1 in Turner et al. 2016). The overall warming trend has been accompanied by significant increase in ocean water temperature west of the Peninsula, as well as region-specific changes in the annual and inter-annual variations in sea ice (Ducklow 2013), which Antarctic organisms, from phytoplankton to seabirds, depend upon for their life cycles, distributions, and population dynamics (Ross et al. 2008, Vernet et al. 2008, Chapman 2004). Concerns that the warming trend will alter the WAP coastal food web (Moline et al. 2004) have prompted large efforts to understand its components' dynamics. The WAP has seen a recent southward shift in the alongshore distribution of phytoplankton biomass, including an increase in diatoms in the southern sub-region (Montes Hugo et al. 2009), whereas cryptophytes increasingly dominate primary production in the northern sub-region (Garibotti et al. 2003). These changes in plankton community composition may alter food web interactions through changes in prey availability and/or total production.

Although in the past 25 years the WAP ecosystem has been studied extensively through the Palmer Long Term Ecological Research (LTER) program (Ducklow et al. 2007), the role of HP in the WAP had been largely neglected, until an extensive first study of the WAP LTER region was conducted during the summer productive season (Garzio et al. 2013). Little is known about HP grazing dynamics in the extensive system of glacio-marine fjords fringing the WAP's western side (Domack & Ishman 1993). These fjords are sites of exchange between the cryosphere and the ocean, and as such are influenced by glacial discharge and may be particularly sensitive to the region's

warming. Despite these embayments being hot spots of benthic biodiversity (Grange and Smith 2013), and locations of occasionally large aggregations of krill and whales (Nowacek et al. 2011, Espinasse et al. 2012), little is known about the structure and function of their ecosystems.

The work presented here quantified grazer-induced mortality and its impact on phytoplankton during two research-cruises performed at two different seasons in the fjords lining the northern part of the WAP. The information contributes to the general understanding of the annual cycle of phytoplankton dynamics in an understudied but potentially important coastal area of the WAP.

The dilution method: dilution series vs. the 2-point method

Since its introduction (Landry & Hassett 1982), the dilution method has become the standard protocol to quantify HP grazing rates, and most of what is known about the role of HP in marine food webs has been obtained through its extensive use (*see* Calbet & Landry 2004). Although the method has facilitated the acquisition of a large data set (Calbet & Landry 2004), its laborious application is also one major impediment to increasing the sampling resolution needed to fill knowledge gaps. Such gaps exist at the geographical, seasonal, and vertical scales (Schmoker et al. 2013), as well as in the empirical investigation of physical, chemical, and/or biological factors driving grazing rate magnitude, hindering predictions about food web responses to climate-driven ocean changes (Caron & Hutchins 2013). Of course geographical gaps can be partly explained by the difficulty to sample vast expanses of ocean. Seasonal gaps, in particular at high

latitudes, partly result from the difficulty of performing work in forbidding winter sea conditions. Nevertheless the logistical effort required by the dilution method to obtain even a single rate measurement precludes acquisition of large sample sizes across the many potentially influencing factors.

In their introduction of the dilution method, Landry & Hassett (1982) suggested that any two dilution levels could be used to estimate grazing rates, providing a “short cut” alternative to the dilution series (later referred to as the 2-point method; Gallegos 1989). Easier and faster to use, the 2-point method offers a capacity to increase rate measurements. It may be quite useful, for example, to investigate the vertical variability of grazing rates through the water column, or the effect of light, temperature, or other climate-related factors on grazing rate magnitude.

Several studies have used the 2-point method (e.g. Landry et al. 1984, 2008, 2009, 2011; Menden-Deuer & Fredrickson 2010). Rates estimated using the 2-point approach are considered conservative (Worden and Binder 2003; Lawrence and Menden-Deuer 2012) and in general do not vary significantly from rates obtained using a linear regression (Worden and Binder 2003; Strom and Fredrickson 2008). Nevertheless, concerns remain regarding the quality of estimates generated by an abbreviated dilution method. The protocols used vary widely among studies, including the dilution level used in the diluted treatment, the number of replicates per dilution, and the method of calculating rates, and there is no general understanding on how these choices may influence the quality of the generated data. Additionally, considering the potential grazers’ functional responses of feeding saturation (Gallegos 1989), it is unclear if the 2-

point method is applicable under any in situ prey abundance, and there is a need for clarifying whether the lowest dilution used for 2-point estimates should be chosen as a function of available prey biomass (i.e. chlorophyll concentration). Therefore, a thorough cost-benefit assessment of the 2-point method is needed so that the method can be more widely trusted and applied. The work presented herein provides an assessment of how accurate 2-point estimates are relative to rates generated using a series of dilutions. More importantly, it provides practitioners with a quantified knowledge of the trade-offs involved in applying a particular protocol.

Importance of species composition and size structure of plankton communities

The ecological and biogeochemical functioning of pelagic ecosystems largely depends on the size structure and taxonomic composition of phytoplankton (Legendre & Le Fevre 1991; Falkowski et al. 1998). Size influences many biological properties of planktonic autotrophs, including growth, respiration, nutrient uptake and other resource acquisition (Finkel et al. 2010), as well as abundance (Irwin et al. 2006), and sinking velocity (Smayda 1970). Herbivory is highly influenced by both the species composition and the size of phytoplankton prey, which determines trophic pathways (Frost 1972; Hansen et al. 1994; Tilmann 2004; Montagnes et al. 2008). Characterization of spatial and temporal fluctuations in the size structure and species composition of phytoplankton is important to understand and predict responses of plankton communities and plankton food webs to environmental changes (Edwards & Richardson 2004; Hays et al. 2005).

Our understanding of plankton processes has been hindered in part by the traditional methods used to monitor phytoplankton communities. In Narragansett Bay (NB), the long running phytoplankton time-series provide invaluable information, but the method used, which relies on microscope particle analysis, presents the disadvantages of being slow, tedious, dependent on analyst expertise (Culverhouse et al. 2003; 2006), and does not resolve ecologically important parameters such as size spectrum and carbon biomass. Microscopy involves analysis of a small volume of seawater (1 mL), which is likely insufficient to characterize phytoplankton diversity (Rodriguez-Ramos et al. 2013).

Several technologies and instruments for the automated counting of plankton organisms have been developed to overcome the limitations of traditional analyses (Benfield et al. 2007). One of these instruments is the FlowCAM, a plankton imaging system that combines flow cytometry and microscopy with a camera (Sieracki et al. 1998). Some of the advantages of the FlowCAM not provided by the presently used method for NB include the ability to derive size spectra, as well as bio-volumes and carbon biomass estimates using recorded cell measurements.

In the work presented in Chapter 4, the FlowCAM was tested for its ability and usefulness to routinely characterize phytoplankton communities, by comparing results of automated and microscopy analyses of phytoplankton samples from Narragansett Bay.

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CHAPTER TWO

This manuscript has been prepared and is being submitted for publication to the journal

Marine Ecology Progress Series

Seasonal similarity in rates of protistan herbivory

In fjords along the Western Antarctic Peninsula

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Running Head: Antarctic seasonal protistan herbivory

ABSTRACT

We quantified phytoplankton growth and herbivorous-protist grazing rates during late austral fall 2013 and late austral spring 2014, in several glacio-marine fjords and connecting channels along the Western Antarctic Peninsula (WAP). To assess the effect of increased temperature on phytoplankton growth and grazing rates, during spring 2014 we also conducted a temperature perturbation experiment for 7 days using a natural plankton assemblage. Austral fall concentrations of chlorophyll-a never exceeded $0.4 \mu\text{g L}^{-1}$, whereas spring chlorophyll-a concentrations indicated a rapid yet patchy development of a phytoplankton bloom, reaching up to $18.5 \mu\text{g L}^{-1}$. During both seasons, rates of protistan grazing were comparatively low, and approx. half of all experiments in both seasons yielded no measurable grazing. Surprisingly, the magnitude of non-zero grazing rates in the fall (0.1 to 0.26 d^{-1}) was larger than during the more productive spring season (0.06 to 0.1 d^{-1}). During spring, positive grazing rates were associated with the dominance of small cells assessed both from fractionated chlorophyll measurements and FlowCAM, while neither predator nor prey biomass were predictive of grazing pressure. Phytoplankton growth rates were higher in the spring (0.06 to 0.93 d^{-1}) than in the fall (-0.01 to 0.19 d^{-1}), resulting in an average accumulation rate that was negative in the fall (-0.02 d^{-1}) but positive in the spring (0.18 d^{-1}). Elevated temperature increased chlorophyll concentration, nutrient limitation of phytoplankton growth, and magnitude of grazing rates. Lack of grazing at ambient temperature may help explain the large blooms that characterize the region during spring and summer. Though there were significant events

of grazer-induced phytoplankton mortality, there was little evidence of seasonal patterns in predation rates.

INTRODUCTION

Phagotrophic protists play critical roles at the base of pelagic food webs (Worden et al. 2015). Their grazing activity represents a primary source of mortality for phytoplankton (Sherr & Sherr 2002, Calbet & Landry 2004). Through grazing, these highly diverse microbial consumers influence primary production by recycling nutrients (Sherr & Sherr 2002) and modulating the phytoplankton community dynamics of abundance, size, and species composition (Verity 1986, Strom et al. 2007, Mariani et al. 2013). As prey to larger zooplankton, they contribute to channeling organic matter to higher trophic levels (Schmidt et al. 2006, Saiz & Calbet 2011). The pivotal position of phagotrophic protists in microbial food webs makes it essential to quantify their grazing impact on primary production, and to constrain factors controlling grazing rates' magnitude, so that we can better understand trophic linkages and biogeochemical cycles.

Studies of protistan herbivory in Antarctic waters have been conducted in various regions of the Southern Ocean (see Table 2 in Garzio et al. 2013). These studies have shown that HP in Antarctic waters can achieve high biomass (Garrison 1991, Caron et al. 2000, Dennett et al. 2001, Calbet et al. 2005), but their distribution can be patchy (Landry et al. 2002, Garzio & Steinberg 2013). HP biomass maxima sometimes occur at intermediate depths within the mixed layer (Burkill et al. 1995, Umani et al. 1998, Calbet et al. 2005). HP assemblages are often dominated by ciliates and athecate dinoflagellates (Burkill et al. 1995, Calbet et al. 2005, Garzio & Steinberg 2013). HP grazing rates reported from Antarctic waters vary among regions. Some studies have shown HP grazing exerting a strong impact on phytoplankton, at times consuming >

100% PP (Tsuda & Kawaguchi 1997, Pearce et al. 2008), whereas others have measured low grazing rates or a low proportion of PP consumed (Burkill et al. 1995, Froneman & Perissinotto 1996, Caron et al. 2000). Understanding of how much protistan grazing rates and grazing impact vary seasonally is limited. To our best knowledge, measurements of protistan herbivory for times of the year outside the productive season only exist for two regions of Antarctica forming unique ecosystems. On the East Antarctic coastline, measurements made throughout the year yielded grazing rates that varied seasonally from averages of 0.3 d^{-1} during austral summer to 0.9 d^{-1} during austral fall, and all but one experiment conducted during the austral spring suggested lack of grazing activity (Pearce et al. 2008). In the Ross Sea, the majority of experiments conducted during four separate cruises covering three different seasons yielded non-significant grazing, and generally low grazing rates irrespective of the season were attributed to the consistently cold Antarctic water temperatures (Caron et al. 2000).

The Western Antarctic Peninsula (WAP) is topographically and climatically distinctive among Antarctic regions (Ducklow et al. 2013). It is also a region where long warming trends have been obscured by decadal variability (Turner et al. 2016). In the latter half of the 20th century, the WAP underwent warming at a rate far exceeding global averages (Turner et al. 2005). The pace of warming in the region has slowed, but despite the cooling that has occurred over the past two decades, there has been an overall warming trend since the beginning of the record (Turner et al. 2016). A coincident rise of sea surface temperatures (Meredith & King 2005) is broadly affecting the biology of the WAP's marine food web, from primary producers (Moline et al. 2004, Montes-Hugo et

al. 2009) to higher trophic levels (Chapman et al. 2004, Ross et al. 2008, Vernet et al. 2008). A first characterization of protistan herbivory within the WAP region has been provided for the productive season, reporting highly variable grazing rates (Garzio et al. 2013). As other coastal regions of Antarctica, PP exhibits extreme seasonal fluctuations, including large spring blooms of diatoms in the seasonal sea ice zone (Ducklow et al. 2013), due to the high dependence of PP on sea ice dynamics (Smith & Nelson 1986, Garibotti et al. 2005). It is likely that large seasonal swings in the abundance, species composition, and nutritional quality of prey at the base of the food web are accompanied by similar seasonal variations in the strength of trophic interactions.

Here we present results of experiments performed to quantify rates of phytoplankton growth and protistan grazing mortality in fjords lining the northern part of the WAP known as the Danco Coast and in the adjacent Gerlache Strait, during two cruises aboard the R/V *Nathaniel B. Palmer*. Fjords are influenced by glacial discharge and may therefore be particularly sensitive to changes in the region's climate (Dierssen et al. 2002). The timing of the cruises created the opportunity to make measurements during two contrasting seasons, once during austral late fall 2013, and a second time during austral late spring 2014. The second cruise provided an opportunity to gain insight into the importance of trophic dynamics on the formation of the austral summer phytoplankton blooms. We also examined the effect of temperature on phytoplankton growth and mortality rates in a temperature perturbation experiment using a plankton community collected in the field.

MATERIALS AND METHODS

Study sites

We quantified rates of phytoplankton growth and protistan-grazing mortality from 17 May – 6 June 2013 and again from 7 – 24 December 2014, in several glacio-marine fjords fringing the WAP between latitudes of 64-65°S and longitudes of 62-63°W (Wilhelmina, Andvord, Paradise, and Flandres Bay). For each bay, we grouped the various locations where sampling took place into sites (Fig. 2.1), with up to three experiments performed at each site (Table 2.1). Other experiments were performed in straits and channels linking the fjords (Fig. 2.1). One additional experiment was performed in 2014 south of the general sampling area (not shown on Fig. 2.1), in Crystal Sound (Lat. 66°54S, Long. 66°51W).

Environmental conditions

At each station, hydrographic data of total depth, temperature, and salinity were collected with a SBE911Plus Seabird Electronics Inc. CTD equipped with sensors of chlorophyll fluorescence (WET Labs AFLT) and PAR (Biospherical Instruments Licor Chelsea). In order to characterize irradiance for the entire duration of each experiment, the ship's continuous data of mast PAR was used, by averaging the 10 % highest PAR values recorded over the daylight hours of the experiments' duration. Mixed layer depth was calculated from a density (σ_t) difference criterion of 0.03 kg m^{-3} , using as reference the near surface (5 m) density values.

Experimental set-up

Rates of phytoplankton growth and protistan grazing-mortality were quantified in a total of 46 experiments. We used the Landry & Hassett dilution method (Landry & Hassett 1982) in 11 out of 20 experiments in 2013, and in 13 out of 26 experiments in 2014, which we further refer to as “dilution series”. The other experiments (9 in 2013, and 11 in 2014) were performed using a 2-point modification of the dilution method (Worden & Binder 2004; Chen 2015; Morison & Menden-Deuer, in review).

Water for the experiments was collected using the CTD rosette sampler from either a depth of 5 m (Fall 2013 dilution series), or from the depth of the CTD fluorescence maximum (Spring 2014), the latter varying between 5 and 12 m. In 2013, simultaneous 2-point experiments were performed on 3 occasions using water from 3 different depths from the same sampling site (5, 18 and 80 m). Water containing the plankton assemblage was gently transferred from the Niskin bottles into 10 or 20 L carboys through a silicone tube to which a 200 μm mesh was affixed to screen out mesozooplankton. We refer to this $< 200\text{-}\mu\text{m}$ water as whole seawater (WSW). Filtered seawater (FSW) was obtained by filtering water collected from the same depth as WSW directly from the Niskin bottles through a 0.2 μm capsule filter (Pall) into separate carboys.

For the dilution series, WSW and FSW were combined to obtain 4 dilution levels each containing 10, 20, 40, 70% WSW in addition to the undiluted treatment. The 2-point experiments included a 10-25 % WSW and a 100 % WSW treatment. Each dilution level was prepared as a stock in a carboy and further siphoned into either 2.4 L (2013 austral

fall) or 1.2 L (2014 austral spring) polycarbonate bottles, depending on in situ chl *a* concentration. Dilutions were generally incubated in duplicate, and in some cases in triplicate. In the fall, replicates of all treatments were amended with macronutrients to a final concentration of 10 μ M and 1 μ M for nitrate and phosphate respectively, and a set of undiluted duplicates was prepared without adding nutrients to serve as a nutrient control. As some of the early experiments yielded phytoplankton growth rates in the 10 % WSW treatment that were substantially lower than in other dilution levels, in 2 subsequent experiments, we tested whether nutrient addition was adversely affecting growth by incubating duplicates of the 10 % WSW treatment without nutrients. In the spring, no nutrients were added to the dilutions, and to check for nutrient limitation, a set of undiluted duplicates were amended with 10 μ M nitrate, 1 μ M phosphate, 10 μ M silicate, and a solution of f/2 trace metals corresponding to a final concentration of 1 nM iron.

In the spring, rate estimates were also determined for the >20 μ m chl *a* fraction measured for the 10% and the 100% WSW dilutions, except for experiments performed in Wilhelmina Bay and Crystal sound, where phytoplankton was dominated by pico- and nano-size cells. Additional 2-point experiments (8 total) were performed using source water that had been screened through a 20 μ m mesh during collection from the Niskin bottles. Except for Wilhelmina Bay, in order to investigate potential trophic cascades these experiments were conducted in parallel with the experiments in which rates were estimated for the >20 μ m fraction.

To provide gentle agitation, bottles were vertically suspended from lines running across the top of deck-board incubators, and incubations were maintained at ambient

temperature with flow-through water. Temperature was recorded at 15 min intervals using Hobo data loggers. In order to recreate the ambient in situ light environment, the deck incubators were covered with a light filter (Lee light blue 118). In general incubations lasted 24 h, with two exceptions during the fall when all or a subset of bottles were incubated for 48 h. Due to the cruises' CTD deployment schedules, in the fall incubations started after sunset, whereas in the spring incubations started in late morning.

Estimation of phytoplankton growth and grazing mortality rates

For all experiments, rates of phytoplankton growth (μ) and protistan-grazing mortality (g) were estimated following Landry & Hassett (1982) from changes in extracted chlorophyll a (chl a). For each experiment, initial (P_0) and final (P_t) chl a concentrations were determined from triplicate subsamples of each dilution stock and of each replicate bottle respectively, in volumes that varied between 60 and 500 ml depending on chl a concentration. Chl a extraction and determination followed Graff & Ryneerson (2011), except that extraction took place at room temperature for 12 hours in 96% ethanol (Jespersen & Christoffersen 1987). Apparent phytoplankton growth rate (k , d^{-1}) in each bottle was estimated using the equation $k = 1/t \ln (P_t - P_0)$, where t is the incubation time in days. A critical assumption of the dilution method is that k be a linear function of the dilution factor. For the dilution series, the linear regression was tested for deviations from linearity using ANOVA at an alpha level of 0.05 (Zar 2010).

We determined μ and g according to the following: (1) If no deviations from linearity was detected, we tested the null hypothesis that the regression slope = 0. If the

regression slope was significantly different from 0, the rates were estimated from the linear regression coefficients (g from the negative slope and μ from the y-intercept) following Landry & Hassett (1982). If the regression slope was not significantly different from 0, g was set to 0 and μ was calculated as the average of k across all dilution levels (Murrell et al. 2002; Chen et al. 2009). For the few cases when the regression slope was > 0 , g was also set to 0 and μ to the average of k in the undiluted bottles. (2) When deviations from linearity were detected, dilution series plots were visually inspected and rates were determined from the regression coefficients of the plots' linear portion following the approach described in (1). If no adjustment was possible (no linear data subset), g was set to 0 and μ was estimated as the average of k across all dilution levels.

For the 2-point experiments, the regression analysis was replaced by a t-test to compare the average k values of the diluted and undiluted treatments, after which the same approach as with linear regression was used to estimate g and μ based whether the p-value was $\leq$ or > 0.05 . Using two dilution levels, g (the slope) was estimated using the equation $g = (k_d - k_1) / (1 - D)$, where the subscripts d and 1 correspond to the diluted and undiluted treatments respectively and D represents the dilution factor in the diluted treatment, and μ was calculated as $\mu = g + k_1$ (Landry et al. 2008). When the diluted treatment used in the 2-point experiment only represented a 10 % fraction of WSW, k_d was used as a conservative estimate of μ (Worden & Binder 2003; Strom & Fredrickson 2008; Lawrence & Menden-Deuer 2012), and g was determined using the equation $g = k_d - k_1$. In case the difference $k_d - k_1$ was positive, g was set to 0 and μ to the average k_1 value.

In both the dilution series and the 2-point methods, we applied realized dilution factors as determined from measured initial chl a concentrations in the dilutions, which varied from the target dilutions by an average of 3 %. We found no significant difference between treatments incubated with and without nutrients (paired t-tests) and thus all undiluted replicates regardless of nutrient treatment were included in the calculation of rates. Accumulation rates (d^{-1}) were estimated using the equation $r = \mu - g$, and grazing impact on phytoplankton as the proportion of PP consumed was estimated using the equation $\% \text{ PP grazed} = g/\mu$ (Calbet et al. 2004). The latter was not estimated when instantaneous phytoplankton growth rates were < 0 . Averages of grazing rate estimates, grazing impact, and accumulation rates are calculated including all ≥ 0 grazing rates. All statistical analyses were performed at an alpha value ≤ 0.5 .

Spatial and size distribution of phytoplankton biomass

To characterize the distribution of phytoplankton biomass across the sampling region, chl a measurements were performed in addition to those made for the dilution experiments (Fig. 2.1). For each geographical area sampled during the two cruises, a total of 18 (2013) and 9 (2014) vertical profiles of phytoplankton biomass were obtained at 11 and 10 separate sites respectively by extracting chl a from discrete water samples collected with the CTD rosette from 4-6 depths. To characterize the size distribution of the phytoplankton community, chl a was measured in 2-5 size fractions, from ~ 0.7 (GF/F) to $20 \mu m$, at all depths for all profiles in 2013, and at the depth of the fluorescence

maximum in 6 of the 2014 profiles, as well as twice with water collected for dilution experiments.

Phytoplankton community composition

To obtain a qualitative description of the nano- and micro-phytoplankton community (> 6 or $10\ \mu\text{m}$), sub-samples of the source water used in dilution experiments were analyzed using a FlowCAM[®], an imaging instrument that provides rapid characterization of plankton composition (at a group level rather than to species). In late fall 2013 samples were analyzed sporadically, as low in situ biomass dominated by picoplankton necessitated large volumes of water (up to 10 L) to be concentrated so analysis could be performed. In 2014, we analyzed duplicate or triplicate sub-samples of undiluted source water used in the dilution experiments until 1000 images per sample were obtained.

Protistan grazers' community composition and biomass

To determine the species composition and biomass of the grazing community, well mixed sub-samples of the undiluted plankton assemblage were collected at the beginning of each experiment and preserved with a 2% acidified Lugol's solution (Menden-Deuer et al. 2001). All 2013 samples were analyzed but one (Wilhelmina cast #23). For 2014, biomass estimates were generated for at least one sample collected from each fjord. When a fjord was sampled both at the beginning and the end of the sampling period (Table 2.1), a second sample was analyzed for temporal comparison.

Phagotrophic protists ($> 10 \mu\text{m}$) were enumerated following the Utermöhl method (1958). In 2013 a settled volume of 100 ml was used. Due to high phytoplankton abundance in the 2014 samples, only 10 ml of sample was concentrated and analyzed (with one exception – Wilhelmina 50 ml).

Organisms were assigned to major taxa, within which some were identified to genus/species and others were categorized into morpho-types. Although many dinoflagellates and ciliates function as mixotrophs (Flynn et al. 2013), because of their phagotrophic capacity, we categorized all of them as herbivorous. Since enumeration of heterotrophic nanoflagellates using the Utermöhl method results in underestimates (Davis & Sieburth 1982), these organisms were not counted, although they are included in the incubations and thus contribute to measured grazing rates.

Linear cell dimensions were measured using ImageJ software (National Institute of Health) of at least 50 or all imaged individuals per morphotype. Cell volumes were calculated from linear dimensions using appropriate geometric shape algorithms, after which an average cell volume and equivalent spherical diameter (ESD) were derived for each morphotype. Biomass estimates for each morphotype was calculated by converting biovolumes into carbon content ($\mu\text{g C L}^{-1}$) applying published conversion factors specific to dinoflagellates and other general plankton groups (Menden-Deuer and Lessard 2000) or to tintinnid ciliates (Verity & Langdon 1984).

Temperature perturbation experiment

To assess the effect of increased temperature on phytoplankton growth and grazing rates, in 2014 we conducted a temperature perturbation experiment using a natural plankton assemblage collected from the Gerlache Strait (G1 on Fig. 2.1). A total of eight 4.5 L bottles containing the undiluted $< 200 \mu\text{m}$ plankton assemblage were incubated for seven days, four bottles in each of two separate incubators: One incubator was kept at ambient water temperature, and the other was gradually heated over a 24 h period using a 1000-watt JBJ True Temp digital controller and heater, in order to reach and maintain a temperature 4°C above ambient. On December 13, the water flow to the ambient tank was interrupted and the tank drained. Although the flow through was promptly restored, the temperature in the incubator rose for approx. 30 min and reached a further 4°C above ambient, but it's unknown whether the water temperature in the incubation bottles was affected. This spike in temperature was not included when plotting the temperature data. Bottles were amended with nutrients as for other experiments conducted in 2014 and described in the previous section. Incubation bottles were sampled every day (60 ml) for chl a. The water removed was replaced with an equal volume of FSW obtained from the water collection site. At Tzero a baseline 2-point dilution experiment was conducted on the day the temperature experiment was set up using the same source water. At Tfinal (day 7) we conducted four 2-point dilution experiments using the pooled water from each set of ambient and heated replicate treatment bottles. Prior to the experiment the pooled water was gently screened through $200 \mu\text{m}$ mesh. The ambient and heated pooled water was divided to allow 2-point experiments under both

ambient and heated conditions. For each 2-point experiment, triplicate 1-L bottles of each 10% and 100% WSW treatments were amended with nutrients, and one bottle of 100% WSW was incubated without the addition of nutrients.

RESULTS

Environmental conditions

During late fall of 2013, daily averaged sea surface temperature (SST) varied from -1.5 to 0.7 °C. Water temperature at collection depth varied between -1.4 and 0° C (Table 2.1). Between the beginning and the end of the sampling period, day length decreased from ~6 to ~4 hours (Table 2.1). There were very few sunny days, the sky was generally overcast, and heavy snow fell on several occasions, conditions that, together with a low sun angle, contributed to overall low PAR: daily averages of Mast PAR (including night records) ranged from 4 to 40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and averages of 10% highest PAR measurements ranged from 30 to 426 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Table 2.1). Mixed layer depth (MLD) at sampling sites was generally shallow (7 to 30 m), with one exception in the Bismarck Strait where MLD reached 60 m (Table 2.1), and stratification was driven by the presence of a layer of fresher but colder water at the surface.

During late spring, SST ranged from -1.7 to 1 °C (Table 2.1). Below-zero T were found in the channels and during our transit to and from Crystal Sound (latitude 66° 54' S). Attempts to reach locations further south were aborted due to the presence of impassable sea-ice. There was no period of total darkness (Table 2.1), and daily averages of Mast PAR measurements ranged from 252 to 941 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with large variation around the daily mean. Averages of the 10% highest PAR values ranged from 882 to 1775 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Table 2.1). At sites where water was collected, MLD ranged from 6 to 20 m (Table 2.1). In the bays, stratification resulted from warmer but

fresher water at the surface, but in the Straits and Crystal Sound, surface water was both fresher and up to 1 °C colder than at 100 m.

Estimates of phytoplankton growth and grazing mortality

In late austral fall 2013, initial chl *a* varied from 0.14 to 0.40 $\mu\text{g L}^{-1}$, with lowest concentrations measured in Wilhelmina Bay at the end of the sampling season (Table 2.2). Phytoplankton growth rates were low, ranging from -0.1 to 0.19 d^{-1} (Table 2.2). Grazing rates were either zero, negative, or undetectable in 60 % of the experiments. Measurable grazing was detected in only 4 out of the 11 dilution series performed, and in 2 out of the 3 multiple-depths experiments conducted in Wilhelmina Bay with water collected at 5 m. Grazing rates from these experiments ranged from 0.11 to 0.26 d^{-1} (Table 2.2). Rates of growth and mortality were either closely coupled, or > 0 estimates of g exceeded estimates of μ . In the remaining 2-point experiments performed in Wilhelmina Bay using water collected at 18 and 80 m, both growth and grazing rates (if detected) were generally low ($\leq 0.2 \text{ d}^{-1}$) except for the first multiple-depth experiment conducted using water collected at 80 m, which yielded growth and grazing estimates of $0.61 \pm 0.1 \text{ d}^{-1}$ (Table 2.2).

During late spring of 2014, initial chl *a* varied from 0.21 $\mu\text{g L}^{-1}$ in Crystal Sound to 18.5 $\mu\text{g L}^{-1}$ in Flandres Bay (Table 2.3). Phytoplankton growth rates ranged from 0.06 to 0.93 d^{-1} , and averaged 0.23 d^{-1} (Table 2.3). Grazing rate estimates were significantly different from zero in about half of the experiments (7 out of 13 dilution series), and

ranged from 0.06 to 0.11 d⁻¹ (Table 2.3). Three experiments yielded negative grazing rates.

During fall 2013, grazing rates often exceeded growth rates (Fig. 2.2), resulting in an overall average balance (i.e. accumulation rate) of -0.02 d⁻¹, and an average >100 % of primary production consumed (Table 2.2). In contrast, during spring 2014, phytoplankton growth rates generally exceeded grazing rates (Fig. 2.2), resulting in an average accumulation rate of 0.18 d⁻¹ (Table 2.3).

In the experiments in which rates were estimated based both on total and > 20 µm chl a, growth rates for the > 20 µm fraction ranged from -0.11 (± 0.06) d⁻¹ to 0.92 (± 0.07) d⁻¹ (Table 2.3) and were significantly higher than corresponding estimates based on total chl a (T-test, $t = 2.185$, $DF = 9$, $p = 0.028$). The mean difference between the two estimates averaged 0.12 (± 0.17) d⁻¹ (Fig. 2.3). Grazing for the > 20 µm fraction was different from 0 in only one of the experiments, in Andvord Bay ($g = 0.33 \pm 0.10$ d⁻¹; Table 2.3). All the other experiments yielded negative grazing rates.

For the experiments performed using source water screened through 20 µm, only the experiment performed at Wilhelmina site W2 on December 22 yielded a grazing estimate different from 0, with a value of 0.11 ± 0.03 d⁻¹, and a corresponding μ of 0.05 ± 0.03 d⁻¹ (Table 2.3). Both μ and g estimates were similar to the estimates based on total chl a, the latter entirely composed of <20 µm cells. All other experiments yielded negative grazing rates.

Plankton abundance and species composition

In late fall, chl a in surface waters (5-20 m) rarely exceeded $0.4 \mu\text{g L}^{-1}$, with little variation among sampled sites (Fig. 2.4-A). In Wilhelmina Bay chl a decreased by 50% from 0.33 to $0.15 \mu\text{g L}^{-1}$ between May 22 and June 5. In the bays and the Gerlache strait, there was hardly any phytoplankton below 100 m, whereas in Bismarck strait and the Palmer basin, chl a concentration at 100 m decreased by less than 1/2 that of surface waters (Fig. 2.5). At all sites sampled, on average 91% of the chl a measured at 5 m and 20 m was contributed by cells $< 10 \mu\text{m}$, and an average of 47% was contributed by cells $< 2 \mu\text{m}$ (Fig. 2.5). In Wilhelmina Bay, cells $> 10 \mu\text{m}$ almost entirely consisted of cryptophytes. There were very few diatom cells, yet this group was represented by many genera including *Thalassiosira*, *Rhizosolenia*, *Guinardia*, *Chaetoceros*, *Pseudonitzschia*, and *Thalassiothrix*. The species *Corethron pennatum* was present in all the bays sampled.

During the spring, chl a concentrations indicated a rapid but patchy development of a phytoplankton bloom (Fig. 2.4-B). Chl a was higher north (3.4 to $12.2 \mu\text{g L}^{-1}$) than south ($0.2 - 1.1 \mu\text{g L}^{-1}$) of Flandres Bay but the difference between locations was not statistically significant (ANOVA, $p = 0.07$). Of the bays, Wilhelmina had the lowest chl a concentration ($3.4 \pm 0.5 \mu\text{g L}^{-1}$), 90% of which was measured in the $< 5 \mu\text{m}$ fraction (Fig. 2.7). Maximal concentrations were measured in Andvord on 11 Dec ($13.5 \mu\text{g L}^{-1}$) and in Flandres on 12 Dec ($18.5 \mu\text{g L}^{-1}$), yet within each bay, biomass was spatially heterogeneous as evidenced by large variations in chl a depending on sampling location (Fig. 2.4-B & Table 2.3). In all bays except Wilhelmina, and at sites in the Gerlache Strait where size distribution was assessed (G1 and G2), an average of 76% of chl a could

be attributed to cells $>20\ \mu\text{m}$ (Fig. 2.7). In contrast in the Bismarck Strait, up to 81% of chl a was found within the $<2\ \mu\text{m}$ fraction (Fig. 2.7).

The species composition of the $>10\ \mu\text{m}$ spring phytoplankton community varied among sites. In Wilhelmina Bay and Bismarck Strait, cryptophytes dominated. Diatoms dominated in Andvord, Flandres, and Paradise bays, but whereas in Andvord Bay long chains of *Chaetoceros* spp. were most abundant, long chains of *Thalassiosira* spp. and other centric diatoms dominated the two other bays, together with species of *Corethron*. Both in Flandres and Paradise, prasinophytes identified as *Pyramimonas* sp. were also abundant. The micro-phytoplankton community was most diverse in the Gerlache strait, composed of all the different diatom species present in the bays. Colonies of *Phaeocystis antarctica* were sometimes present in samples, but never abundant.

In both sampling seasons, the protistan grazer community was dominated by athecate dinoflagellates (Fig. 2.8). In the fjords, aloricate ciliates and thecate dinoflagellates were rare and tintinnid ciliates almost absent. During fall, grazer's biomass in the fjords varied from a bay average of $3.3 (\pm 0.9)\ \mu\text{g C L}^{-1}$ in Andvord Bay to a bay average of $6.0 (\pm 1.1)\ \mu\text{g C L}^{-1}$ in Flandres Bay (Fig 2.8-A). In Wilhelmina Bay, grazers' biomass decreased between the beginning and the end of the sampling season, from an average of $4.3 (\pm 0.4)\ \mu\text{g C L}^{-1}$ to an average of $2.2 (\pm 0.9)\ \mu\text{g C L}^{-1}$. In Bismarck strait, grazers' biomass was $17.6\ \mu\text{g C L}^{-1}$, 4.5 times greater than the fjords' overall average biomass. Dinoflagellates dominated the fall grazers' community both in abundance and biomass, representing an average of 77% of both the numerical abundance and the total biomass in the fjords (Fig. 2.8-B), $\frac{3}{4}$ of which could be attributed

to numerous athecate forms, principally of the genera *Gyrodinium* and *Gymnodinium*. Oligotrich ciliates accounted for ¼ of total biomass. In Bismarck strait, the ratio dinoflagellates:ciliates was similar to that in the fjords, with dinoflagellates dominating the grazing community both in numerical abundance (82%) and biomass (69%), but dinoflagellate thecate forms were more predominant than in the fjords, contributing 53% of the dinoflagellate biomass (Fig. 2.8-B). During spring, the numerical abundance of HP was similar to their abundance in the fall (data not shown), but their biomass was greater, reflecting an increase in the average size of grazers. Spring grazers' biomass varied over an order of magnitude, from 6.2 µg C L⁻¹ in Andvord Bay on December 10th, to 52.3 µg C L⁻¹ in Flandres Bay (Fig 2.8-C). Based on the samples collected ten days apart in two of the fjords, there was a tendency for grazers' biomass to increase with time. As during austral fall, athecate dinoflagellates generally dominated the grazer community, with < 20 µm gymnoid forms being particularly abundant on the first visit to Wilhelmina Bay. In Flandres Bay a few large *Protoperidinium* spp. and tintinnid ciliates contributed most of the biomass. In general there were few aloricate ciliates, except in Paradise Bay where a few but large *Strombidium*-type oligotrichs contributed 91% of the biomass (Fig. 2.8-D).

Effect of increased temperature on phytoplankton biomass and process rates

A significantly higher than ambient temperature was consistently maintained in the temperature perturbed incubator, relative to ambient conditions. Temperature fluctuations were observed in both treatments. Ambient temperature varied from -1.7 to 2.4 °C, due to diurnal fluctuations in sea surface temperature as well as fluctuations

during ship's transit. The average temperature in the ambient treatment was -0.3°C ($\pm 1^{\circ}\text{C}$ SD). The elevated temperature treatment had an average temperature of 4.5°C ($\pm 2^{\circ}\text{C}$ SD). After an initial 14-hour warming period, the overall warming relative to ambient averaged 5.1 (± 1.2 SD) $^{\circ}\text{C}$.

Although chl a increased substantially over the 7-day incubation period regardless of the temperature treatment (Fig. 2.9), the increase was larger when temperature was raised (Paired one-tailed t-test, $t = 2.86$, $\text{DF} = 6$, $p = 0.014$). Chl a was 4.0 (± 0.24 SD) $\mu\text{g L}^{-1}$ at the beginning of the experiment, 74% of which could be attributed to the $>20 \mu\text{m}$ fraction. Chl a increased to 35.5 (± 6.2 SD) $\mu\text{g L}^{-1}$ and 49.2 (± 2.4 SD) $\mu\text{g L}^{-1}$ on day 7 at ambient and elevated temperature respectively (Fig. 2.9). On Day 3, $> 90\%$ of chl a was $>20 \mu\text{m}$ in both the ambient and heated incubations, dominated by large chains of diatoms, and this fraction represented almost all of the chl a on Day 7. Based on daily chl a measurements, the overall average net growth rate at ambient temperature was 0.31 (± 0.15 SD) d^{-1} , whereas at elevated temperature, the overall average net growth rate was 0.36 d^{-1} , albeit with large variations from day to day (± 0.39 SD).

On the first day (T_0), no grazing was detected ($g = 0$), and instantaneous phytoplankton growth rate (μ) was estimated as 0.31 ± 0.08 d^{-1} (Table 2.4). On day 7, no grazing was detected when ambient water was incubated at ambient temperature (AA), whereas in all treatment subjected to elevated temperature, grazing rates were 0.40 , 0.31 , and 0.41 d^{-1} for ambient to heated (AH), heated to ambient (HA), and heated to heated (HH) respectively (Table 2.4). Net growth rates were lower in the undiluted bottles incubated without the addition of nutrients than in nutrient amended bottles, indicating

nutrient limitation, which was stronger for phytoplankton that had been incubated for 7 days under increased temperature (Table 2.4). Estimates of μ were the highest for the AH treatment irrespective of whether nutrients were added or not ($\sim 0.7 \text{ d}^{-1}$; Table 2.4).

DISCUSSION

This study is one of few conducted during different seasons to quantify phytoplankton mortality rates due to protistan grazing, in a region of Antarctica particularly sensitive to climate-change. Not only are seasonal quantifications of plankton trophic dynamics critical to understand triggers of phytoplankton bloom and assess the hypothesized role of HP in the yearly cycles of primary production (e.g. Behrenfeld 2014; Schmoker et al. 2013), such data also contribute to establishing an essential year-round baseline of process rates against which potential climate-driven changes may be detected.

Overall, we found no seasonal signal in the magnitude of protistan herbivory rates. Grazer-induced phytoplankton mortality rates were low across the two sampling seasons, rarely exceeding 0.1 d^{-1} if mortality was detected at all. The grazing rates we measured are among the lowest reported for Antarctica (see Garzio et al. 2013 and their Table 2), except for rates measured in the Ross Sea, which never exceeded 0.26 d^{-1} (Caron et al. 2000). Nevertheless grazing rates from the present study are within the same order of magnitude as the estimated average of 0.16 d^{-1} for polar habitats (Calbet & Landry 2004) and of 0.14 d^{-1} for the polar southern biogeographic region (Schmoker et al. 2013), although the estimated proportion of primary production grazed for these regions (59 and 53 % respectively) is higher than the average spring estimate from the present study (40 %).

In late fall and late spring, a similar proportion of experiments yielded no grazing. During late austral fall, 40 % of experiments (including multiple depths) indicated some

grazing, a proportion comparable to that of our late spring study (50 %). It is not rare that studies of protistan herbivory in Antarctic waters report non-significant grazing rate estimates for a substantial proportion of experiments, up to 75 % in the Palmer LTER region (Garzio et al. 2013) and up to 67% in the Ross Sea (Caron et al. 2000).

Although there was a lack of a seasonal signal in grazing rates, a marked seasonality of the phytoplankton growth was measured, resulting in a seasonal difference in the sign of the phytoplankton biomass accumulation rate derived from our experiments. In the fall, the average accumulation rate was negative, generally a result of low or negative phytoplankton growth, suggesting that grazing was not the principal agent of phytoplankton mortality. In the spring, phytoplankton growth rates exceeded grazing rates in 85 % of the experiments, which is consistent with previous findings in the WAP summer (Garzio et al. 2013). Our estimates of net accumulation rates are consistent with the observed phytoplankton biomass as indicated by chl a concentration and suggest that seasonal variations in phytoplankton growth rather than grazing pressure drive net accumulation rates. Provided there are no other losses, e.g. through mixing, this decoupling between protistan herbivory and phytoplankton growth may have contributed to the observed developing blooms that characterize the seasonally high productivity of the WAP system (Vernet 2008).

Methodological factors may have affected our ability to detect grazing. Winter low chl a lowers the sensitivity of the dilution method because the rate estimation relies on measuring very small changes in chl a occurring over the experiment's duration (Dolan & McKeon 2005). Results of our fall study suggest this may have been the case,

as >0 grazing rates were associated with days when PAR was relatively higher than on other days, which stimulated some phytoplankton growth, potentially enhancing our ability to detect grazing. Difficulty to distinguish signal from inherent variability may not only affect the ability to detect grazing, it can also result in overestimation of both instantaneous rates of growth and grazing due to a large effect of tiny increases in chl *a* on the estimated net growth. This is evident from results of one experiment performed in Wilhelmina Bay with water collected at a depth of 80 m, which yielded questionable estimates of *g* and μ of 0.6 d^{-1} . A frequent attempt to remedy the sensitivity methodological issue is to increase the incubation time in order to increase the signal (e.g. Caron et al 2000, Garzio et al. 2013). In the two instances in which we left all or an extra set of bottles to incubate for 48 instead of 24 h, the additional incubation time did not improve our ability to detect grazing, so we proceeded with the 24 h incubation protocol. Our decision was also motivated by the fact that longer incubations likely magnify bottle effects, shifts in community composition, and potential nutrient limitation. Substantial alterations in the abundance and composition of the grazer community have been observed in 72 h incubations (Garzio et al. 2013). Such changes may lead to unreliable or anomalous estimates of grazing (First et al. 2007).

Noteworthy are the significant but positive slopes (i.e. negative estimates of grazing rate) obtained for three out of the four spring experiments in which initial chl *a* was the highest ($8 \text{ to } 18 \mu\text{g L}^{-1}$). Anomalous negative grazing rates have been reported not only during blooms of *Phaeocystis* spp. both in the Arctic (Calbet et al. 2011) and the Antarctic (Caron et al. 2000), but also during blooms dominated by diatoms (Sherr et al.

2013). In one seasonal study performed on the East Antarctic Coast near Davis Station, all the dilution experiments conducted during spring yielded both negative phytoplankton growth and grazing mortality rates (Pearce et al. 2008). One proposed hypothesis to explain positive regression slopes in dilution experiments is that blooming phytoplankton can produce inhibitory metabolites that may be released during preparation of the filtered seawater (Stoecker et al. 2015). This process may have caused the negative grazing coefficients we observed. Toxic metabolites may also have impacted the 2-point experiments on the $< 20 \mu\text{m}$ fractions, most of which yielded negative grazing rates. In dense bloom conditions, further clarification of the processes involved may advance our understanding of the complex chemical interactions among phytoplankton and their grazers and of their influence on blooms and ocean carbon flow (Stoecker et al. 2015).

An important aspect of the dilution method that may compromise measurements of phytoplankton growth rates is the difficulty of replicating the in situ light environment and the potential for phytoplankton cells to photo-acclimate during incubations (Behrenfeld et al. 2015). If incubations are performed at a higher light level than the one experienced by the cells in situ, chl *a* per cell may decrease, yielding an underestimate of phytoplankton net growth rates, resulting in misestimates of μ (Caron et al. 2000). Photo acclimation would not have affected grazing estimates, assuming it is independent of dilution, yet it could have contributed to negative grazing rates if self-shading due to high biomass in the undiluted treatment resulted in differential levels of photo acclimation across dilutions. We do not have data to provide evidence that photo acclimation was not occurring in our incubations, however we controlled the intensity and spectral quality of

the light in our incubators to match the in situ light environment. Furthermore, mixed layer depths were generally shallow, presumably retaining cells within a layer receiving replicable irradiance, which should have prevented any substantial loss of chl a to occur during incubation.

Grazing mortality rates in the global ocean are often positively correlated to phytoplankton standing biomass (Calbet & Landry 2004), although several studies found no relationship between initial chl a concentration and measured grazing rates (Strom et al. 2001; Sherr et al. 2009, Menden-Deuer & Fredrickson 2010, Lawrence & Menden-Deuer 2012). Bulk prey abundance, as assessed by chl a concentration, was not a determinant of grazing magnitude in our study, since both absent and low grazing were similarly observed across seasons at chl a concentration that ranged over two orders of magnitude. Caron et al. (2000) came to a similar conclusion based on the low rates of phytoplankton mortality they observed across a wide gradient of chl a concentration. In their study, high chl a concentrations were dominated by colonies of *Phaeocystis antarctica*. The authors suggested that the inadequate dilution of grazers included within the gelatinous colonies could have contributed to the low rates of grazing, but this was not the case in our study, as we observed few colonies of *P. antarctica*.

During spring, grazing was generally observed when the size distribution of phytoplankton biomass was most similar to winter conditions, i.e. dominated by small cells. This observation suggests that most of the measurable grazing during spring was occurring on the pico- to nano-size fractions, which is consistent with previous Antarctic studies that have indicated a grazing preference on small cells: in the WAP, Garzio et al.

(2013) found higher grazing rates on pico- and nano-plankton than on total phytoplankton, and in the Ross Sea, Caron et al. (2000) noted that grazing only occurred when chl a concentration was $< 1 \mu\text{g L}^{-1}$, indicative of a dominance of small phytoplankton cells (Irigoien et al. 2005). In the present study, grazing on small cells may also have been occurring in bays dominated by diatoms, but the $> 20 \mu\text{m}$ phytoplankton fraction grew at higher rates than those measured for total chl a, which may have obscured any observable chl a signal of grazing and contributed to non-significant grazing rates. Feeding preference for small prey may explain the lack of seasonality in grazing rate magnitude.

Low grazing magnitude could not be explained by a lack of grazers. Grazers' abundance was similar and at some sites greater than what has been reported for the North Atlantic in spring (Gifford et al. 1995, Morison & Menden Deuer 2015). Low grazing in the presence of a substantial standing biomass of HP could indicate that these organisms were relying on mixotrophy, which is widespread among HP (Flynn et al. 2013), and particularly among dinoflagellates (Stoecker 1999). Dinoflagellates, which dominated the grazing community in this study, could also have consumed prey other than phytoplankton, including bacteria. Dinoflagellates have been shown to feed on a wide variety of prey (Jeong et al. 2010), including heterotrophic nanoflagellates, naked and tintinnid ciliates, and other dinoflagellates. Dinoflagellates also feed on heterotrophic bacteria (HB). Their grazing impact on bacteria can be comparable to that of heterotrophic nanoflagellates (Jeong et al. 2010), the latter being generally considered as the major consumers of heterotrophic bacteria (Sherr & Sherr 1994). Drivers of the

dynamics of HB in the WAP are mostly understood in terms of physical and bottom up (resource) controls (Ducklow et al. 2012, Saba et al. 2014, Kim & Ducklow 2016), whereas knowledge of the importance of bacterivory is limited, although it has been invoked as a potential factor controlling the usually low ratio of bacterial to primary production (Bird & Karl 1999). One study found HP as a group to exert a grazing pressure on bacteria greater than on phytoplankton (Garzio et al. 2013), yet the importance of bacteria and other prey sources for different groups of HP in the WAP remains to be determined.

Despite the low rates of grazing measured during spring, the average biomass of HP in bays dominated by diatoms increased over the period of time (~10 days) separating repeated sampling. This increase mostly reflected an increase in the average size of the grazers, suggesting grazers' response to the increasing dominance of large phytoplankton cells. The development of phytoplankton blooms in the fjords however suggests that the grazer community did not grow rapidly enough to exert a feeding impact restricting PP.

Low water temperature has been postulated as a major factor limiting the growth rates and thus the grazing impact of HP, driving the decoupling between phytoplankton growth and losses leading to blooms at high latitudes (Rose & Caron 2007). Yet significant grazing has previously been observed at near freezing temperatures (Lawrence & Menden-Deuer 2012). Moreover, maximal growth rates of Arctic species of ciliates and athecate dinoflagellates have been found to occur at temperatures $<5^{\circ}\text{C}$, indicating these organisms may be adapted to the ambient temperatures in which they naturally occur (Franze & Lavrentyev 2014).

In the present study, a direct limiting effect of temperature on herbivory remains questionable, as grazing rates in a range of magnitude were obtained from experiments conducted at similarly low temperatures. Yet increasing water temperature produced significant increases of grazing rates. We measured substantial increases in mortality rates on day 7 in all but the ambient to ambient treatment. Although our data do not provide an understanding of the mechanism involved, these results are consistent with the expected effects of increased temperature on physiological rate processes (Eppley 1972, Alcaraz et al. 2014). Together with the measurements made in the field, these results suggest that while low ambient temperature ($< 5^{\circ}\text{C}$) may set an upper limit on the magnitude of grazing rates, it cannot universally account for the variability of this magnitude, suggesting that other factors likely play a role.

Elevated temperature also had a positive effect on phytoplankton nutrient utilization. Nutrient limitation was evident in the non-amended treatments of the dilution experiments conducted on day 7, except for the incubation of the ambient plankton community at elevated temperature. Phytoplankton growth rates indicated a rapid response to sudden increase in temperature but a lesser response after acclimation to higher temperature, and differences were consistent irrespective of nutrient addition, indicating that absolute temperature may be more important than the stress imparted by a sudden transition. Importantly the proportion of primary production consumed (i.e. the $g:\mu$ ratio) was $< 100\%$ in the ambient acclimated community subjected to elevated temperature but the ratio was $>100\%$ in the heat acclimated community, irrespective of the incubation temperature treatment.

The present study is the first to examine the role of herbivorous protists in the WAP at different seasons and contributes to a baseline of rates necessary for detecting potential climate-driven changes in the dynamics of the WAP plankton food web. Consistent with previous findings, spring phytoplankton grazing-mortality rates in our study never exceeded growth rates, which may help explain the large blooms that characterize the region during spring and summer. Despite a large seasonal difference in phytoplankton standing biomass, we found no seasonal difference in the magnitude of grazing rates. These results suggest a lack of predators' functional response in the WAP, which is contrary to the positive relationship between prey biomass and ingestion rates usually assumed when describing zooplankton grazing in ecological and biogeochemical models. Instead, plankton population dynamics and ultimately phytoplankton biomass accumulation rates in the WAP region may be best predicted as a function of plankton community composition. Results also underline the need to extend measurements for the global ocean to less productive seasons in order to verify whether the assumed enhancing effect of prey abundance on grazing rates is always observed in the field.

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Table 2.1. Locations and environmental characteristics of sampling stations where water was collected for dilution experiments conducted along the Western Antarctic Peninsula during austral fall 2013 and austral spring 2014. Mixed layer depth (MLD) was calculated from a density (σ_t) difference criterion of 0.03 kg m^{-3} , using as reference the near surface (5 m) density values. Temperature and salinity data are from depth of sample collection. Photosynthetically available radiation (PAR) values represent the average 10 % highest values of daylight hours over the duration of the experiment. Day length is for Anvers Island and may be approximate.

Date	Cast #	Location	Site	Latitude	Longitude	Total Depth (m)	MLD (m)	T (°C)	Salinity (PSU)	PAR ($\mu\text{E m}^{-2} \text{ s}^{-1}$)	Day Length (H:min)
<i>Austral late fall 2013 Dilution series</i>											
17-May	002	Wilhelmina	W2	-64 37.200	-62 14.360	523	n/a	n/a	n/a	85	5:55
20-May	013	Wilhelmina	W3	-64 40.915	-62 13.949	530	20	-1.4	33.56	39	5:37
21-May	023	Wilhelmina	W1	-64 32.097	-62 14.063	619	7	-1.1	33.57	96	5:32
22-May	032	Wilhelmina	W1	-64 32.039	-62 14.002	627	30	-0.5	33.76	341	5:26
23-May	035	Andvord	A3	-64 50.783	-62 36.856	297	11	-1.2	33.58	140	5:20
24-May	043	Andvord	A1	-64 48.312	-62 43.436	323	22	-0.6	33.79	426	5:15
25-May	050	Andvord	A1	-64 48.698	-62 42.272	346	16	-1.1	33.63	92	5:09
27-May	052	Antvord	A1	-64 48.636	-62 43.049	340	19	-1.3	33.73	80	4:58
29-May	056	Flandres	F2	-65 02.992	-63 18.753	258	17	-0.8	33.63	38	4:48
30-May	064	Flandres	F1	-65 00.185	-63 18.616	746	13	-0.8	33.63	57	4:43
31-May	068	Bismarck Strait	BS	-64 52.494	-63 47.948	553	60	-0.4	33.87	46	4:38
<i>Austral late fall 2013 - 2-point multiple depths experiments (Wilhelmina Bay)</i>											
4-Jun	080	5 m	W2	-64 37.127	-62 14.314	386	14	-1.2	33.80	79	4:21
4-Jun	080	18 m	W2	-64 37.127	-62 14.314	386	14	-0.7	33.96	79	
4-Jun	080	80 m	W2	-64 37.127	-62 14.314	386	14	-0.3	34.31	79	
5-Jun	096	5 m	W2	-64 36.970	-62 14.387	362	15	-1.4	33.74	36	4:17
5-Jun	096	18 m	W2	-64 36.970	-62 14.387	362	15	-1.1	33.81	36	
5-Jun	096	80 m	W2	-64 36.970	-62 14.387	362	15	0.0	34.31	36	
6-Jun	103	5 m	W1	-64 35.329	-62 13.337	496	15	-0.9	33.77	30	4:13
6-Jun	103	18 m	W1	-64 35.329	-62 13.337	496	15	-0.8	33.86	30	
6-Jun	103	80 m	W1	-64 35.329	-62 13.337	496	15	-0.1	34.29	30	
<i>Austral late spring/early summer 2014</i>											
7-Dec	006	Wilhelmina	W3	-64 41.498	-62 15.052	553	8	0.1	33.96	882	20:58
8-Dec	015	Wilhelmina	W3	-64 41.499	-62 15.121	534	10	0.4	33.89	1414	21:04
9-Dec	026	Wilhelmina	W3	-64 41.634	-62 16.673	475	9	0.6	33.80	1729	21:09
9-Dec	028	*Gerlache Strait	G1	-64 31.987	-62 23.911	679	20	-0.05	33.97	1286	21:09
10-Dec	030	Andvord	A2	-64 49.471	-62 38.961	433	10	0.2	33.92	1700	21:14
11-Dec	042	Andvord	A2	-64 49.477	-62 38.719	432	7	0.4	33.64	1770	21:19
12-Dec	046	Flandres	F3	-65 03.322	-63 19.362	328	6	0.07	33.63	1713	21:24
14-Dec	051	Crystal Sound	CS	-66 53.958	-66 51.011	585	6	-1.2	33.62	1082	21:32
18-Dec	071	Bismarck Strait	BS	-64 53.724	-63 43.146	509	13	-1.0	33.39	1775	21:43
19-Dec	082	Flandres	F3	-65 02.823	-63 13.443	300	10	-0.04	33.83	1775	21:44
20-Dec	105	Flandres	F3	-64 59.612	-63 17.267	391	6	0.5	33.37	1769	21:45
21-Dec	122	Andvord	A2	-64 48.891	-62 40.326	374	9	0.5	34.07	1030	21:45
22-Dec	131	Wilhelmina	W2	-64 37.389	-62 14.689	556	6	1.3	33.76	1417	21:45
24-Dec	144	Paradise	P	-64 51.152	-62 55.600	254	11	0.8	33.85	n/a	21:43

* 2 x 2-Point experiments 7 days apart (Temperature experiment)

Table 2.2. Initial Chlorophyll a concentration (Chl a, $\mu\text{g L}^{-1}$), as well as rates (per day) of phytoplankton growth (μ), grazing mortality (g), and phytoplankton accumulation (r), and grazing impact as proportion of primary production consumed (% PP) estimated from dilution experiments conducted in the Western Antarctic Peninsula during austral fall 2013. Rates are given $\pm$ one standard deviation of the mean rate obtained from replicate incubation bottles.

Date	Site	Chl a	μ	g	r	% PP
<i>Dilution series</i>						
17-May	W2	0.39 (0.01)	0.18 (0.02)	0.16 (0.03)	0.02 (0.04)	89
20-May	W3	0.38 (0.01)	-0.01 (0.06)	undetectable	n/a	n/a
21-May	W1	0.31 (0.01)	-0.01 (0.03)	< 0	-0.01 (0.03)	n/a
22-May	W1	0.28 (0.01)	0.03 (0.04)	0.13 (0.06)	-0.10 (0.07)	433
23-May	A3	0.35 (0.01)	-0.07 (0.08)	0	-0.07 (0.08)	n/a
24-May	A1	0.28 (0.03)	0.15 (0.05)	0.26 (0.08)	-0.11 (0.09)	173
25-May	A1	0.34 (0.01)	0.05 (0.02)	0	0.05 (0.02)	0
27-May	A1	0.23 (0.03)	0.10 (0.08)	0	0.10 (0.08)	0
29-May	F2	0.24 (0.01)	0.06 (0.08)	undetectable	n/a	n/a
30-May	F1	0.23 (0.02)	0.19 (0.03)	0.19 (0.05)	0.00 (0.04)	100
31-May	BS	0.40 (0.02)	-0.08 (0.04)	0	-0.08 (0.04)	n/a
		Average	0.05 (0.02)	0.08 (0.01)	-0.02 (0.02)	132
<i>2-point multiple depths experiments (Wilhelmina Bay)</i>						
4-Jun	W2 - 5m	0.14 (0.00)	0.01 (0.03)	< 0	0.01 (0.03)	0
4-Jun	W2 - 18 m	0.10 (0.00)	0.20 (0.11)	0.16 (0.10)	0.04 (0.15)	80
4-Jun	W2 - 80 m	0.04 (0.00)	0.61 (0.12)	0.61 (0.13)	0.00 (0.18)	100
5-Jun	W2 - 5m	0.17 (0.01)	0.07 (0.07)	0.12 (0.06)	-0.05 (0.09)	171
5-Jun	W2 - 18 m	0.13 (0.00)	-0.02 (0.04)	0	-0.02 (0.04)	0
5-Jun	W2 - 80 m	0.03 (0.00)	-0.12 (0.05)	< 0	-0.12 (0.05)	0
6-Jun	W1 - 5m	0.14 (0.01)	0.02 (0.02)	0.11 (0.02)	-0.09 (0.02)	550
6-Jun	W1 - 18 m	0.12 (0.01)	0.02 (0.04)	0	0.02 (0.04)	0
6-Jun	W1 - 80 m	0.03 (0.00)	-0.08 (0.07)	0	-0.08 (0.07)	0

Table 2.3. Total and fractionated (> 20 µm and < 20 µm) initial chlorophyll a (Chl a) concentration [$\mu\text{g L}^{-1}$ (+/- one standard deviation of the mean of triplicate measurements)] and rates of phytoplankton growth (μ) and grazing mortality (g) estimated from dilution experiments performed along the Western Antarctic Peninsula in December (austral spring) 2014, as well as accumulation rates (d^{-1}) and proportion of primary production consumed (% PP). Rates and chl a for the >20 µm fraction are based on the dilution series conducted on total chl a. Rates and chl a for the < 20 µm fraction were measured in separate 2-point experiments. Rates are given per day (+/- one standard deviation). Sites locations are shown on Fig. 2.1.

Date	Site	Total Chl a	μ (total chl)	g (total chl)	r (total chl)	% PP	>20 µm Chl a	>20 µm μ	>20 µm g	<20 µm Chl a	<20 µm μ	<20 µm g
<i>Austral late spring 2014 Dilution series</i>												
7-Dec	W3	4.0 (0.17)	0.25 (0.02)	0.11 (0.04)	0.14 (0.04)	44	n/a	n/a	n/a	4.1 (0.04)	0.28 (0.12)	0
8-Dec	W3	2.9 (0.31)	0.14 (0.01)	0.06 (0.02)	0.08 (0.02)	43	n/a	n/a	n/a	n/a	n/a	n/a
9-Dec	W3	3.8 (0.26)	0.15 (0.05)	0	0.15 (0.05)	0	n/a	n/a	n/a	n/a	n/a	n/a
9-Dec	*G1	4.0 (0.24)	0.31 (0.08)	0	0.31 (0.08)	0	3.0 (0.68)	0.36 (0.02)	<0	n/a	n/a	n/a
10-Dec	A2	3.3 (0.37)	0.11 (0.05)	0	0.11 (0.05)	0	2.1 (0.57)	0.36 (0.01)	<0	1.1 (0.00)	-0.02 (0.03)	<0
11-Dec	A2	3.7 (0.35)	0.93 (0.03)	0.10 (0.04)	0.83 (0.05)	11	2.6 (0.26)	0.92 (0.07)	<0	n/a	n/a	n/a
12-Dec	F3	18.5 (n/a)	0.08 (0.03)	<0	0.08 (0.03)	0	16.0 (n/a)	-0.11 (0.06)	0	1.2 (0.03)	-0.14 (0.02)	0
14-Dec	CS	0.2 (0.01)	0.06 (0.03)	0.11 (0.04)	-0.05 (0.05)	183	n/a	n/a	n/a	n/a	n/a	n/a
18-Dec	BS	1.3 (0.06)	0.21 (0.03)	0.11 (0.04)	0.10 (0.05)	52	0.1 (0.01)	0.36 (0.04)	<0	1.0 (0.03)	0.25 (0.01)	<0
19-Dec	F3	9.8 (0.83)	0.20 (0.06)	<0	0.20 (0.06)	0	6.9 (0.11)	0.28 (0.05)	<0	2.2 (0.07)	0.20 (0.07)	<0
20-Dec	F3	5.3 (0.33)	0.22 (0.12)	undetectable	n/a	n/a	2.7 (0.16)	0.44 (0.07)	<0	1.3 (0.03)	0.20 (0.05)	<0
21-Dec	A2	7.6 (0.29)	0.22 (0.05)	<0	0.22 (0.05)	0	6.6 (0.12)	0.42 (0.09)	0.33 (0.10)	n/a	n/a	n/a
22-Dec	W2	3.1 (0.22)	0.05 (0.02)	0.08 (0.03)	-0.03 (0.04)	160	0.06 (0.00)	0.47 (0.03)	<0	3.1 (0.18)	0.05 (0.03)	0.11 (0.03)
24-Dec	P	12.2 (0.09)	0.22 (0.02)	0.10 (0.03)	0.12 (0.04)	45	7.8 (0.55)	0.22 (0.03)	0	3.5 (0.35)	0.17 (0.03)	<0
		Average	0.23 (0.02)	0.05 (0.01)	0.18 (0.02)	41		0.37 (0.02)	0.03		0.12 (0.02)	0.01

* Initial 2-point experiment in 7-day temperature perturbation

Table 2.4. Estimates of rates of phytoplankton grazing mortality (g) and instantaneous growth (μ) with (+ N) or without (No N) the addition of nutrients, at the beginning (Day 0) and the end (Day 7) of a 7-day temperature perturbation experiment, during which source water was incubated at ambient (A) or elevated (+4 °C above ambient; H) temperatures. On day 7, ambient (A) and heated (H) 7-day incubation water was used in four 2-point dilution experiments performed at either ambient (A to A and H to A) or elevated water temperature (A to H and H to H). Rates are per day ($\pm$ 1SD of the mean of triplicate incubation bottles). Ration g: μ represents proportion (%) of primary production consumed in situ (No N).

	A to A (Day 0)	A to A (Day 7)	A to H (Day 7)	H to H (Day 7)	H to A (Day 7)
g	NS	NS	0.40 (0.09)	0.41(0.04)	0.31 (0.11)
μ + N	0.31(0.08)	0.23 (0.06)	0.72 (0.07)	0.29 (0.02)	0.12 (0.04)
μ No N	N/A	0.02 (N/A)	0.66 (N/A)	0.02 (N/A)	-0.22 (N/A)
g : μ	N/A	N/A	61%	>100 %	>100 %

Figure 2.1. Locations along the West Antarctic Peninsula where sampling was performed. Open symbols: chlorophyll profiles and/or dilution experiments; closed symbols: chlorophyll profiles only.

Figure 2.2. Rates of phytoplankton growth (circles) and grazer-induced mortality (diamonds) from dilution experiments performed along the Western Antarctic Peninsula during (A) austral fall 2013 and (B) austral spring 2014. Error bars represent one standard deviation of the mean. Solid horizontal lines represent the average phytoplankton biomass accumulation rate. For figure clarity, one extreme phytoplankton growth value (0.93 d^{-1}) was omitted from panel B.

Figure 2.3. Differences (d^{-1}) between specific growth rates based on $>20\ \mu\text{m}$ and total chlorophyll a in dilution experiments performed along the Western Antarctic Peninsula during austral spring 2014. On December 24 there was no difference between total and $>20\ \mu\text{m}$ growth rates.

Figure 2.4. Average surface chlorophyll a concentration along the Western Antarctic Peninsula during fall 2013 (A) and spring 2014 (B). Error bars represent one standard deviation of the mean of N samples (given in parentheses on x-axis). Note the different scale on y-axis for each panel.

Figure 2.5. Vertical profiles and size distribution of chlorophyll a along the Western Antarctic Peninsula during austral fall 2013. Note that all fractions do not appear at all locations.

Figure 2.6. Vertical profiles of chlorophyll a in bays (left panels) and channels (right panels) along the Western Antarctic Peninsula during austral spring 2014. Note differences among vertical profile panels in scale of x-axis.

Figure 2.7. Size distribution of chlorophyll a in bays (left panels) and channels (right panels) along the Western Antarctic Peninsula during austral spring 2014. Total chlorophyll a ($\mu\text{g L}^{-1}$), cast number, and collection depth (m) corresponding to depth of CTD fluorescence maximum indicated inside the rings.

Figure 2.8. Initial biomass of herbivorous protists in dilution experiments conducted in fjords along the Western Antarctic Peninsula during austral fall 2013 (A and B) and spring 2014 (C and D) expressed as μg of Carbon L^{-1} (A and C) and percent of total (B and D). BS= Bismarck Strait.

Figure 2.9. Evolution of chlorophyll a concentration ($\mu\text{g L}^{-1}$) sampled each day (T0-T7) of the seven-day temperature perturbation experiment in incubations performed at ambient (white columns) and elevated (grey columns) temperatures. Error bars represent standard deviation of the mean of four replicate incubation bottles.

CHAPTER THREE

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Doing more with less?

A cost-benefit analysis of sampling effort vs. data quality in protistan grazing-rate measurements

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ABSTRACT

The dilution method is the standard protocol to quantify phytoplankton grazing-mortality rates and has been key in developing an understanding of protistan grazing. Yet the method's laborious application limits the achievable sampling resolution needed to fill knowledge gaps in deciphering the environmental and biotic drivers of protistan grazing and its impact on net primary production. We assessed the reliability of an abbreviated method known as the 2-point, by analyzing a dataset of 77 dilution experiments performed using 4-5 dilutions in a wide range of geographic regions, chlorophyll concentrations, temperatures, and plankton species composition, in order to enable practitioners to make informed choices based on how experimental design affects rate-estimate accuracy. Overall, we found that rate-estimates for either phytoplankton growth (μ) or grazer-induced mortality (g) obtained using only two dilution levels did not substantially deviate from those obtained when using multiple dilutions, and that their accuracy was satisfactory and similar in magnitude to the inherent error associated with the dilution-series estimates ($\pm \sim 0.1 \text{ d}^{-1}$). Increasing biomass in the dilute treatment from 10 to 40% increased the magnitude and variance of deviations from dilution series rates approximately 2-fold for both μ and g . Recognition of the biomass vs. accuracy tradeoff gives practitioners leeway in working with less dilute samples for greater procurable biomass at the expense of better constrained estimates. Increasing replication from 1 to 2 bottles in each dilution increased average accuracy of 2-point estimates 2-fold, but adding a 3rd bottle did not bring significant improvement. Although estimates should be best interpreted based on groups of experiments rather than individually, the 2-point

abbreviated sampling design can facilitate acquisition of high-resolution data on predation rates across seasons, latitudes, and in response to environmental conditions in the ocean - all critical factors necessary to parameterize protistan herbivory in global biogeochemical models.

Introduction

Microzooplankton occupy a pivotal position in pelagic food webs. As active herbivores, the dominantly protistan grazers exert significant influence on the species composition, size structure, and abundance of phytoplankton (Verity 1986; Strom et al. 2007; Mariani et al. 2013), thereby affecting primary production (Banse 2013) and the flow of carbon in the ocean (Legendre & Le Fevre 1995; Legendre & Rassoulzadegan 1995; Calbet & Landry 2004). Key agents of geochemical cycles (Buitenhuis 2010) and important constituents of the microbial loop (Azam 1983), phagotrophic protists also are prey to meso- and macro-zooplankton (Schmidt et al. 2006; Saiz & Calbet 2011), acting as an important trophic link channeling otherwise unavailable primary production up the food web. Thus quantification of these predators' feeding rates is crucial to understanding trophic linkages and biogeochemical cycles.

Although the significance of grazing by herbivorous protists has been widely recognized, empirical measurements have been challenging. Since its introduction, the dilution method (Landry & Hassett 1982) has become the standard protocol to quantify protistan-grazing rates in mixed plankton assemblages (*see* Calbet & Landry 2004). The dilution method presents several advantages: it is conceptually simple; it requires limited manipulation of the fragile plankton assemblage; and it measures both instantaneous rates of phytoplankton growth (μ) and grazer-induced mortality (g). Nevertheless, the method still carries limitations associated with the incubation process, such as the difficulty to recreate the in situ light field or level of turbulence (McManus 1995, Ross et al. 2011). Furthermore, preparing the dilutions requires manipulation of large volumes of seawater,

and pre- and post incubation analyses are extensive. The logistical effort required by the dilution method to obtain even a single rate measurement precludes acquisition of the large sample sizes needed to fill knowledge gaps with respect to seasonal, environmental and regional variations in grazing rates (see review by Schmoker et al. 2013). The method's logistics also limit empirical investigations of physical, chemical, and/or biological covariates of grazing rate magnitude, hindering predictions about food web responses to climate-driven ocean changes (Caron & Hutchins 2013).

In their introduction of the dilution method, Landry & Hassett (1982) suggested that any two dilution levels could be used to estimate grazing rates, providing a “short cut” alternative to the dilution series. Easier and faster to use, this “2-point method” (Gallegos 1989) offers the capacity to increase measurement frequency and efforts have been made to promote its wider application (Strom et al. 2006, Strom and Fredrickson 2008, Chen 2015). Several studies have used the 2-point method (e.g. Landry et al. 1984, 2008, 2009, 2011; Worden & Binder 2003, Menden-Deuer & Fredrickson 2010). Rates estimated using the 2-point approach are considered conservative (Worden and Binder 2003; Lawrence and Menden-Deuer 2012) and in general do not vary significantly from rates obtained using a linear regression (Worden and Binder 2003; Strom and Fredrickson 2008). Yet protocols vary widely among studies, including the dilution-level used, the method of calculating rates, and the number of replicates per dilution. Chen (2015) subjected an extensive global data set to analysis and concluded that replication in the 2-point dilution method was not absolutely necessary, i.e. that one could use only one bottle per end-point.

One significant concern regarding the 2-point approach is that, in contrast to measurements made along a dilution gradient, using two points does not allow detection of deviations from linearity, potentially violating a critical assumption of the dilution method, i.e. that grazing pressure is a linear function of dilution. Deviations from linearity may result from feeding thresholds (Lessard & Murrell 1998), from grazer mortality in a very dilute treatment (Dolan et al. 2000), or from a feeding saturation response at high food levels (Gallegos 1989), all of which can yield misestimates of grazing rates. Although methods to estimate rates from non-linear data have been repeatedly discussed (e.g. Gallegos 1989, Evans & Paranjape 1992, Landry et al. 1995, Moigis 2006), the frequency of non-linearity in dilution series experiments is largely unknown, as deviations are not routinely reported. Thus if linearity is either not assessed or deviations not reported, some advantages of using a full dilution series may not compensate for the amount of labor associated with its application. As a first step, a thorough assessment of the accuracy of the 2-point method in estimating both the phytoplankton growth rate (μ) and heterotrophic protist grazing rate (g) is needed so that it can be more widely trusted and applied.

Here we build on previous work assessing the 2-point method (Chen 2015) to add to the understanding on how protocol choices may influence the quality of the data. We do not aim to be prescriptive in the application of the method, but rather to quantify the consequences on the means and variations of rate-estimates when practitioners make specific choices regarding number of dilutions, dilution level, and replication. We analyzed a data set of unpublished results from dilution experiments to compare estimates

of phytoplankton growth and grazing mortality obtained using only two points to those obtained using linear regression of a dilution series. We assessed the effect of level of dilution and of replication on the accuracy and the variance of 2-point estimates, related these effects to in situ conditions of phytoplankton biomass, and addressed the linearity concern, with the ultimate goal of providing a qualitative and quantitative analysis of trade-offs in applying the 2-point method.

Methods

Data source

We used an existing data set consisting of unpublished results of 77 dilution experiments performed by our laboratory at different times of year and in diverse marine environments. Some experiments were conducted at sea aboard research vessels: in the North Atlantic (NA) during early spring (N= 15), and along the West Antarctic Peninsula (WAP) during austral late fall (N= 11) and austral late spring (N= 13). The remaining experiments (N=38) were conducted onshore at the University of Rhode Island Graduate School of Oceanography during summer, with water collected from Narragansett Bay (NB) at the site of the NB long-term phytoplankton time series (N=36; <http://www.gso.uri.edu/phytoplankton/>), or from a dock in Jamestown, RI (N=2).

Details of the methods used for setting up the experiments and chlorophyll a (chl a) measurements have been previously described (Morison and Menden-Deuer 2015). Water was collected either from the surface or subsurface, often at the apparent chl a maximum, using either Niskin bottles mounted on the ships' CTD rosette, or by bucket grabs (NB). To exclude larger grazers, the collected water was gently screened using a 200 μm mesh, except for 8 experiments in NB when the mesh used was 150 μm . We refer to this screened fraction as “whole seawater” (WSW). A portion of the collected water was gravity-filtered through a 0.2 μm membrane capsule (Pall) to obtain filtered seawater (FSW). FSW and WSW were mixed in various proportions to achieve a series of dilutions. The number of dilutions varied from 4-5, except for a subset of experiments (NA; N= 6), in which only 3 dilution levels were used. Once prepared the various

dilutions were dispensed by siphoning into clear 1 or 2 L polycarbonate bottles. The number of replicates per dilution level varied (2-3). In most cases, all bottles were amended with macronutrients (N, P, and Si) except when in situ nutrients were assumed to be in excess. To serve as nutrient controls, additional 100% replicates were incubated without added nutrients. In a few cases these additional nutrient controls were also included at the 10% WSW dilution level. At sea, incubations took place in on-deck tanks at depth-adjusted irradiance simulated by using either screen mesh or blue light filter. Onshore, incubation bottles were strapped to rotating plankton wheels. Incubation vessels were kept at ambient temperature by flow-through water. Initial Chl a concentration in the 77 experiments ranged from 0.17 to 18.5 $\mu\text{g L}^{-1}$, and incubation water temperature that varied from -1 °C to 26 °C.

Estimation of phytoplankton growth and grazing rates

For each experiment, the apparent phytoplankton growth rate (k) in each replicate of each dilution level was calculated from 24-hour changes in chl a:

$$k = 1/t * \ln (P_t/P_0) \quad (\text{Equation 1})$$

where t = incubation time (in units of day), and P_0 and P_t represent the chl a concentration at the beginning and end of the experiment respectively. From the replicates of each dilution level, an average k value was calculated $\pm$ one standard deviation of the mean (SD). The effect of nutrient addition was assessed by comparing k in nutrient amended and non-amended replicates using a paired t-test. If a significant nutrient effect on k was detected, nutrient controls were excluded when calculating average k to ensure that the

nutrient treatment was the same within all levels of the dilution series. When no effect of nutrient addition was detected, all WSW replicates, amended or not, were combined.

When based on the full series of dilutions, rates of instantaneous phytoplankton growth (μ) and grazing mortality (g) were determined following the Landry & Hassett method (1982), in which the coefficients of a linear regression of k vs. the respective dilution factor yield g and μ , from the negative slope and y-intercept respectively. For all experiments, the hypothesis that the regression slope = 0 was tested. When a regression slope was not significantly different from zero ($p > 0.05$), g was set to 0, and the average k of all dilution levels was used as an estimate of μ (Murrell et al. 2002; Chen et al. 2009). We subsequently tested whether the linear regression data deviated from linearity using ANOVA (Zar 2010). If such deviations were detected, dilution series plots were visually inspected and a set of adjusted rates was obtained by estimating g from regression of the linear portion of the data only, and μ from the equation $\mu = g + k_1$ (Landry et al. 1984).

For 2-point grazing-rate estimates, we used as one endpoint the average k in the non-diluted treatment, and as the other endpoint the average k in either one of three dilution levels corresponding to a fraction of WSW of 0.1 (± 0.02), 0.22 (± 0.04), and 0.44 (± 0.07). In what follows, we refer to 2-point rate estimates as μ^* and g^* followed by a subscript indicating the dilution level used (e.g. μ^*_{10} and μ^*_{20} for growth rates estimated respectively using 10% and 20% WSW dilutions).

When using the most diluted treatment ($\sim 10\%$ WSW), we assumed that the average k in this most dilute treatment served as a reasonable estimate of phytoplankton

instantaneous growth rate, i.e. $\mu^*_{10} = k_{0.1}$ (Worden & Binder 2004, Strom & Fredrickson 2008, Menden-Deuer & Fredrickson 2010). To calculate the corresponding 2-point grazing rates, we followed procedures by Landry et al. (1984) and the following equations:

$$g^*_{10} = \mu^*_{10} - k_1 \quad (\text{Equation 2})$$

where k_1 refers to the average k in the 100% WSW treatment.

For estimating grazing rates using either the ~20 or ~40 % WSW dilution level, we used the equation:

$$g^*_{20} \text{ (or } g^*_{40}) = (k_d - k_1) / (1 - x) \quad (\text{Equation 3})$$

where k_d represents the average k in the diluted treatment, and x is the corresponding fraction of WSW (Landry et al. 1984). Once g^*_{20} or g^*_{40} were obtained, corresponding 2-point phytoplankton growth rates were then determined from the equation:

$$\mu^*_{20} \text{ (or } \mu^*_{40}) = g^*_{20} \text{ (or } g^*_{40}) + k_1 \quad (\text{Equation 4})$$

Comparison of 2-point estimates and regression rates

We compared 2-point and dilution series estimates in two ways. First, using dilution series rates obtained without testing the regressions for deviations from linearity but rather taking them at face value, which we refer to as “blind” dilution series rates. Second, using “adjusted” dilution series rates, i.e. those obtained after testing for deviations from linearity and adjusted as described above. For dilution series that met the linearity assumption, no adjustment was needed and the rates used in both “blind” and “adjusted” comparisons were the same.

For both phytoplankton growth and grazing-mortality rates, we evaluated the agreement between the 2-point and the dilution series μ and g estimates, by performing a model II linear regression analysis for each of the 2-point μ^* estimates (i.e. μ^*_{10} , μ^*_{20} and μ^*_{40}) vs. each “blind” and “adjusted” dilution series μ , and similarly of 2-point g^* estimates vs. each “blind” and “adjusted” dilution series g , which resulted in a total of 12 comparison regressions. Regression slopes <1 indicate a tendency for the 2-point method to underestimate rates, whereas slopes >1 indicate rate overestimates. A perfect agreement would result in a regression slope of 1, although a significant y-intercept would indicate a consistent bias. We used a one-sample t-test to compare the Major Axis slope and y-intercept of each comparison regression to theoretical values of 1 and 0 respectively (Zar 2010).

Dilution series for which the “blind” linear regression yielded a positive slope, i.e. negative g ($N=7$) were not further included in the comparisons, reducing the initial number of experiments used in the present analysis to 70. In several cases, g estimates based on dilution series were ≥ 0 whereas their counterpart 2-point estimates were <0 ($N=11, 8$, and 15 for g^*_{10} , g^*_{20} , and g^*_{40} respectively). Although <0 g estimates are anomalous, they were retained in the comparisons since neglecting them would bias the comparisons’ outcome towards better agreement. These <0 g estimates were however not included in the calculation of 2-point g averages. Excluded from the “adjusted” comparison were dilution series exhibiting such deviations from linearity that no adjustment was possible ($N=6$).

Assessing the accuracy of 2-point estimates

To assess the accuracy of the 2-point method, i.e. how much 2-point estimates deviated from the dilution series estimates, we calculated the difference between each 2-point rate estimate and the corresponding rate estimate from the “blind” dilution series. We investigated how accuracy was affected by the choice of the diluted treatment by comparing calculated differences for each treatment using ANOVA followed by a Tukey multi-comparisons test. Furthermore, we used the mean error (SD) associated with dilution series rates as a criterion of assessing measurement variation against which mean differences could be compared. To assess whether differences between the two methods were acceptable, their range was measured against the range within which 95% of the errors (SD) from full dilution series rates were predicted to occur, which we calculated as $= \pm \text{mean error} + t_{(95\%, \text{DF})} \times \text{error SD}$. Finally, we considered whether linearity (or deviations thereof) as well as initial chl a concentration affected the magnitude of the differences between the two methods.

Assessing the variability of 2-point estimates

To assess the variability of 2-point estimates, for each experiment we calculated all the 2-point estimates that could be obtained from all the possible pairings of each k_d and k_1 values, and generated coefficients of variation (CV, as %) from the average and SD of these estimates. This was repeated for each of the three diluted treatments used, and the three groups of CVs were then compared in a Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn’s multiple-comparisons test. The latter is a

conservative post-hoc test that applies a Bonferroni correction required because of the many comparisons made. Prior to analysis, CVs outliers were removed from the data using the Median Absolute Deviation (MAD) method (Leys et al. 2013), which is more robust than assessing outliers based on the number of standard deviations.

Assessing the effect of replication on the accuracy of 2-point estimates

We assessed the effect of replication by comparing how much estimates of μ^*_{10} and g^*_{10} based on a different number of replicates departed from dilution series rate-estimates. Instead of using a random approach for selecting replicates (see Chen 2015), we used a “worst-case” scenario approach, as we wanted to quantify and compare the maximum extent of deviations between 2-point and dilution series estimates as a function of number of replicates. Thus for our analysis, we selected replicates that yielded 2-point rate estimates that were most different from the ones obtained through regression of the dilution series rates. The number of comparisons possible depended on the different replication schemes used in the different experiments (Table 3.1). We compared differences based on either one or two replicates (i.e. pairs) using a Wilcoxon matched-pairs signed rank test (N= 69). We compared differences based on up to three replicates for a subset of 24 and 28 experiments for g^*_{10} and μ^*_{10} respectively using a one-way ANOVA with repeated measures, followed by a Holm Sidak’s multiple comparisons test when data were normally distributed (μ^*_{10}), and for the non-normally distributed g^*_{10} we used the non-parametric alternative Friedman test.

All statistical analyses were performed at an alpha value ≤ 0.05 . Average values are presented $\pm$ one standard deviation of the mean.

Results

Comparison of dilution-series and 2-point estimates of μ

Overall, μ and g rate-estimates based on 2-point and those based on dilution series followed a similar distribution (Table 3.2). Estimates of μ obtained from dilution series without testing for deviations from linearity (“blind”, $N=70$) ranged from -0.16 to 2.34 d^{-1} and averaged $0.78 (\pm 0.71) \text{ d}^{-1}$. Averages of 2-point μ^* estimates across dilution levels were similar to the averages of dilution-series rates (Table 3.2), whereas their range increased with increasing fraction of WSW in the diluted treatment, from -0.17 to 2.29 d^{-1} for μ^*_{10} , to -0.35 to 2.40 d^{-1} for μ^*_{20} , and -0.35 to 2.49 d^{-1} for μ^*_{40} . Dilution-series estimates had comparable ranges and averages, irrespective of whether deviations from linearity were tested for (“adjusted”, $N=61$), or not (Table 3.2). “Adjusted” estimates of μ ranged from -0.16 to 2.43 d^{-1} and averaged at $0.83 (\pm 0.73) \text{ d}^{-1}$. Notably, variance was nearly identical on both types of dilution-series estimates.

Comparison regressions showed good agreement between estimates of μ based on dilution series and those based on only two dilution levels (Fig. 3.1). Slopes from comparison regressions of μ varied from 0.95 to 1.04 and from 0.93 to 1.01 in the comparisons of 2-point vs. “blind” and “adjusted” estimates respectively. In most cases, comparison slopes were not significantly different from 1 ($p \geq 0.15$; Table 3.3), except for estimates of μ^*_{10} , for which values of regression slopes indicated an underestimation of 5% and 7% from “blind” and “adjusted” dilution-series rates respectively (Table 3.3). In some cases, the y-intercepts of the comparison regression were significantly different from 0 , however their magnitudes were negligible (data not shown).

Comparison of dilution-series and 2-point estimates of g

Grazing-mortality estimates (g) varied more substantially. “Blind” dilution-series estimates of g ranged from 0 to 1.22 d⁻¹ and averaged 0.28 ($\pm$ 0.30) d⁻¹. The range of 2-point g^* estimates was similar to that of dilution series rates (Table 3.2), but their average varied with the fraction of WSW in the diluted treatment, from 0.31 ($\pm$ 0.20) d⁻¹ for g^*_{10} (N= 58) to 0.25 ($\pm$ 0.33) d⁻¹ for g^*_{20} (N= 48) and 0.19 ($\pm$ 0.34) d⁻¹ for g^*_{40} (N= 50), not including negative estimates (see Methods). “Adjusted” dilution-series estimates of g had a range similar to “blind” estimates (0 to 1.40 d⁻¹) but had a higher average of 0.36 ($\pm$ 0.34) d⁻¹. Like for μ , the magnitude of the variance was similar for both types of dilution-series g estimates.

Comparison regressions indicated good agreement between estimates of g based on dilution series and those based on only two dilution levels, although data were more variable than for μ (Fig. 3.1 G-L). Slopes for g varied from 0.91 to 1.14 in the “blind” comparison and from 0.76 to 1.02 in the “adjusted” comparison (Table 3.3). Most slopes were not significantly different from 1 ($p \geq 0.14$), except for estimates of g^*_{10} , with an offset indicating an underestimation of 9 % in comparison with “blind” dilution series rates, and of 24 % in comparison with “adjusted” dilution series rates (Table 3.3). As for μ , y-intercepts of comparison regressions for g were sometimes different from 0 but had negligible magnitudes.

Departure from a 1:1 agreement between the 2pt and regression based estimates was larger for g than for μ . This was in part due to negative estimates of g^* that often

corresponded to dilution-series g of 0 (10 out of 11 negative g^*_{10} rates, 7 out of 8 negative g^*_{20} , and 11 out of 15 negative g^*_{40} ; Fig. 3.1). This can be expected since anomalously negative 2-point estimates of g can be obtained when calculating differences between two points along a non-significant regression slope.

Accuracy of 2-point estimates

To assess the accuracy of the 2-point estimates, we quantified how much they deviated from “blind” dilution series rates as a function of the proportion of biomass in the diluted treatment. Increasing plankton biomass increased both the range of differences between the two methods, and the proportion of differences outside the interval containing 95 % of errors associated with dilution series rates (Fig. 3.2). Compared to dilution series, the 2-point approach generally underestimated μ , by an average of 0.02 ($\pm$ 0.07), 0.01 ($\pm$ 0.11), and 0.07 ($\pm$ 0.15) d^{-1} for μ^*_{10} , μ^*_{20} , and μ^*_{40} respectively. The average deviations between 2-point and dilution-series estimates were similar in magnitude to the average error (SD) of 0.05 ($\pm$ 0.03) d^{-1} associated with dilution series estimates of μ .

The range of differences was narrowest for μ^*_{10} and largest for μ^*_{40} (Fig. 3.2). For μ^*_{10} , most differences (93 %) fell within the 95% range of errors (SD) associated with rates from dilution series ($\pm$ 0.11 d^{-1}). Variance of differences increased with increasing proportion of WSW in the diluted treatment, resulting in a greater proportion of differences falling outside the 95% SD range (23 % and 51 % for μ^*_{20} and μ^*_{40} respectively).

Compared to dilution series, the 2-point approach underestimated g , by an average of $0.04 (\pm 0.07)$, $0.04 (\pm 0.15)$, and $0.10 (\pm 0.19) \text{ d}^{-1}$ for g^*_{10} , g^*_{20} , and g^*_{40} respectively. In comparison, dilution-series estimates of g had an average error (SD) of $0.06 (\pm 0.03) \text{ d}^{-1}$.

The narrowest range of differences for grazing rates was found for g^*_{10} and the range increased with increased plankton biomass in the diluted treatment used (Fig. 3.2). For g^*_{10} , a large proportion (90 %) of the differences fell within the 95% range of SD associated with rates from dilution series ($\pm 0.13 \text{ d}^{-1}$), but this proportion decreased with increasing fraction of WSW in the diluted treatment to 71 % and 48 % for g^*_{20} and g^*_{40} respectively.

For all g^* and for μ^*_{20} and μ^*_{40} , more than half of the differences (66-69 % and 54-69 % respectively) were negative, indicating a tendency for the 2-point approach to yield underestimates. For μ^*_{10} , approx. half the differences were either positive (52 %) or negative (48 %), indicating that the 2-point approach can also yield overestimates of μ .

Variability of 2-point estimates

The range of CVs for 2-point estimates increased with increasing plankton biomass in the diluted treatment (Fig. 3.3), and CVs were substantially (3 to 4-fold) lower for μ than for g . CVs for μ ranged from 0.3-30.5 %, 0.2-30 %, and 0.2-54 % for μ^*_{10} , μ^*_{20} , and μ^*_{40} respectively, and their corresponding medians of 6.4 %, 5 %, and 10 % differed significantly from each other ($p = 0.038$; Fig. 3.3). CVs for g ranged from 2.7-81

%, 1.4-123 %, and 0.5-205 % for g^*_{10} , g^*_{20} , and g^*_{40} respectively. Median CV for g^*_{10} (19.8 %) was significantly smaller than for both g^*_{20} (34 %) and g^*_{40} (42 %).

Effects of replication on the accuracy of 2-point estimates

We investigated the effect of the number of replicates included in the calculation of 2-point rates μ^*_{10} and g^*_{10} on their maximal difference from dilution-series rates. For both estimates, increasing the number of replicates from 1 to 2 significantly decreased the range of differences (Fig. 3.4) and reduced the absolute magnitude of the median difference (Table 3.4) by $1/3$ (μ^*_{10}) to $1/2$ (g^*_{10}). Further increasing the number of replicates to 3 in the subset of data available with a complete set of triplicates also reduced differences (Table 3.4), but the reduction was only significant for g^*_{10} and not for μ^*_{10} (Fig. 3.4).

Effects of non-linearity in dilution series data

Out of the 70 dilution-series experiments available for analysis, 28 (39 %) exhibited statistically significant deviations from linearity. Visual inspection of the regression plots after data testing indicated a feeding saturation response in 17 experiments. Other deviations from linearity were associated with low net apparent growth rates in the 10% WSW dilution (N= 7). Differences between 2-point and dilution-series estimates tended to be greater for non-linear than for linear data (Table 3.5) and varied over a range that was on average 1.6-fold (for g) to 2.2-fold (for μ) greater (Fig. 3.5). There was no correlation between initial chl *a* concentration and either the

occurrence of non-linearity or the effect of the fraction of WSW in the diluted treatment on the magnitude of the differences between 2-point and dilution-series rates (Fig. 3.5).

Discussion

Predation by herbivorous protists is generally accepted as the major loss factor in phytoplankton production (Banse 2013) and has been hypothesized as a contributing factor in driving large-scale phenomena, such as the North Atlantic Spring Bloom (Behrenfeld 2010; Behrenfeld & Boss 2014). To test these hypotheses, we need to gain a deeper understanding of the critical process of phytoplankton grazing mortality and its drivers in the global ocean. This necessitates acquiring a higher resolution dataset of grazer-induced mortality rates from diverse geographic locations, seasons, and environmental and biological conditions. Rate estimates are also needed at higher than daily resolution in order to match observations from autonomous in situ assets. Acquisition of more frequent grazing-rate estimates could be more easily achieved if experimental logistics can be reduced without sacrificing measurements' accuracy. Using a large and diverse dataset of dilution experiments, we compared estimates of both phytoplankton growth and grazing-mortality rates obtained using a series of dilutions with those obtained using only two, and are now able to provide recommendations about the reliability and applicability of the abbreviated method.

Overall, we found that rate estimates for either phytoplankton growth or grazer induced mortality obtained using only two dilution levels did not substantially deviate from those obtained when using multiple dilutions, and that their accuracy was satisfactory and similar in magnitude to the inherent error associated with the dilution-series estimates, supporting findings by Chen (2015). Phytoplankton growth rates (μ) estimates based on either two or a series of dilutions were essentially equivalent.

Similarly, grazer-induced mortality rates (g) based on two points were overall equivalent to estimates based on a series of dilutions, or if deviations did occur, they were conservative, meaning they provided an underestimate.

The agreement between the two methods was most robust when assigning to μ the value of k in the treatment containing the smallest fraction of plankton biomass (~10% WSW), thereby confirming results from several previous investigations (Strom et al. 2006, Strom and Fredrickson 2008, Chen 2015). Yet an increase in plankton biomass in the dilute treatment may be desirable for post-incubation flow-cytometry or DNA analysis (e.g. Landry et al. 2008, Taniguchi et al. 2012), or may be necessary to achieve a detectable signal in very low plankton biomass conditions, such as wintertime or the oligotrophic ocean. Our results indicate a biomass vs. accuracy tradeoff: when doubling plankton biomass in the diluted treatment from 10 to 20 %, average accuracy of 2-point estimates was maintained and the increase in variance was not statistically significant, but doubling biomass further (to 40%) increases 2 to 3-fold the proportion of estimates with deviations outside the range of errors from dilution series, i.e. the chance that 2-point estimates may not be acceptable based on dilution-series errors.

Both the analyses of effect of replicates and variability (CVs) indicate that inclusion of at least duplicates is strongly advisable, in contrast with previous recommendations that only one replicate can be used (Chen 2015). Based on the mere fact that critical values of the t-distribution substantially increase as degrees of freedom decrease - by a factor of three when increasing degrees of freedom from one to two -,

using duplicates will substantially, that is by 3-fold, decrease confidence intervals of 2-point estimates.

Using only two dilutions greatly reduces sampling effort, including time needed to prepare the dilutions, and measurements of chl a. Furthermore, when performed at sea, the 2-point method can save at least 60 % of collected seawater just by reducing the number of bottles from 12-15 to four, leaving more seawater to be shared among investigators or for increasing the number of dilution experiments that can be done in parallel. The agreement between the two methods is, however, based on the inclusion of a group of experiments in the analysis, and as has been noted before (Chen et al. 2015), does not always hold for individual 2-point estimates, a proportion of which showed departures from the corresponding dilution-series estimates outside acceptable bounds. The need for multiple experiments may seem to defeat the initial purpose of the 2-point approach (i.e. that of minimizing sampling logistics). Nevertheless, in a heterogeneous environment one would want to make multiple measurements across gradients of depth, light, or temperature, even when using a full dilution series. Making multiple measurements would thus be more practical with the 2-point method, with the added benefit of obtaining greater confidence in the yielded estimates.

Previous comparison of estimates obtained by dilution series and 2-point approaches performed in the field have attributed their good agreement to the dependably linear response of phytoplankton net growth to dilution (Landry et al. 2011). In our comparison, experiments with non-linear data indeed yielded the largest differences between estimates from the two methods. Nevertheless, despite the widespread concern

associated with non-linear data, which represented a substantial fraction of our total data set (1/3), their inclusion in our analysis did not significantly alter the outcome, nor did it bias the data to either under- or overestimates of rates. Thus, the theoretical significance of losing the ability to assess non-linearity with the 2-point method is not relevant in terms of practical changes of rate estimates as long as groups of experiments are considered.

The ability to predict under what conditions a non-linear response to dilution is likely to occur would be an asset to decide when a two-point approach may be inappropriate. However, generally applicable, strong predictors of non-linearity remain elusive (Chen et al. 2014). Chl-a is often considered a major driver of non-linearity: functional responses of feeding saturation are often associated with coastal or estuarine conditions of high chl a (e.g. Gallegos 1989, Strom et al. 2001, Teixeira and Figueiras 2009), and non-linearity could also occur when low chl a concentration renders detection of signal difficult for some dilution levels. We found no relationship between chl a concentration and deviations from linearity, the latter occurring across the entire range of chl a data, suggesting that neither feeding saturation nor lack of signal, i.e. chl a concentration, is a reliable predictor. Several factors may contribute to obscure the relationship between functional responses and ambient chl a. Chen et al. (2014) found microzooplankton grazing half-saturation constants to be highly variable and suggested that this variability may reflect the ability of grazers to acclimate to and fully exploit the ambient prey abundance by minimizing feeding thresholds/food satiation in low/high prey abundance. How fast grazers in situ acclimate to a patchy and fluctuating prey

environment, including in terms of phytoplankton species, is largely unknown. Moreover, heterotrophic protists are highly selective grazers. Species-specific feeding preferences rather than absolute concentration of bulk measurements of prey abundance (Chl a or carbon) may be stronger determinants of feeding saturation and thresholds (Menden-Deuer & Kiorboe, in review), as well as stronger drivers of realized grazing (Lawrence & Menden-Deuer 2012). Furthermore, predators may rely on prey sources other than those measured by chl a, such as bacteria (Strom et al. 2007), and often are mixotrophic (Flynn et al. 2012). Non-linear responses have also been attributed to changes in the composition and abundance of the predator community during incubations (Dolan et al. 2000, Dolan & McKeon 2004, Agis et al. 2007), or to trophic cascades within the grazing community (Calbet & Saiz 2013). While deviations from linearity may stem from many factors other than prey abundance, there is a definite need to identify whether patterns of non-linearity exist. This may be possible from an exploration of the continuously growing global dataset of grazing-rate measurements and of supporting environmental and biological data.

While we found robust consistency between phytoplankton growth-rate estimates from either method, such estimates do not necessarily reflect in situ rates. Whether they are performed using only two or multiple dilutions, grazing experiments carry limitations associated with incubations, one of the most challenging being failure to replicate the in situ light environment in a mixed layer (Ross et al. 2011). This can result in pigment change artifacts, which makes it difficult to interpret the chl a signal (see Behrenfeld et al. 2015). Light-mediated changes in μ alter the ratio of primary production consumed

(i.e. $g:\mu$, Morison & Menden-Deuer 2015). Poorly constrained estimates of μ would also jeopardize estimations of phytoplankton accumulation rate ($\mu - g$), an important metric for understanding the annual dynamics of phytoplankton biomass (Behrenfeld et al. 2014). Combining chl-a based measures of phytoplankton growth with other alternate methods, including cell counts performed using flow-cytometry and/or microscopy (Landry et al. 2008, Taniguchi et al. 2012), does not completely overcome the inability of fixed-depth incubations to mimic phytoplankton cells' light history. Thanks to its simpler execution, the 2-point approach could be used to address the challenge of replicating in situ conditions by making it possible to simultaneously conduct incubations exposed to different simulated light levels, from which rates estimates for the mixed layer closer to “true” in situ rates could be derived.

Recommendations

When using the 2-point approach, 2-point estimates can be best constrained relative to those from a full dilution series by using a diluted endpoint with low plankton biomass (~10%). If a larger fraction of plankton biomass is needed for additional sample analyses beyond chl-a, or if there is a concern that low in situ biomass (low chl-a), may influence the sensitivity of the method, the experimenter can now, based on our analysis, weigh the trade offs in choosing the appropriate diluted treatment. A most dilute treatment will constrain the rates best relative to a full dilution experiment, whereas decreasing dilution will maintain a statistically equivalent estimate of the mean rate, but increase the variance. Finally, using duplicates rather than triplicates at each end-point

(total 4 bottles) should significantly reduce variability of 2-point estimates (compared to using a single replicate) and still afford considerable logistical advantages. Although limited, our data indicate that increasing the number to three bottles per endpoint may not be worth the extra sampling effort, as it may not drastically improve how constrained the estimates would be in comparison to dilution-series rates.

Conclusion

Extensive characterization of the impact of grazing on phytoplankton dynamics exist for the Equatorial Pacific and has been achieved with ample use of the 2-point approach (i.e. Landry et al. 2003, 2011a, 2011b). It is time for the approach to be extended to other important regions of the global ocean and to seasons outside annual productivity peaks, particularly in view of recent hypothesis on the role of grazing in the annual cycle of phytoplankton (Behrenfeld 2014).

The application of the 2-point sampling design can facilitate acquisition of higher-resolution data on predation rates across seasons, latitudes, and in response to multiple environmental conditions in the ocean – all critical factors necessary to parameterize protistan herbivory in global biogeochemical models.

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Table 3.1. Summary of location, season, and experimental set-up of dilution experiments used to compare 2-point and dilution series rates. NA = North Atlantic, NB = Narragansett Bay, WAP= West Antarctic Peninsula, Sp= spring, S= summer, Asp= Austral spring, AF= Austral fall.

Location	Season	# of experiments	# of dilution levels	# of replicates			Total # of bottles
				10%	100%	Other dilution levels	
NA	Sp	6	3	2	2	2	7
NA	Sp	1	4	0	2	2	10
NA	Sp	8	5	2	2	2	12
NB	S	1	4	2	2	2	8
NB	S	8	4	3	3	3	12
NB	S	16	5	2	2	2	10
NB	S	4	5	3	2	2	11
NB	S	9	5	3	3	2	12
WAP	AF	1	5	2	2	2	12
WAP	AF	8	5	3	3	2	14
WAP	AF	2	5	3	3	2	16
WAP	Asp	13	5	2	2	2	12
	Total	77					

Table 3.2. Summary of phytoplankton growth (μ) and grazing mortality (g) rate estimates obtained using either regression analysis of dilution series data, or the 2-point method with three different diluted treatments containing an average of either 10, 20, or 40 % undiluted seawater. “Blind” dilution series rates are those obtained from the regression coefficients without testing the regression for deviations from linearity. For experiments that exhibited deviations from linearity, dilution series rates were “adjusted”, i.e. obtained from the linear portion of the data only.

	Blind Dilution series	Adjusted Dilution series	2-point estimates per average minimum fraction of WSW (SD)		
			10% (2)	22% (4)	44% (7)
<i>Growth rates (μ)</i>			μ^*_{10}	μ^*_{20}	μ^*_{40}
Range	-0.16 - 2.34	-0.16 - 2.43	-0.17 - 2.29	-0.35 - 2.40	-0.35 - 2.49
Average	0.78 (0.71)	0.83 (0.73)	0.77 (0.68)	0.81 (0.78)	0.71 (0.75)
N	70	61	69	56	67
<i>Grazing rates (g)</i>			g^*_{10}	g^*_{20}	g^*_{40}
Range	0 - 1.22	0 - 1.40	0 - 1.09	0 - 1.26	0 - 1.40
Average	0.28 (0.30)	0.36 (0.34)	0.31 (0.20)	0.25 (0.33)	0.19 (0.34)
N	70	61	58	48	50

Table 3.3. Regression coefficients from analysis comparing 2-point and dilution series rate estimates of phytoplankton growth, and grazer-induced mortality (shaded columns). Comparisons are made for regression data that were not-adjusted for deviations from linearity (blind) and for those where an adjustment was made (adjusted). The number of experiments available for analysis (N) varied according to the diluted treatment used in the 2-point method, due to differences among experiments in dilution series set-up.

	μ^*_{10} vs μ	g^*_{10} vs. g	μ^*_{20} vs μ	g^*_{20} vs. g	μ^*_{40} vs μ	g^*_{40} vs. g
<i>2-point vs. "blind" regression</i>						
Regression slope	0.95	0.91	1.03	1.01	1.04	1.14
Slope SE	0.011	0.025	0.021	0.067	0.026	0.09
R ²	0.99	0.95	0.98	0.81	0.96	0.71
Testing H ₀ : Slope = 1	S	S	NS	NS	NS	NS
P-value	1.49E-04	4.16E-04	0.19	0.87	0.15	0.14
N	69	69	56	56	67	67
<i>Difference between 2-point and "blind" regression</i>						
Average	-0.017	-0.036	-0.014	-0.036	-0.074	-0.099
SD	0.073	0.069	0.114	0.151	0.152	-0.192
<i>2-point vs. "adjusted" regression</i>						
Regression slope	0.93	0.76	1.01	0.91	1.00	1.02
Slope SE	0.018	0.06	0.018	0.087	0.025	0.090
R ²	0.98	0.76	0.98	0.70	0.97	0.70
Testing H ₀ : Slope = 1	S	S	NS	NS	NS	NS
P-value	1.17E-04	4.82E-05	0.71	0.30	0.89	0.99
N	61	61	50	50	59	59

Table 3.4. Comparison of maximum range and magnitude of differences obtained between 2-point and dilution series estimates as a function of the number of replicates (1 or 2= left two columns; or 1, 2, or 3= right two columns) in the 2-point estimation.

Differences	$\mu*10$		$g*10$		$\mu*10$			$g*10$		
	1	2	1	2	1	2	3	1	2	3
Replicates	1	2	1	2	1	2	3	1	2	3
N	69	69	69	69	28	28	28	24	24	24
Minimum	-0.35	-0.29	-2.97	-0.26	-0.28	-0.26	-0.18	-2.97	-0.26	-0.18
Median	-0.03	0.00	-0.09	-0.05	-0.04	0.02	-0.004	-0.12	-0.09	-0.03
Maximum	0.26	0.17	0.28	0.21	0.26	0.17	0.11	0.28	0.10	0.11
Mean	-0.01	-0.01	-0.09	-0.05	-0.01	-0.006	-0.01	-0.19	-0.09	-0.03
SD	0.11	0.09	0.38	0.10	0.12	0.10	0.06	0.61	0.09	0.08

Table 3.5. Summary of differences between 2-point and dilution-series estimates of phytoplankton growth (μ^*) and grazer-induced mortality (g^*) for linear (L) and non-linear (NL) data.

	μ^*_{10}		μ^*_{20}		μ^*_{40}	
	L	NL	L	NL	L	NL
N	41	28	30	26	41	26
Min	-0.10	-0.29	-0.16	-0.29	-0.40	-0.42
Max	0.10	0.16	0.07	0.37	0.11	0.31
Mean	-0.01	-0.02	-0.01	-0.01	-0.07	-0.08
SD	0.04	0.11	0.05	0.16	0.12	0.20

	g^*_{10}		g^*_{20}		g^*_{40}	
	L	NL	L	NL	L	NL
N	41	28	30	26	41	26
Min	-0.14	-0.23	-0.21	-0.34	-0.51	-0.53
Max	0.13	0.11	0.14	0.43	0.13	0.35
Mean	-0.02	-0.05	-0.02	-0.05	-0.08	-0.10
SD	0.05	0.09	0.07	0.20	0.14	0.24

Figure 3.1. 2-point vs. dilution series based rate estimates (d^{-1}): 2-point phytoplankton growth rates (μ^*) vs. corresponding “blind” (1st column) or “adjusted” (2nd column) dilution-series rates (μ), and 2-point grazing mortality rates (g^*) vs. corresponding “blind” (3rd column) or “adjusted” (4th column) dilution series rates (g). Each row of plots corresponds to 2-point estimates obtained using a diluted treatment with a 10 %, 20 %, or 40 % fraction of WSW. Diagonal solid lines represent linear model II regression Major Axis (MA) and diagonal dashed lines represent 95% CI limits of MA.

Figure 3.2. Differences (d^{-1}) between 2-point and corresponding “blind” dilution series estimates for phytoplankton growth rates (μ , left panel) and grazing mortality rates (g , right panel), as a function of fraction of plankton biomass in the diluted treatment. Lines in the middle of the boxes represent median values and whisker extends to 25th (lower) and 75th (upper) percentile $\pm 1.5 \times$ interquartile range. Values higher/lower than upper/lower limits of whiskers are plotted as individual points. Percentiles (P) are computed as $P = k/(n+1)$, where k is the rank starting at 1 and n is the sample size. Dotted lines represent upper and lower limits of interval containing 95% of the error (SD) associated with dilution series rates. Treatments marked with a different letter were significantly different from each other (ANOVA).

Figure 3.3. Coefficient of variation (%) of 2-point estimates of phytoplankton growth (upper panel) and grazer-induced mortality (lower panel). Note different scales of y-axes. Box plots as in Fig. 3.2.

Figure 3.4. Differences (d^{-1}) between 2-point estimates and dilution series estimates for rates of phytoplankton growth (left panels) and grazing mortality (right panels), as a function of number of replicates used in the 2-point estimation. Treatments marked with a different letter were significantly different from each other (see methods for statistical analyses performed). Box plots as in Fig. 3.3. For clarity, one outlier (-2.97) was omitted from left panels.

Figure 3.5. Differences (d^{-1}) between 2-point and dilution-series estimates as a function of initial chlorophyll a concentration ($\mu\text{g L}^{-1}$), for phytoplankton growth rates (μ , left panels) and grazer-induced mortality rates (g , right panels). Open and solid symbols indicate linear and non-linear dilution-series data respectively

CHAPTER FOUR

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Evaluating FlowCAM to characterize phytoplankton communities, with examples from Narragansett Bay

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Running Head: FlowCAM characterization of Narragansett Bay Phytoplankton

ABSTRACT

The feasibility of automating measurements of plankton abundance and species-richness using a FlowCAM (FC) was assessed by comparing results of FC and microscopy analyses of 43 weekly samples from the Narragansett Bay plankton long-term time series. FC's precision was further assessed by quantifying the variability among FC replicate outputs of average biovolumes and size-distribution. The satisfactory performance of FC was qualitative rather than quantitative. Qualitatively, FC reproduced seasonal trends and identified shifts in abundance and biomass, but missed several occurrences of high abundance of *Skeletonema*, potentially due to pre-filtering of samples. Quantitatively, FC mis-estimated particle abundance by a median factor of 2, usually overestimating or underestimating counts when flagellates or chain-forming diatoms respectively dominated microscopy counts. The number of taxa identified by either method differed by an average of 3, and taxa shared by both methods represented an average of 36% of combined taxa, meaning that taxa were missed by both FC and microscopy. FC was most precise estimating total abundance (17% CV), average biovolume (15 and 28% CV), and size-distribution, but less so in estimates of abundance of large particles (42% CV) and in taxonomic characterization. Some of FC shortcomings, especially regarding taxonomic composition, should be overcome by re-considering the need to pre-filter samples and by increasing the volume processed through the FC. FC can provide a rapid insight into the structure of phytoplankton communities yet needs to be combined with microscopy if high taxonomic resolution and/or high count-accuracy are needed.

INTRODUCTION

Understanding what factors regulate phytoplankton communities is essential to understanding the ecological and biogeochemical functioning of marine ecosystems (Legendre & Le Fevre, 1995; Falkowski et al., 1998; Worden et al. 2015). Such an objective requires sustained characterization of the size structure and species composition of phytoplankton, so that ultimately responses of plankton communities and food webs to environmental changes can be predicted.

Understanding of plankton processes has been hindered in part by the methods used to monitor phytoplankton. Enumeration of samples has traditionally relied on microscopy. Microscopic analysis of plankton is time consuming, long time spent at the microscope can result in fatigue and thus increase the risk of errors, and the pool of needed taxonomic experts is dwindling (Culverhouse et al., 2003; 2006). To overcome the limitations of traditional analyses, several technologies and instruments for the automated counting of plankton organisms have been developed (Benfield et al., 2007).

The FlowCAM[®] (FC) enumerates plankton particles by combining flow cytometry, microscopy, and image analysis (Sieracki et al., 1998), simultaneously delivering 30 different properties (including size and biovolume) for each particle, which can be used in particle identification. Several studies have assessed FC for its ability to provide estimates of abundance comparable to microscopy for both mono- and mixed cultures (Sieracki et al., 1998; Buskey & Hyatt, 2006), as well as for natural samples (See et al., 2005; Ide et al., 2008). The instrument has also been assessed for its ability to accurately measure the size of plankton (Sieracki et al., 1998) and the size spectra and

taxonomic composition of plankton communities (Alvarez et al., 2011; 2013). The reviews found FC to be satisfactory, albeit after substantial consideration given to sample preparation and analysis protocol, and based on usually low taxonomic resolution.

Narragansett Bay (NBay) is a productive estuary located on the east coast of North America and is the site of one of the world's longest-running Plankton Time Series (NBayPTS). Weekly collection and microscopy analysis of plankton samples provide invaluable information that has been essential in understanding the dynamics of the bay's phytoplankton community (Smayda, 1957; 1973; 1998; Karentz & Smayda, 1984; 1998; Lawrence & Menden-Deuer, 2012; Canesi & Ryneerson, 2016) and in detecting long-term changes (Smayda & Borkman, 2004; 2008; Borkman & Smayda, 2009; Windecker, 2010). Weekly microscopy analyses provide data against which the performance of FC can be tested. In addition, some ecologically important parameters such as size distribution and biomass in terms of Carbon are presently not accounted for using microscopy, whereas size and biovolumes are automatic FC outputs.

The objective of this work was to assess the ability of the FC to characterize the dynamics of plankton communities, by assessing the similarity between FC and microscopy in the analysis of the nano- and micro-plankton communities of NB.

METHODS

Study site and sampling program

Samples for NBayPTS are collected once a week from the same site (Station II, 41° 34.2'N, 71° 23.4'W) for temperature, salinity, turbidity, nutrients, size-fractionated chlorophyll a, and light microscopy analysis of abundance and species composition of the microplankton community (size >10 µm). Data are publicly available at the program website (<http://www.gso.uri.edu/phytoplankton/>). For the analysis presented here, 43 samples from NBayPTS collected between 10/14/2014 to 10/14/15 were used. Seawater samples were collected by bucket grab of surface water and subsamples were transferred to opaque 1L bottles for transport to the laboratory. Both microscopy and FC analyses were performed on live, un-concentrated samples.

Microscopy counts

Results of microscopy counts were obtained from the online database. Phytoplankton cells were enumerated under a compound microscope using a Sedgewick-Rafter counting chamber (total volume= 1ml). Organisms' identification was based on morphological features observed under the microscope and organisms were assigned to the highest taxonomic level possible. Organisms unidentifiable to the genus or species level were assigned to taxonomic categories. For the samples analyzed these categories included pinnate diatoms, unidentified dinoflagellates, and flagellates.

FlowCAM analysis

Operators of the FC were trained on the basic use of the instrument. Over the course of the sampling year, four different operators processed the samples. We used a

Benchtop B3 Series FC. The instrument counts and images particles contained in a seawater sample that is drawn through a glass chamber, the flow cell, by means of a syringe pump. The flow cell is mounted in front of a microscope lens. The camera images particles passing within the field of view. Particles contained within each image frame are then extracted and separated into individual images. For each captured particle, a series of particle properties are recorded, and particle concentration is determined automatically.

Live seawater samples were analyzed using the FC fluorescence-trigger mode, which only images particles exceeding a fluorescence threshold, and thus is best to target the autotrophic community in live samples. The depth of the flow cell used sets the upper size limit of particles to be analyzed, while the lower size limit depends on the smallest size that the magnification can resolve and can be specified by the operator. Therefore, to get an overview of the plankton community in the particle size range of interest ($>10\ \mu\text{m}$), samples were analyzed in two size-fractions using two flow cells of different depths at two magnifications (Table 4.1). One sub-sample of seawater analyzed at 40x magnification using a 4x objective and a standard $300\ \mu\text{m}$ flow cell, initially setting a minimum size filter of $25\ \mu\text{m}$, which was reduced to $20\ \mu\text{m}$ starting May 2015. Another sub-sample of seawater analyzed at 100x magnification using a 10x objective and a standard $100\ \mu\text{m}$ flow cell, setting a minimum size filter of $6\ \mu\text{m}$. Prior to analysis the subsamples were filtered through a $200\ \mu\text{m}$ and $100\ \mu\text{m}$ mesh for 4x and 10x respectively. Thus further references in the present manuscript to either 4x or 10x FC analyses apply to $\geq 25\text{-}200\ \mu\text{m}$ and $6\text{-}100\ \mu\text{m}$ particles respectively, except when

specified otherwise. Note that lower limits of the particle size-ranges are FC estimates (see last paragraph of this section about FC size-related statistics), and differ from how the upper limits are determined, which is based on the mesh size of the filters used.

Typically, each sample was processed through the FC in triplicates. The analysis was generally terminated when the particle count reached 500 cells, in order to ensure that counts obtained were consistent among weekly samples regardless of particle concentration. There were some exceptions (Table 4.1), when the time of analysis of highly dilute samples was limited to 45 minutes irrespective of the counts obtained, and in some cases only one FC run per sample was performed. For a few samples, only one magnification/flow cell was used. Image files were visually inspected and non-living/detritus particles or bubbles were removed. For the post-May 2015 4x samples, only cells $\geq 25 \mu\text{m}$ were used for consistency with previous samples. Processing the data post analysis resulted in total counts that were < 500 particles (Table 4.1).

Statistics extracted from the FC files included particle concentration (particle ml^{-1}), size distribution, and average biovolume. All size-related measurements were based on the FC area-based diameter size algorithm (ABD). ABD is the diameter of a solid circle formed by merging all the pixels considered part of the particle, and has been suggested to be the better choice for morphologically complex species such as chain-forming diatoms (Jakobsen & Carstensen, 2011). Diameter estimates are used by the FC to generate estimates of particles biovolume, which in the software version available at the time of the study were based on the algorithm for the volume of a sphere.

Assessment of the FC

The functionality of the FC was assessed in relation to data available from the NBayPTS. This includes determination of phytoplankton numerical abundance and biomass, taxonomic composition (taxonomic richness and most abundant taxa), and size structure, as well as a measure of consistency among FC analyses of aliquots of the same sample, both replicated and conducted at two different magnifications.

Phytoplankton abundance/biomass

FC particle counts (particles mL^{-1}) were compared to microscopy cell counts (cells m^{-1}). NBayPTS microscopy analysis does not include measurements of size and/or biomass. Chlorophyll a data from the NBayPTS was used as a proxy for biomass to investigate any relationship between the size-distribution of chlorophyll a with the abundance of the two size ranges analyzed by FC at the two magnifications.

Taxonomic composition

In order to assess the ability of the FC to describe the species composition of the phytoplankton community, a qualitative analysis of a subset of 14 samples was performed. Samples were selected to reflect the high dynamic range of chlorophyll a concentration and to represent seasonal dynamics and monthly variability of phytoplankton abundance (Table 4.2). For the taxonomic analysis, results of FC10x analyses were used, as they provided both better coverage and better image resolution of diatoms, which constituted the bulk of the microscopic enumeration. For each replicate of each selected sample, images were visually inspected to determine taxonomic richness (i.e. how many different taxa were identified) and which taxa were dominant. Results of

the analysis were then compared to the corresponding microscopy data. Genera were used as the highest taxonomic level in the comparison, except for flagellates, which in the microscopy analysis were treated as one taxon (see the section ‘microscopy counts’ above). For the microscopy cell counts, a taxon was considered dominant based on its abundance and irrespective of cell size, if it represented >10 % of the total cell counts.

Size structure

The size structure of the plankton community was examined against fractionated chlorophyll concentration using the average estimates of biovolume extracted from FC particle statistics for both 4x and 10x analyses.

FC 10x data for the 14 selected samples were used to characterize the size-structure of the phytoplankton community. For each replicate of a sample, particles counts were binned into five size categories (6-10 μm , 10-20 μm , 20-40 μm , 40-60 μm , and >60 μm).

Reproducibility of output

Analyzing the size data separately for each replicate allowed to evaluate the reproducibility of data acquired by the FC. This was further assessed by comparing replicates for counts and species composition as well as the effect of magnification on the resulting counts of particles within the same size range. For the latter one sample was used (10/14/2014), and particles were binned as described above for the 10x analysis, whereas for the 4x analysis, bin sizes included 25-40 μm , 40-60 μm , 60-80 μm , and >80 μm .

RESULTS

Environmental conditions

During the sampling year, NB was characterized by large variations of sea-surface temperature, from below-zero temperatures for six weeks in mid-winter to $>20^{\circ}\text{C}$ for nine weeks in mid-summer. The lowest recorded SST was -1.3°C on 2/24/2015 and the highest SST was 22.3°C on 9/8/2015. Fall/early winter and spring/early summer were periods of steady decrease and increase in SST respectively (Table 4.2). Chlorophyll a concentrations for the same period exhibited a highly dynamic range (Table 4.2) that varied between $1\ \mu\text{g L}^{-1}$ (5/12/15) and $20.3\ \mu\text{g L}^{-1}$ (9/21/15).

What follows is an assessment of the functionality of the FC, comparing results of FC analyses to microscopy and other data obtained for the NBayPTS. This includes determination of (1) phytoplankton numerical abundance (in cells mL^{-1} for microscopy and particles mL^{-1} for FC), (2) taxonomic richness measured as the number of different taxa identified, and (3) most abundant taxa. In addition, we present (4) results of FC analysis of the size structure of the phytoplankton community, and (5) variability among FC outputs of analyses that were replicated and those that were conducted both at 4x and 10x.

1. Phytoplankton numerical abundance

Replicate averages of 10x FC particle counts (FC10x) were compared to cell counts from microscopy (MC), as they targeted a comparable particle size range.

Replicate averages of FC10x varied from $61 (\pm 19)$ to $5609 (\pm 1270)$ particles mL^{-1}

(5/12/15 and 6/16/15 respectively), and averaged 1536 particles ml^{-1} (± 1640 SD), whereas MC varied from 49 to 7502 cells ml^{-1} (5/19/15 and 6/23/15 respectively; Fig. 1A) and averaged 1320 cells ml^{-1} (± 1572 SD). Lowest abundance of FC10x on 5/19/15 corresponded to a sample with the second to lowest abundance counted under the microscope (50 cells ml^{-1}). Maximum FC10x and MC were made a week apart in June 2015.

The slope of the relationship between MC and FC10x was 0.35 ± 0.16 ($R^2 = 0.11$, $p = 0.042$, $DF = 37$) and was significantly different from the 1:1 line ($p = 0.0003$; Fig. 4.1B). In half of the samples, FC underestimated abundance by a median factor of 1.7. In the other half of samples, FC overestimated abundance by a median factor of 2.3. MC were lowest relative to FC10x (7-10 times lower) on two consecutive weeks in September 2015 when small flagellates, dinoflagellates, and a Euglenid sp. dominated the phytoplankton community. MC were highest relative to FC10x (14 times higher) 5/5/15 when chains of *Leptocylindrus minimus* were the most numerically abundant organisms. In fact in $\frac{3}{4}$ of samples, microscopy underestimated total abundance relative to FC when flagellates were identified by microscopy as the numerically most abundant taxonomic group, whereas microscopy counts were higher than those from FC when diatom chains were identified as the dominant taxa. There was a significant positive correlation between FC10x and total chlorophyll a concentration (Fig. 1C; Pearson $r = 0.53$, $R^2 = 0.28$, $p = 0.0005$, $N = 40$), whereas no significant correlation was found between MC and total chlorophyll a (Fig. 1C; Pearson $r = 0.19$, $R^2 = 0.03$, $p = 0.24$, $N = 42$).

Replicate averages of FC4x varied from 3 to 126 (± 40) particles ml^{-1} on 2/12/15 and 6/23/15 respectively (Fig. 4.2) and averaged 31 ml^{-1} (± 31 SD). Lowest concentration (< 10 particles ml^{-1}) was recorded during late fall 2014. The highest FC4x was observed the same day as the highest concentration of MC. When MC were lowest, concentration from FC4x was also low ($< 10 \text{ ml}^{-1}$). There was a weak but significant positive correlation between the concentration of $\geq 25 \mu\text{m}$ particles analyzed with 4x and $> 20 \mu\text{m}$ chlorophyll a (Fig. 4.3; Pearson $r = 0.56$, $R^2 = 0.31$, $N = 41$, $p = 0.0001$).

2. Species composition

In 24 out of the 43 weeks sampled using microscopy, diatoms numerically dominated the phytoplankton community (Table 4.3). The genus *Skeletonema* was the most numerically abundant for 14 out of these 24 weeks, and represented between 32 and 91 % and an average of 64% of total counts. At other times, dominant taxa were represented by only three other genera, *Chaetoceros* (6 weeks, 32-92%), *Thalassiosira* (two weeks, 47%), and *Leptocylindrus* (two weeks, 76 and 98%). During the remaining 19 weeks, the most abundant organisms were flagellates. In 14 weeks when flagellates dominated, one or more genera of diatoms still represented $> 10\%$ of the total abundance (Table 4.3).

For 14 samples, we compared the microscope analysis to the FC output both in terms of species richness (i.e. number of different taxa identified) and most abundant taxa. The number of taxa per sample (Fig. 4.4) ranged from 5 to 21 for microscopy (10/6/15 and 9/21/15 respectively) and from 8 to 19 for FC (7/20/15 and 1/21/15

respectively). In half of the samples, the number of taxa from the microscopy analysis exceeded that of the FC, while in five samples, FC number of taxa was greater, and for the two remaining samples the number of taxa for microscopy and FC were equal (Fig. 4.4). The two different analyses did not always identify all the same taxa. Taxa common to microscopy and FC varied from 2 to 10 and averaged 47% of the highest number of taxa recorded for a specific sample by either method (Fig. 4.4).

3. Dominant taxa

For eight out of the 14 samples analyzed, based on counts for microscopy and visual inspection of images for FC, the two types of analyses returned the same dominant taxon (Table 4.3). For the remaining six samples, either missing or undistinguishable from the FC output were diatom genera, particularly the genus *Skeletonema*, identified by microscopy as the most abundant cells and constituting an important proportion (26-82%) of the microscope counts (Table 4.3).

4. Size structure

The FC analysis provided an opportunity to investigate the weekly variability in the size structure of the phytoplankton community. The average biovolume in each weekly sample varied between $419 \mu\text{m}^3$ on 7/8/15 and $10213 \mu\text{m}^3$ on 10/14/14 and over the sampling period it averaged $1911 \pm 2039 \mu\text{m}^3$ (Fig. 4.5). The minimum and maximum average biovolume respectively coincided with highest (100%) and lowest (14%) proportion of $\leq 20 \mu\text{m}$ chlorophyll a (Fig. 4.5). The average biovolume decreased

significantly as the proportion of $\leq 20 \mu\text{m}$ chlorophyll a increased (Fig. 4.6; Pearson $r = -0.54$, $R^2 = 0.29$, $N = 40$, $p = 0.0003$).

We compared the size distribution of 14 selected samples analyzed with FC at 10x. In general the 6-10 μm fraction represented the largest proportion of total FC10x, ranging between 44 and 80 % of the total counts (Fig. 4.7). There was one exception on 9/21/15, the 10-20 μm fraction contributed the largest proportion of the total counts, i.e. 63 % vs. 30% for the 6-10 μm fraction, possibly due to the high numerical abundance of single cells of *Thalassiosira* sp. The 20-40 μm size fraction contributed between 1 % and 14 % of the total counts and 5 % on average. Particles $>40 \mu\text{m}$ were usually low in abundance, representing <1 % on average (Fig. 4.7). Based on the FC output, $< 20 \mu\text{m}$ particles represented an average of 95 ± 5 % of the total FC10x, whereas for the same samples, the $< 20 \mu\text{m}$ chlorophyll a fraction varied between 57 % and 99 % and averaged 75 ± 13 % of total chlorophyll a, not including an extreme value of 14 % on 10/14/14 (Fig. 4.8).

5. Reproducibility of FC output

Samples of which replicate aliquots were analyzed with the FC were used to investigate the variability of several FC summary statistics. Estimates of particle concentrations from replicated analyses had median coefficients of variation (CV) of 17 % (both 4x and 10x total counts), 42 % (10x $\geq 25 \mu\text{m}$ counts), and CV for average biovolumes had median values of 15 % and 28 % for $\geq 25 \mu\text{m}$ (4x) and $\geq 6 \mu\text{m}$ (10x) particles respectively (Fig. 4.9). For all samples, particles $\geq 25 \mu\text{m}$ were enumerated

using both 4x or 10x, which returned unequal results, with no consistency in which analysis returned a higher count (Fig. 4.10).

The variability of replicated FC outputs on size distribution for all samples was not quantified but appeared generally low, except for the least abundant size classes (Fig. 4.7). Only one sample was used to compare the effect of replication on the consistency of the size distribution output, and no significant difference was found among replicates of each separate 4x and 10x analysis (one way ANOVA, $p > 0.06$ for 4x and $p > 0.34$ for 10x). Yet for the same sample, the abundance of particles in the 10x 20-40 μm size bin was about $\frac{1}{2}$ lower than the abundance of particles in the 4x 25-40 μm size bin, and $> 40 \mu\text{m}$ particles were almost completely absent from the 10x analysis (Fig. 4.11).

Finally, the same selected sample was used to examine the reproducibility of FC in terms of taxonomic composition. Ten diatom genera, one unidentified pennate diatom, and one unidentified tintinnid species were distinguished (Table 4.4). Out of these twelve taxa, four were common to all replicates, five were common to two replicates, and three occurred in only one replicate (Table 4.4).

DISCUSSION

Instruments for the automatic enumeration of plankton organisms are promising tools developed to replace the traditional microscopic approach. In this study we considered the suitability of the automatic FC for characterizing phytoplankton communities of natural samples. We compared estimates of phytoplankton numerical abundance and taxonomic composition obtained by light microscopy and FC using weekly plankton samples collected for the long-term NBayPTS covering an entire yearly cycle. The wide variety of phytoplankton abundance and taxonomic composition represented in the samples allowed us to dependably determine the functionality of the FC for general applications of phytoplankton study, and to make recommendations regarding whether the FC should be integrated into the NBayPTS.

We found that the FC was able to satisfactorily summarize the overall seasonal dynamics in the bay's phytoplankton abundance and species composition in a qualitative rather than quantitative manner. FC reproduced seasonal trends observed using microscopy and chlorophyll analysis, generally identifying parallel shifts in abundance and biomass. FC exhibited good precision, delivering outputs of abundance estimates, average biovolumes, and size distribution that were consistent among replicate analyses. Yet in detailed weekly side-by-side comparisons, microscopy and FC yielded different estimates of abundance and different descriptions of taxonomic composition, and the resolution of FC images only enabled taxonomic resolution at the genus level at best, and only for particles $\geq 15 \mu\text{m}$.

Differences between microscopy and average FC weekly estimates of abundance often depended on whether flagellates or chain-forming diatoms were numerically the most abundant (based on microscopy counts) in the phytoplankton community. Previous comparisons between FC and microscopy have also reported significant differences in estimates of plankton abundance in natural samples (See et al., 2005). Others have found minimal differences in abundance (Ide et al., 2008; Alvarez et al., 2014) but applied a more lenient threshold (≤ 2 -fold) to assess the similarity between FC and microscopy estimates, based on published ‘intra-method’ variability for microscopy (Alvarez et al., 2014), or used low taxonomic resolution, only distinguishing autotrophic and phagotrophic protists (Ide et al., 2008). Furthermore the microscopy analysis in these studies was done on Lugol’s preserved samples. In contrast, the microscopy analysis of NBayPTS samples was conducted on live samples, which may have resulted in underestimates when small flagellated and other swimming organisms were abundant, due to the difficulty to accurately enumerate moving particles under a microscope. In contrast, FC generally underestimated total abundance relative to microscopy when chain-forming diatom species dominated, either due to removal of chains by filtration or to the difference in the way the two methods count chain-forming organisms.

Automated counting of chain-forming diatoms presents a particular challenge, since microscopy counts the number of cells present whereas FC and other imaging systems usually count the number of chains. This challenge may explain why in our study FC generally underestimated counts relative to microscopy when chain-forming diatoms numerically dominated samples. Early tests of FC performance reported highest

differences between microscopy and FC counts of cultures for a chain-forming diatom species (Sieracki et al., 1998). To our knowledge, comparative studies do not routinely address how researchers deal with counting chain-forming diatoms (Ide et al., 2008; Alvarez et al., 2014). Addressing the problem invariably requires manual counting of the number of cells per imaged chain (Sieracki et al., 1998). It may then be possible to calibrate the manual counts against some automatically generated particle property (e.g. duration of fluorescence signal, Olson & Sosik, 2007 for the Imaging FlowCytobot), an approach we did not attempt. Nevertheless, information of diatom chain-length gained from FC images is valuable as it may have ecological relevance, for example in predator-prey interactions (Bergkvist et al., 2012).

The lack of an exact overlap between the particle size ranges sampled by FC and microscopy may have further contributed to the differences in their estimates of abundance. Filtering of samples prior to FC analysis using the 10x objective/100 μm flow cell combination likely removed larger particles, which may also explain both the observed mismatch between the $< 20 \mu\text{m}$ FC and chlorophyll fractions, and the failure of FC to identify *Skeletonema* sp. when they were numerically abundant and represented a large proportion of the microscopy counts. Our recent experience using the FC has shown that it may not always be necessary to pre-filter a sample through a 100 μm mesh in order to process it through a 100 μm flow cell, as elongated particles that would be retained on the filter can pass through the flow cell without clogging when the flow aligns the particle's long axis parallel to the flow cell. Prior microscopic inspection of the sample should help determine if and what filtering is needed. Though clogging increases the

handling time, analysis of unfiltered samples may yield increased species diversity and test runs seem advisable.

The differences we found between microscopy and FC in the number and type of identified taxa illustrate the need to increase sampling effort to assess species richness and/or diversity of less abundant species (Rodriguez-Ramos et al., 2014). The need to increase sample volume to detect rare species is common to both microscopy and FC, and helps explain why taxa identified by either method did not always overlap. Counting a sufficient number of particles requires increasing the volume analyzed, which should be more feasible on the FC than with microscopy. Nevertheless, we found that the time of FC analysis at 4x to reach $500 \geq 25 \mu\text{m}$ particles increased up to 10-fold, sometimes requiring a whole hour, when samples were naturally dilute. Ideally then, for a more thorough taxonomic analysis of micro-plankton, samples should be concentrated before analysis. Previous assessment of the ability of the FC to reliably describe the size structure of phytoplankton communities also pointed out the need to concentrate samples of micro-plankton in order to obtain the required particle counts (Alvarez et al., 2011). As mentioned by the same authors, concentrating samples however may result in cell damage and cell loss, but this could be minimized by reverse filtration (Dodson & Thomas 1978). Nevertheless it may be difficult to determine what concentration is required without knowing the natural cells density beforehand (Alvarez et al., 2011). The latter again is a problem common to microscopy and FC, dealing with plankton that cover a vast size range and thus abundance.

FC estimates of $\geq 6 \mu\text{m}$ particle abundance generally exhibited a variability among replicates about twice that found for analysis of mono-specific laboratory cultures (Sieracki et al., 1998; Alvarez et al. 2011). However coefficients of variation (15 %) were similar to or less than those obtained for natural samples with microscopy, which can reach up to ~ 50 % when counts are repeated by a different person (Vuorio et al., 2007). Counting precision increases with increasing the threshold number required for each individual taxon (Lund et al., 1958; Vuorio et al., 2007). Thus given the trade offs of counting precision and the vast range in abundance typically encountered in plankton samples, the time needed to replicate the FC analysis of a sample may be better applied to processing a higher volume once.

Although a semi-automatic classification of plankton samples into distinct taxa is possible using previously created image libraries, this approach was not used in the present study due to the substantial effort needed to build the digital training sets. The FlowCAM image recognition software has been applied with unsatisfactory results in its ability to correctly identify particles (Buskey & Hyatt, 2006). Detailed analyses have used classification techniques that relied on external image analysis algorithms, demanding a substantial amount of preparatory work leading to the creation of several training sets (Alvarez et al., 2012). Regardless, particle properties used for proper identification are dependent on good image resolution. We found it difficult to visually determine the general taxonomic identity of particles $< 15 \mu\text{m}$ with the 10x objective— and even larger particles often could not be identified beyond genus level.

Despite some of the shortcomings discussed here, the FC presented definite advantages. Undisputable assets of FC analysis include the ability to analyze live samples in near real time, and the generation of an image for each particle. Although microscopic analysis can resolve the taxonomic identity of cells better than FC, it relies on the expertise of the taxonomist counting the sample. Consequently, microscopic analysis can become highly subjective. On the other hand FC analysis has the potential to be operator independent, through development of a standard operating protocol. Since each particle is imaged, the imaged sample can become part of a voucher collection that can be re-examined at any time. Information regarding each analysis is automatically saved, thus even an operator without taxonomic expertise can run samples and the acquired data can be analyzed later.

In addition, FC records many image properties, including particle size and biovolume, which can be further used to generate estimates of biomass in terms of carbon, measurements that are not routinely performed with microscopy, including for the microscopy analysis of NBayPTS samples. FC analyses of phytoplankton size distribution are likely more standardized than those obtained with microscopy, since FC measures each particle, whereas with microscopy, a limited number of cells are usually measured. The average biovolume estimated for each taxon from these measurements is then applied to all the cells counted, not taking into account the size variability within populations of the same species. Although at the time of the present study, FC biovolume estimates were based on equating all cells to a sphere, our laboratory collaborated with Fluid Imaging to develop a series of algorithms to reflect the diversity of shapes among

phytoplankton species, which should be available in the latest version of the software. FC can effectively describe plankton size spectra provided a minimum number of cells are counted within a particular size range, which has been estimated at about 9000 cells for effectively sampling the 20-200 μm size range (Alvarez et al., 2011). Although this may require a substantial processing time, once initiated the analysis only requires minimal supervision and can be automated by continuous feeding of the sample into the instrument (Jakobsen & Carstensen, 2011).

CONCLUSIONS

FC can rapidly assess plankton community composition in near real time, although taxonomic identification based on the images is often restricted to genus level, particularly for $<15 \mu\text{m}$ particles. FC allows for rapid order-of-magnitude estimation of biomass thanks to the biovolume estimates delivered concurrently with image analysis, measures that require additional effort when done on the microscope. Overall, FC is a valuable tool to rapidly gain insight into the size structure and taxonomic composition of phytoplankton communities and the number of discrete samples can be increased to support higher spatial or temporal sample coverage. When specific, rare taxa need to be detected (e.g. harmful algal bloom detection), or counts with accuracy within a 2-fold margin are essential, microscopic and FC analyses need to be combined.

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Table 4.1. Summary of Narragansett Bay surface seawater samples analyzed using the FlowCAM, including date of analysis, size of the objective used, flow rate (ml min⁻¹), volume of sample processed (ml), duration of analysis (min:sec), and number of particles counted after removing unwanted particles (non-living/detritus and bubbles).

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
10/14/14	4x	0.75	6.4	08:06	362
10/14/14	4x	0.75	6.1	07:42	389
10/14/14	4x	0.75	6.2	07:50	445
10/14/14	10x	0.75	1.9	02:23	460
10/14/14	10x	0.75	2.0	02:28	456
10/14/14	10x	0.75	1.9	02:23	440
10/21/14	4x	0.75	9.9	12:36	412
10/21/14	4x	0.75	9.9	12:34	381
10/21/14	4x	0.75	9.1	11:28	366
10/21/14	10x	0.75	6.3	07:58	349
10/21/14	10x	0.75	5.6	07:02	415
10/21/14	10x	0.75	5.5	07:00	442
10/28/14	4x	0.75	49.7	63:16	412
10/28/14	4x	0.75	48.5	61:36	408
10/28/14	4x	0.75	49.7	63:18	406
10/28/14	10x	0.75	2.8	03:32	514
10/28/14	10x	0.75	2.1	02:38	459
10/28/14	10x	0.75	2.2	02:46	456
11/4/14	4x	0.75	49.3	62:37	415
11/4/14	4x	0.75	32.3	40:58	250
11/6/14	4x	0.75	49.4	62:44	422
11/5/14	10x	0.75	8.1	10:12	406
11/5/14	10x	0.75	3.8	04:53	435
11/5/14	10x	0.75	4.1	05:13	459
11/12/14	4x	2	1.8	45:22	226
11/13/14	4x	3	99.6	28:37	160
11/13/14	4x	3	99.6	28:40	191
11/11/14	10x	0.75	3.7	04:40	421
11/11/14	10x	0.75	3.2	04:05	422
11/11/14	10x	0.75	3.2	04:02	397

Table 4.1. Page 2

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
11/20/14	4x	3	38.9	10:48	169
11/20/14	4x	3	38.1	11:14	118
11/20/14	4x	3	43.6	12:42	130
11/20/14	10x	1.5	6.3	03:50	382
11/20/14	10x	1.5	4.0	02:24	448
11/20/14	10x	1.5	4.5	02:45	442
12/2/14	4x	2	99.2	45:20	214
12/2/14	10x	1	6.8	06:25	448
12/2/14	10x	1	3.6	03:26	433
12/2/14	10x	1	4.3	03:59	433
12/10/14	4x	2	99.6	45:36	188
12/10/14	10x	1	5.4	05:02	427
12/10/14	10x	1	3.7	03:29	442
12/10/14	10x	1	3.5	03:18	410
12/17/14	4x	2	99.2	45:30	226
12/17/14	10X	1	5.7	05:26	451
12/17/14	10x	1	5.0	04:40	471
12/17/14	10x	1	5.2	04:50	448
1/21/15	4x	2	77.0	35:08	282
1/21/15	4x	2	97.4	44:32	296
1/21/15	10x	1	3.0	02:47	415
1/21/15	10x	1	3.0	02:50	417
1/21/15	10x	1	2.8	02:38	435
1/26/15	4x	2	77.2	34:57	365
1/26/15	4x	2	31.5	14:24	373
1/26/15	4x	2	26.1	12:07	408
1/26/15	10x	1	2.2	02:06	412
1/26/15	10x	1	2.0	01:51	408
1/26/15	10x	1	1.9	01:48	383
2/4/15	4x	2	73.0	33:10	266
2/4/15	4x	2	99.3	45:24	307
2/4/15	10x	1	2.8	02:41	431
2/4/15	10x	1	2.4	02:14	397
2/4/15	10x	1	4.6	04:20	381

Table 4.1. Page 3

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
2/12/15	4x	2	99.4	45:24	189
2/12/15	10x	1	1.8	01:42	490
2/12/15	10x	1	1.5	01:30	442
2/12/15	10x	1	1.7	01:35	429
2/19/15	4x	2	82.8	37:46	339
2/19/15	4x	2	72.1	32:48	304
2/19/15	4x	2	29.5	13:34	340
2/19/15	10x	1	2.3	02:07	359
2/19/15	10x	1	2.0	01:53	397
2/24/15	4x	2	74.5	33:45	218
2/24/15	4x	2	99.2	45:23	327
2/24/15	10x	1	3.1	02:52	382
2/24/15	10x	1	1.5	01:27	434
2/24/15	10x	1	1.6	01:32	436
3/4/15	4x	2	33.9	15:12	400
3/4/15	10x	1	2.2	02:05	470
3/4/15	10x	1	2.4	02:17	500
3/4/15	10x	1	1.1	01:03	498
3/10/15	4x	2	17.1	07:49	409
3/10/15	4x	2	15.9	07:17	485
3/10/15	4x	2	15.4	07:03	478
3/10/15	10x	1	1.6	01:32	500
3/10/15	10x	1	1.5	01:28	500
3/10/15	10x	1	1.4	01:20	500
3/23/15	4x	2	27.5	12:36	477
3/23/15	4x	2	17.0	07:47	460
3/23/15	4x	2	15.4	07:04	455
3/23/15	10x	1	3.6	03:26	468
3/23/15	10x	1	3.2	03:00	475
3/23/15	10x	1	2.6	02:29	465
4/1/15	4x	2	12.1	05:32	471
4/1/15	4x	2	11.1	05:08	444
4/1/15	4x	2	9.0	04:11	443

Table 4.1. Page 4

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
4/1/15	10x	1	2.0	01:52	463
4/1/15	10x	1	1.4	01:20	464
4/1/15	10x	1	1.3	01:15	462
4/6/15	4x	2	25.3	11:35	355
4/6/15	4x	2	27.4	12:36	355
4/6/15	4x	2	21.7	09:57	360
4/6/15	10x	1	2.2	02:03	476
4/6/15	10x	1	2.3	02:11	488
4/6/15	10x	1	2.0	01:53	472
4/13/15	4x	n/a	n/a	n/a	213
4/21/15	4x	0.75	19.9	25:19	257
5/5/15	4x	0.8	23.2	27:37	143
5/5/15	4x	0.8	17.7	21:02	93
5/5/15	4x	0.8	13.5	16:01	99
5/5/15	10x	0.53	6.6	11:57	315
5/5/15	10x	0.53	7.0	12:40	316
5/5/15	10x	0.1	1.0	09:47	295
5/12/15	10x	0.1	4.9	48:37	115
5/12/15	10x	0.5	5.0	09:34	128
5/12/15	10x	0.5	5.0	09:46	124
5/19/15	4x	0.8	25.4	30:21	137
5/19/15	10x	0.1	2.1	20:36	474
5/19/15	10x	0.1	2.3	22:34	974
5/26/15	4x	0.8	16.0	18:58	226
5/26/15	4x	0.8	16.4	19:30	226
5/26/15	4x	0.8	9.4	11:04	216
5/26/15	10x	0.4	5.0	12:04	1400
5/26/15	10x	0.2	0.9	04:30	415
5/26/15	10x	0.2	0.8	04:06	500
6/1/15	4x	0.8	9.5	11:18	74
6/1/15	4x	0.8	10.2	12:07	100
6/1/15	4x	0.8	7.9	09:21	98

Table 4.1. Page 5

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
6/1/15	10x	0.1	0.3	02:56	498
6/1/15	10x	0.1	0.3	02:50	368
6/1/15	10x	0.1	0.3	03:06	455
6/8/15	4x	0.8	7.3	08:36	82
6/8/15	4x	0.8	13.0	15:23	46
6/8/15	4x	0.8	15.5	18:22	107
6/8/15	10x	0.1	0.5	04:47	449
6/8/15	10x	0.1	0.5	05:12	446
6/8/15	10x	0.1	0.5	04:42	463
6/16/15	4x	0.8	7.3	08:36	171
6/16/15	4x	0.8	13.0	15:23	229
6/16/15	10x	0.1	0.5	04:47	500
6/16/15	10x	0.1	0.5	05:12	500
6/16/15	10x	0.1	0.5	04:42	500
6/23/15	4x	0.8	4.7	05:36	243
6/23/15	4x	0.8	4.4	05:15	209
6/23/15	4x	0.8	3.4	04:01	271
6/23/15	10x	0.3	0.4	01:11	457
6/23/15	10x	0.3	1.3	04:23	1464
6/23/15	10x	0.3	0.4	01:13	387
7/1/15	4x	0.8	5.6	06:42	138
7/1/15	4x	0.8	25.5	30:16	334
7/1/15	4x			35:48	162
7/1/15	10x	0.1	0.2	02:00	485
7/1/15	10x	0.1	0.4	04:30	500
7/1/15	10x	0.1	0.3	02:56	422
7/8/15	4x	1.5	3.8	02:21	21
7/8/15	4x	1.5	3.8	02:24	23
7/8/15	4x	1.5	3.8	02:22	25
7/8/15	10x	0.1	0.3	03:25	463
7/8/15	10x	0.1	0.2	02:12	454
7/8/15	10x	0.1	0.2	01:53	464
7/14/15	4x	0.8	12.2	14:32	81
7/14/15	4x	0.8	8.2	09:49	69
7/14/15	4x	0.8	5.1	06:06	52

Table 4.1. Page 6

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
7/14/15	10x	0.1	0.7	07:07	408
7/14/15	10x	0.1	0.6	06:03	410
7/14/15	10x	0.1	0.6	05:43	398
7/20/15	10x	0.1	0.7	06:40	442
7/20/15	10x	0.1	0.8	07:33	427
7/27/15	4x	0.8	12.2	16:19	100
7/27/15	4x	0.8	4.3	05:45	81
7/27/15	4x	0.8	3.6	04:48	90
7/27/15	10x	0.1	0.3	02:08	469
7/27/15	10x	0.1	0.2	01:45	429
7/27/15	10x	0.1	0.2	01:59	421
8/3/15	4x	0.7	4.2	05:44	27
8/3/15	4x	0.7	2.9	03:54	28
8/3/15	4x	0.7	1.1	01:32	27
8/3/15	10x	0.1	0.4	03:41	426
8/3/15	10x	0.1	0.3	02:59	415
8/3/15	10x	0.1	0.3	02:35	412
8/10/15	4x	0.8	9.5	11:14	154
8/10/15	10x	0.1	0.3	03:19	399
8/10/15	10x	0.1	0.3	03:06	378
8/10/15	10x	0.1	0.3	03:08	381
9/8/15	4x	0.8	4.6	05:25	119
9/8/15	4x	0.8	4.0	04:47	131
9/8/15	4x	0.8	4.1	04:49	118
9/8/15	10x	0.1	0.5	05:07	443
9/8/15	10x	0.1	4.0	39:36	3392
9/8/15	10x	0.1	0.4	03:53	453
9/15/15	4x	0.8	11.3	13:26	129
9/15/15	10x	0.1	0.3	03:21	500
9/15/15	10x	0.1	0.3	03:21	500
9/15/15	10x	0.1	0.4	03:32	500
9/15/15	10x	0.1	1.0	10:14	1330
9/15/15	10x	0.1	1.0	09:48	1174
9/21/15	4x	0.8	3.2	03:44	129
9/21/15	4x	0.8	2.5	03:02	116
9/21/15	4x	0.8	2.1	02:26	132

Table 4.1. Page 7

Date	Objective size	Flow rate ml min ⁻¹	Volume processed (ml)	Analysis duration (min:sec)	Particle count (total)
9/21/15	10x	0.1	0.2	02:22	486
9/21/15	10x	0.1	0.2	01:45	484
9/21/15	10x	0.1	0.2	01:44	490
9/29/15	4x	0.8	7.2	08:25	80
9/29/15	4x	0.2	2.6	12:45	47
10/6/15	4x	0.5	12.2	23:41	59
10/6/15	4x	0.3	2.7	08:48	107
10/6/15	10x	0.2	2.3	11:05	463
10/6/15	10x	0.2	2.1	10:06	457
10/6/15	10x	0.2	1.8	08:52	462
10/14/15	4x	0.8	4.4	05:13	202
10/14/15	4x	0.8	2.8	03:18	77
10/14/15	4x	0.8	5.2	06:11	120
10/14/15	10x	0.2	0.8	03:53	486
10/14/15	10x	0.1	0.5	05:14	494
10/14/15	10x	0.1	0.4	03:41	491

Table 4.2. Sea surface temperature (SST; °C), total chlorophyll a ($\mu\text{g L}^{-1}$), and <20 μm chlorophyll a fraction ($\mu\text{g L}^{-1}$ and %) on dates when seawater samples were collected from Narragansett Bay for microscope and FlowCAM analysis of the plankton community. Rows in bold font represent 14 samples selected for analysis of size distribution and taxonomic composition.

Date	SST (°C)	Total Chl a ($\mu\text{g L}^{-1}$)	<20 μm Chl a ($\mu\text{g L}^{-1}$)	<20 μm Chl a (% total)
14-Oct-14	16.51	8.43	1.20	14.2
21-Oct-14	16.61	4.25	1.19	28.0
29-Oct-14	14.44	3.45	1.90	55.1
4-Nov-14	11.86	3.58	1.94	54.2
12-Nov-14	11.27	1.75	1.65	94.3
20-Nov-14	8.01	2.78	2.06	74.1
2-Dec-14	7.54	2.44	1.76	72.1
10-Dec-14	5.65	1.96	1.48	75.5
17-Dec-14	4.92	1.46	1.31	89.7
21-Jan-15	3.62	2.47	1.50	60.7
26-Jan-15	2.32	5.34	3.19	59.7
4-Feb-15	-0.47	3.12	1.47	47.1
12-Feb-15	-0.86	5.03	3.18	63.2
19-Feb-15	-0.42	3.30	2.94	89.1
24-Feb-15	-1.31	7.54	4.59	60.9
4-Mar-15	-0.94	9.75	6.06	62.2
10-Mar-15	-0.68	5.16	3.62	70.2
23-Mar-15	3.62	3.16	2.56	81.0
1-Apr-15	2.96	8.70	4.98	57.2
6-Apr-15	3.79	2.28	1.38	60.5
13-Apr-15	5.96	2.66	2.38	89.5
21-Apr-15	8.03	1.58	1.03	65.2
5-May-15	8.82	1.43	1.37	95.8
12-May-15	13.05	0.98	0.68	69.4
19-May-15	13.62	1.65	1.68	100.0
26-May-15	14.39	2.34	2.71	100.0
1-Jun-15	17.11	4.33	3.93	90.8
8-Jun-15	16.66	3.83	4.09	100.0
16-Jun-15	18.32	5.16	3.71	71.9
23-Jun-15	19.81	6.87	5.10	74.2
1-Jul-15	20.17	7.95	7.85	98.7
8-Jul-15	21.56	5.76	6.83	100.0
14-Jul-15	22.02	2.98	2.69	90.3
20-Jul-15	22.91	3.03	2.51	82.8
27-Jul-15	N/A	8.70	7.72	88.7
3-Aug-15	22.91	6.73	5.99	89.0
10-Aug-15	22.86	9.69	7.91	81.6
8-Sep-15	23.22	5.84	4.16	71.2
15-Sep-15	22.45	7.58	5.97	78.8
21-Sep-15	21.52	20.29	16.02	79.0
29-Sep-15	19.66	7.88	7.09	90.0
6-Oct-15	16.13	1.65	1.38	83.6
14-Oct-15	16.82	15.99	10.77	67.4

Table 4.3. Numerically dominant plankton taxa determined from microscopy cell counts of 43 water samples collected from the Narragansett Bay. Dominant taxa were compared to those identified via visual inspection of FlowCAM images for 14 selected samples (rows in bold font). For microscopy, abundance for all samples is presented as proportion (%) of total counts (all taxa included), and of counts excluding flagellates for the selected samples only.

Date	Microscopy All taxa included		Microscopy Flagellates excluded		FlowCAM
	Dominant Taxon	Abundance (%)	Dominant Taxon	Abundance (%)	Dominant Taxon
10/14/14	<i>Chaetoceros</i>	92	<i>Chaetoceros</i>	99	<i>Chaetoceros</i>
10/21/14	<i>Chaetoceros</i>	85			
10/29/14	Flagellates	55			
10/29/14	<i>Chaetoceros</i>	35			
11/4/14	Flagellates	52			
11/4/14	<i>Chaetoceros</i>	19			
11/4/14	<i>Skeletonema</i>	11			
11/4/14	<i>Leptocylindrus</i>	10			
11/12/14	Flagellate	71	<i>Prorocentrum</i>	46	Flagellates
11/12/14	<i>Prorocentrum</i>	13	<i>Heterocapsa</i>	27	
11/20/14	<i>Chaetoceros</i>	37			
11/20/14	Flagellates	36			
11/20/14	<i>Skeletonema</i>	13			
12/2/14	Flagellates	50			
12/2/14	<i>Chaetoceros</i>	26			
12/2/14	<i>Skeletonema</i>	10			
12/10/14	Flagellate	35	<i>Skeletonema</i>	31	<i>Chaetoceros</i>
12/10/14	<i>Skeletonema</i>	20	<i>Chaetoceros</i>	27	<i>Myrionecta</i>
12/10/14	<i>Chaetoceros</i>	18	<i>Thalassiosira</i>	26	
12/10/14	<i>Thalassiosira</i>	17			
12/17/14	Flagellates	76			
12/17/14	<i>Skeletonema</i>	12			
1/21/15	<i>Skeletonema</i>	82	<i>Skeletonema</i>	91	<i>Skeletonema</i>
1/21/15	Flagellates	11			
1/26/15	<i>Skeletonema</i>	91			
2/4/15	<i>Skeletonema</i>	72			
2/4/15	Flagellates	14			
2/12/15	<i>Skeletonema</i>	49	<i>Skeletonema</i>	74	<i>Skeletonema</i>
2/12/15	Flagellates	33			Pennate diatoms
2/19/15	<i>Skeletonema</i>	67			

Table 4.3. Page 2

Date	Microscopy All taxa included		Microscopy Flagellates excluded		FlowCAM
	Dominant Taxon	Abundance (%)	Dominant Taxon	Abundance (%)	Dominant Taxon
2/19/15	Flagellates	17			
2/24/15	Flagellates	42			
2/24/15	<i>Skeletonema</i>	40			
3/4/15	<i>Skeletonema</i>	52	<i>Skeletonema</i>	62	<i>Chaetoceros</i>
3/4/15	<i>Thalassiosira</i>	16	<i>Thalassiosira</i>	19	<i>Thalassiosira</i>
3/4/15	Flagellates	15	<i>Chaetoceros</i>	13	<i>Rhizosolenia</i>
3/4/15	<i>Chaetoceros</i>	11			
3/10/15	Flagellates	37			
3/10/15	<i>Skeletonema</i>	27			
3/10/15	<i>Thalassiosira</i>	16			
3/10/15	<i>Chaetoceros</i>	12			
3/23/15	<i>Chaetoceros</i>	32			
3/23/15	<i>Skeletonema</i>	29			
3/23/15	<i>Thalassiosira</i>	28			
4/1/15	<i>Thalassiosira</i>	47	<i>Thalassiosira</i>	52	<i>Thalassiosira</i>
4/1/15	<i>Chaetoceros</i>	21	<i>Chaetoceros</i>	23	
4/1/15	<i>Skeletonema</i>	13	<i>Skeletonema</i>	15	
4/1/15	Flagellates	10			
4/6/15	<i>Chaetoceros</i>	52			
4/6/15	Flagellates	16			
4/6/15	<i>Thalassiosira</i>	10			
4/13/15	<i>Skeletonema</i>	42			
4/13/15	<i>Chaetoceros</i>	25			
4/13/15	<i>Leptocylindrus</i>	13			
4/13/15	<i>Thalassionema</i>	10			
4/21/15	<i>Leptocylindrus</i>	76			
4/21/15	<i>Chaetoceros</i>	10			
5/5/15	<i>Leptocylindrus</i>	98			
5/12/15	<i>Chaetoceros</i>	50	<i>Chaetoceros</i>	60	Aloricate ciliates
5/12/15	Flagellates	17	<i>Gyrodinium</i>	12	
5/12/15	<i>Gyrodinium</i>	10			
5/19/15	Flagellates	65			
5/19/15	<i>Thalassiosira</i>	15			
5/19/15	Dinoflagellates	13			
5/26/15	Flagellates	75			
5/26/15	<i>Heterocapsa</i>	13			

Table 4.3. Page 3

Date	Microscopy All taxa included		Microscopy Flagellates excluded		FlowCAM
	Dominant Taxon	Abundance (%)	Dominant Taxon	Abundance (%)	Dominant Taxon
6/8/15	<i>Skeletonema</i>	83			
6/8/15	<i>Thalassiosira</i>	28			
6/16/15	<i>Skeletonema</i>	82	<i>Skeletonema</i>	87	Flagellates
6/23/15	<i>Skeletonema</i>	32			
6/23/15	<i>Thalassiosira</i>	24			
6/23/15	Flagellates	23			
6/23/15	<i>Leptocylindrus</i>	10			
7/1/15	<i>Skeletonema</i>	81			Aloricate ciiates
7/1/15	<i>Thalassiosira</i>	13			
7/8/15	<i>Skeletonema</i>	74			
7/8/15	Flagellates	12			
7/8/15	<i>Thalassiosira</i>	11			
7/14/15	Flagellates	64			
7/20/15	Flagellates	62	<i>Dactyliosolen</i>	78	Flagellates
7/20/15	<i>Dactyliosolen</i>	30			
7/27/15	Flagellates	47			
7/27/15	<i>Skeletonema</i>	28			
8/3/15	<i>Skeletonema</i>	34			
8/3/15	Flagellates	24			
8/3/15	<i>Cylindrotheca</i>	11			
8/3/15	<i>Leptocylindrus</i>	11			
8/10/15	<i>Skeletonema</i>	54	<i>Skeletonema</i>	67	Thalassionema
8/10/15	Flagellates	19	<i>Thalassiosira</i>	14	
8/10/15	<i>Thalassiosira</i>	11			
9/8/15	Flagellates	51			
9/8/15	<i>Euglena</i>	21			
9/15/15	Flagellates	33			
9/15/15	<i>Thalassiosira</i>	18			
9/15/15	<i>Prorocentrum</i>	15			
9/21/15	<i>Thalassiosira</i>	47	<i>Thalassiosira</i>	62	Thalassiosira
9/21/15	Flagellates	25	<i>Skeletonema</i>	13	<i>Prorocentrum</i>
9/21/15	<i>Skeletonema</i>	10			
9/29/15	Flagellates	65			
9/29/15	<i>Dactyliosolen</i>	27			

Table 4.3. Page 4

Date	Microscopy All taxa included		Microscopy Flagellates excluded		FlowCAM
	Dominant Taxon	Abundance (%)	Dominant Taxon	Abundance (%)	Dominant Taxon
10/6/15	Flagellates	52	<i>Prorocentrum</i>	47	Flagellates
10/6/15	<i>Prorocentrum</i>	22	<i>Skeletonema</i>	19	
10/14/15	Flagellates	70			
10/14/15	<i>Chaetoceros</i>	10			

Table 4.4. Example of FlowCAM triplicate analysis of taxonomic composition of a Narragansett Bay plankton sample collected on 14/10/2014. Identified genera are listed in alphabetical order, and taxa are marked present (1) or absent (0).

Taxon	Replicate 1	Replicate 2	Replicate 3
<i>Asterionellopsis</i>	1	1	1
<i>Chaetoceros</i>	1	1	1
<i>Ditylum</i>	1	1	0
<i>Eucampia</i>	1	0	1
<i>Guinardia</i>	1	0	1
<i>Lauderia</i>	1	0	1
<i>Leptocylindrus</i>	1	1	1
<i>Odontella</i>	0	0	1
<i>Pleurosigma</i>	1	0	0
<i>Thalassionema</i>	1	1	1
Unid. Pennate diatom	1	1	0
Tintinnid	0	0	1

Figure 4.1. Phytoplankton abundance in weekly samples collected from Narragansett Bay between Fall 2014 to Fall 2015 analyzed using microscopy (cells ml^{-1}) and Flowcam (particles ml^{-1}). Panel A: Time series of total counts; panel B: 10x FlowCAM counts (y-axis) vs. microscopy counts (x-axis). Panel C: Counts (cells ml^{-1}) obtained from microscopy (open symbols) and FlowCAM (solid symbols) as a function of total chlorophyll a ($\mu\text{g L}^{-1}$). Solid line on panel B represents line of best linear fit and dashed line represents 1:1 ratio line. For clarity, error bars of replicated 10x counts were left out from panel A, and one extreme value was omitted from panel B (14.3 on May 5, 2015).

Figure 4.2. Time series of numerical abundance of $\geq 25 \mu\text{m}$ plankton cells (cells ml^{-1}) analyzed using the FlowCAM with a 4x objective in weekly samples collected from Narragansett Bay between October 2014 and October 2015. Error bars represent one standard deviation of the mean of typically triplicate and sometimes duplicate counts. No error bar means sample was processed only once.

Figure 4.3. $>20\ \mu\text{m}$ chlorophyll vs. abundance of $\geq 25\ \mu\text{m}$ particles (ml^{-1}) counted using FlowCAM at a 4x objective.

Figure 4.4. Number of different taxa determined by microscopy and Flowcam from analysis of 14 selected Narragansett Bay plankton samples, and number of taxa shared by the two methods.

Figure 4.5. Time series of the proportion (%) of chlorophyll a in $>20\ \mu\text{m}$ (dark bars) and $>20\ \mu\text{m}$ (white bars) fractions, together with the average 10x FlowCAM biovolume (μm^3 ; solid line plot) in weekly samples collected from Narragansett Bay from October 2014 to October 2015. For clarity, error bars for replicated measures of biovolume are omitted.

Figure 4.6. Average biovolume (μm^3) output by the 10x FlowCAM analysis of $\geq 6 \mu\text{m}$ particles as a function of the proportion of chlorophyll a $\leq 20 \mu\text{m}$ for weekly samples collected from Narragansett Bay from October 2014 to October 2015.

Figure 4.7. Size distribution of $\geq 6 \mu\text{m}$ phytoplankton abundance in 14 selected Narragansett Bay samples as relative counts (top panel) and % (bottom panel).

Figure 4.8. Proportion of FlowCAM 10x counts contributed by < 20 μm particles (white bars) and of chlorophyll a contributed by < 20 μm fraction (grey bars) in 14 selected Narragansett Bay samples.

Figure 4.9. Variability among replicates expressed as coefficient of variation (%) in FlowCAM outputs, including counts made with 4x ($\geq 25 \mu\text{m}$ particles), counts made with 10x ($\geq 6 \mu\text{m}$ and $\geq 25 \mu\text{m}$ separately), and estimates of average biovolume. Lines in the middle of the boxes represent median values and whisker extends to 25th (lower) and 75th (upper) percentile $\pm 1.5 \times$ interquartile range. Values higher/lower than upper/lower limits of whiskers are plotted as individual points. Percentiles (P) are computed as $P = k/(N+1)$, where k is the rank starting at 1 and N is the sample size.

Figure 4.10. Numerical abundance of $\geq 25 \mu\text{m}$ particles (particles ml^{-1}) examined 4x (x-axis) and 10x (y-axis). The dashed line represents 1:1 ratio.

Figure 4.11. Example of variability among replicates of FlowCAM size distribution of particles examined at 10x (upper panel) and 4x (lower panel). Rep= replicate. Sample used is from 10/14/2014.

CHAPTER FIVE

CONCLUDING REMARKS

Phagotrophic protists have been established as the main consumers of phytoplankton production in the ocean (Sherr & Sherr 2002, Calbet & Landry 2004, Schmoker et al. 2013), and as such occupy a pivotal position in pelagic food webs. Nevertheless rates of protistan grazing and the impact of protistan herbivory on primary production vary at a number of spatial and temporal scales (Schmoker et al. 2013), yet the extent and drivers of this variability remain to be identified. This dissertation addressed some of the gaps in the understanding of protistan trophic interactions by gathering field measurements and observations and by evaluating modifications and alternatives to current methods used to estimate grazing rates and characterize plankton communities.

One surprising finding of our seasonal study of protistan herbivory in the Western Antarctic Peninsula was the seasonal similarity in the occurrence of grazing and in the magnitude of grazing rates despite almost two orders of magnitude difference in phytoplankton biomass. Furthermore, rates tended to be greater during the less productive season of austral fall. Measurable grazing was generally associated with dominance of pico- and nano-phytoplankton, highlighting that size structure rather than total abundance influenced whether and how much grazing occurred. This is important since zooplankton grazing is often modeled as a functional response, i.e. dependent on prey density (Leles 2016), thus inferring diminished losses of phytoplankton due to grazing during periods when phytoplankton biomass is lowest. Such a conjecture may not always apply, as is

evidenced by our findings for the WAP region where protistan grazing exhibited a bimodal distribution of no measurable grazing or low rates across two seasons. Our findings also underline the need to extend measurements for the global ocean to less productive seasons in order to verify whether the assumed enhancing effect of prey abundance on grazing rates is generally observed in the field, so we can better understand the role of grazing in the yearly dynamics of phytoplankton biomass. A recent hypothesis has been proposed that is challenging traditional concepts of bloom initiation by attributing an important role to grazing (Behrenfeld & Boss 2014). Although the difficulty to obtain year-round measurements of grazing rates makes them scarce, if we are to validate hypotheses about the role of predation in the yearly cycle of primary production, knowledge of grazing magnitude at different times of year is particularly critical.

Grazing rates measured in Antarctic waters are generally lower than in other parts of the global ocean (Calbet & Landry 2004). An hypothesis has been proposed to explain these low grazing rates, according to which polar water temperature constrains the growth rates of HP relative to that of autotrophs, potentially explaining the occurrence of massive phytoplankton blooms observed in polar ecosystems (Rose & Caron 2007). These conclusions were derived from a compilation of growth measurements of cultured protists, while in the field significant grazing has previously been measured at near freezing temperatures (Lawrence & Menden-Deuer 2012) and HP have been found to be able to grow at the ambient temperatures in which they naturally occur (Franze & Lavrentyev 2014). Based on results within this dissertation, it remains questionable if low

water temperature limited the degree of herbivory in the Western Antarctic Peninsula, as grazing rates in a range of magnitude were obtained from experiments conducted at similarly low temperatures. Nevertheless, significant increases in grazing rates in response to manipulated elevated water temperature could not be ignored. Together with the measurements made in the field, these results suggest that while low ambient temperature ($< 5^{\circ}\text{C}$) may have set an upper limit on the magnitude of grazing rates, it could not universally account for the variability of this magnitude, suggesting that other factors likely played a role. Elucidation of the role of temperature on the growth rates and feeding activities of phagotrophic protists will require more studies using natural assemblages.

In order to better document the spatial and seasonal variability of grazing rates and to identify driving factors, a greater sampling resolution of grazing rate measurements is needed. This requires a reduction of the sampling effort associated with the dilution technique, the most widely used method to quantify grazing rates. After being assessed, an abbreviated version of the method that uses two instead of a series of four to five dilutions was found to yield rate estimates of both phytoplankton growth and grazer-induced mortality that did not substantially deviate from those obtained when using multiple dilutions. The two-point rates accuracy was similar in magnitude to the inherent error associated with the dilution-series estimates, supporting the usefulness of the abbreviated method.

One practical application of the two-point method has been to quantify the variability of grazing with depth (Landry et al. 2011). The coupling between

phytoplankton growth and grazing mortality rates was found to vary with depth (Landry et al. 2011). Values estimated from one depth are typically applied to the whole water. Such estimations have served as the basis to the established importance of protistan grazing (Calbet & Landry 2004, Schmoker et al. 2013), and the estimated global average of a protistan grazing impact representing ~67% of primary production consumed by protistan grazers is readily incorporated in carbon budgets (Steinberg & Landry 2017). Incubations conducted at one light level (one depth) may result in mis-estimates of instantaneous phytoplankton growth rates for the mixing layer (Ross et al. 2011). This caveat may be further exacerbated if photo-acclimation occurs during incubation (Behrenfeld et al. 2015), ultimately affecting the estimation of the proportion of primary production consumed. Experiments using the two-point approach can be replicated at various depths, representing an effective way to address this important issue. The 2-point approach has been applied to that effect in situ (Landry et al. 2011), and our group has started using the 2-point in shipboard incubations under different light levels.

Ultimately the measurements obtained from the dilution method reflect bulk rates, making it difficult to determine the mechanisms of association between predator and prey. Realization of the many levels of diversity of marine protists and of the feeding strategies and feeding preferences of protistan predators (Caron et al. 2012, Worden et al. 2015) has focused attention to the importance of representing this complexity in modeling studies, yet these are suffering from a lack of reliable empirical data (Anderson 2005, Leles 2016). A first step in gathering empirical data would be to routinely characterize plankton communities along with grazing rate measurements, which enables

to discover ecological patterns of species occurrence and distribution. My assessment of the FlowCAM showed that routine characterization of plankton species composition and size distribution is now more feasible using this automated system, giving the opportunity to observe patterns of association between species composition and level of coupling between phytoplankton growth and mortality terms. Such observations can provide a platform to test hypotheses and fuel further investigations beyond simple characterizations, which are needed to reveal the influence of the functional diversity and ecology of phagotrophic protists on protistan herbivory (Weiss et al. 2016).

Expanding the spatial and temporal resolution of protistan grazing rate measurements and further investigating the factors influencing grazing magnitude is essential to increase the availability of reliable parameters to be used in plankton models and to underpin the importance of phagotrophic protists in pelagic foodwebs.

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