A simple adjustment to test reliability of bacterivory rates derived from the dilution method

Bernadette Pree,*[1] Constanze Kuhlisch,[2] Georg Pohnert,[2] Andrey F. Sazhin,[3] Hans Henrik Jakobsen,[4] Maria Lund Paulsen,[1] Marc E. Frischer,[5] Diane Stoecker,[6] Jens C. Nejstgaard,[7] Aud Larsen[8]

[1]Department of Biology, University of Bergen, Bergen, Norway
[2]Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Jena, Germany
[3]Laboratory of Plankton Ecology, P.P. Shirshov Institute of Oceanology, Russian Academy of Sciences, Moscow, Russia
[4]Department of Bioscience, Aarhus University, Roskilde, Denmark
[5]Skidaway Institute of Oceanography, University of Georgia, Savannah, Georgia
[6]Horn Point Laboratory, University of Maryland Center for Environmental Science, Cambridge, Maryland
[7]Department 3, Experimental Limnology, Leibnitz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Stechlin, Germany
[8]Uni Research Environment and Hjort Centre for Marine Ecosystem Dynamics, Bergen, Norway

Abstract

Quantification of grazing losses of marine heterotrophic bacteria is critical for understanding nutrient and carbon pathways in aquatic systems. The dilution method is a commonly used experimental approach for quantifying bacterivory. However, valid estimates of grazing rates obtained using this method depend on several methodological assumptions including that the method does not influence specific growth rates of bacteria. Here, we hypothesize that filtration during the set-up of a dilution experiment has the potential to release allelochemicals from phytoplankton cells and thereby stimulate or inhibit bacterial growth with the consequence of biased grazing estimates. We tested this hypothesis during a natural *Phaeocystis pouchetii* bloom at two different locations within an Arctic fjord. Results from the dilution experiments suggest higher gross growth rate and grazing impact for bacteria in the outer fjord compared with the inner fjord. However, specific growth rates estimated by bacterial production cell^{-1} were significantly elevated in dilutions of water from the outer fjord but not the inner fjord. The analysis of dissolved metabolites in the seawater from both experiments prior and after filtration revealed altered metabolic profiles after filtration at both stations. As unaffected specific growth of prey on dilution is one of three fundamental assumptions of the dilution method, we conclude that it is important that empirically estimated bacterial specific growth rates be routinely included when using the dilution method to quantify bacterivory.

Marine heterotrophic prokaryotes (subsequently referred to as bacteria) are important players on a global scale in biogeochemical processes, such as nutrient uptake, carbon cycling and remineralization. Whereas methods for determination of bacterial abundance, community composition and activity are well established today, quantification of bacterial interactions with other components of the microbial food web and their dynamics remain a major challenge in the field. Grazing by heterotrophic nanoflagellates (HNF) and microzooplankton on bacteria is, together with viral lysis, the main cause of bacterial mortality (Proctor and Fuhrman 1990; Sherr and Sherr 1994; Suttle 2007). Direct measurement of bacterivory is challenging and a range of experimental methods have evolved, which can be categorized into three approaches. First, the use of fluorescent-labeled (Sherr et al. 1987) or radio-labeled (Lessard and Swift 1985) bacteria, second, the use of size fractionation (Wright and Coffin 1984) or dilution (Anderson and Rivkin 2001; Evans et al. 2003; Pearce et al. 2010; Pearce et al. 2011) to uncouple predator and prey, and third, the direct inspection of food vacuoles of predators (Dolan and Simek 1999). Often, the different approaches give results which are not necessarily

*Correspondence: bernadette.pree@uib.no

Table 1. Definitions of bacterial growth rates (d^{-1}).

Net growth rate (k)	Change of bacterial numbers over time in presence of grazers and viruses, often referred to as *apparent growth* (Landry and Hassett 1982)
Gross growth rate (μ)	Change of bacterial numbers over time in absence of grazers and viruses, in dilution experiments μ corresponds to the *y*-axis intercept (Landry et al. 1995)
Specific growth rate ($\mu_{specific}$)	Rate of biomass production per unit biomass as estimated from leucine incorporation (biomass per time unit per volume) of bacterial abundance (biomass or cell concentration per volume). Due to short incubation time (1 h typically), it is usually used as an estimate for gross growth rate (Ducklow 2000; Kirchman 2001), and sometimes referred to as *independent growth measure* (e.g., Pasulka et al. 2015)

comparable because each approach contains shortcomings not resolved yet (Vaque et al. 1994).

In comparison to the methods using labeled bacteria or food vacuole inspection, the major advantage of size fractionation and the dilution technique is their ability to estimate both grazing and growth of the prey community (net and gross growth). In size fractionation experiments growth and grazing are based only on comparison between predator-free incubations and untreated incubations. The dilution method includes a gradient of dilution with predator-free water and thereby, theoretically results in a more accurate estimate of grazing and growth rates.

The dilution method (Landry and Hassett 1982) was designed to estimate growth and grazing losses of phytoplankton but has also been applied to determination of bacterial growth and bacterivory (Anderson and Rivkin 2001; Evans et al. 2003; Pearce et al. 2010, 2011). For both prey organisms, weaknesses of the dilution method include the need for experimental manipulation and the typically long incubation time of 24 h (Landry 1994; Schmoker et al. 2013). Several studies revealed that preparation of dilutions by addition of whole seawater (WSW) to filtered seawater (FSW) can result in changed grazer behavior (Moigis 2006), differences in bacterial community composition (Agis et al. 2007), and enrichment of organic and inorganic nutrients (Ferguson et al. 1984). During some phytoplankton blooms, such as *Phaeocystis pouchetii* which is known to release organic material [review by Alderkamp et al. (2007)], preparation of FSW can cause release of dissolved metabolites that can inhibit phytoplankton growth in diluted treatments (Stoecker et al. 2015). Measurements of bacterivory may be more sensitive to experimental manipulation and long incubation times than measurements of herbivory because of the great potential of bacteria to be both stimulated and/or inhibited by released metabolites or nutrients and the relatively rapid response times of bacteria compared with phytoplankton assemblages.

In this study, we applied the dilution technique to measure bacterivory during a *P. pouchetii* bloom in coastal Arctic waters. Two locations within a fjord (inner and outer fjord, subsequently referred to as IF and OF) were selected for setting-up dilution experiments. We tested the hypotheses

that (1) 0.2 μm filtration of seawater during the set-up of a dilution experiment alters the metabolic profile of the water with a chance to release allelochemicals and as a consequence (2) the addition of 0.2 μm filtered water to the diluted treatments impacts specific growth of bacteria (see Table 1 for definition of bacterial growth) and thereby violating one of the central assumptions of the dilution technique (Landry and Hassett 1982). To address these hypotheses, we measured bacterial concentrations and bacterial production (Smith and Azam 1992) in the WSW and diluted treatments as well as comparing metabolic profiles of the WSW and the FSW used for dilution.

This combination of biological and chemical measurements allowed us to evaluate the reliability of the dilution technique for measuring bacterivory during *P. pouchetii* blooms and to recommend procedures for applying the dilution technique to measurement of bacterivory.

Materials and procedures
Theoretical outline of dilution method for bacterivory

The dilution technique uses incubations of WSW and dilutions (typically 1–4 dilutions in fraction 10%, 25%, 50%, 75% of sample to WSW) to derive estimates of net growth rate (= apparent growth rate, k) at different densities of grazers based on bulk measurements of biomass or cell counts. Landry and Hassett (1982) state 3 crucial assumptions of the method, first that growth rate of prey (μ) is not affected by dilution, second, that grazing loss (g) is proportional to grazer abundance (D=dilution factor). Third, growth of prey is assumed to be exponential, as described in Eq. 1 (Landry and Hassett 1982), where k is apparent growth rate and P_t and P_0 biomass/concentration of prey at the end and start of the incubation.

$$k = \frac{1}{t}\ln\left(\frac{P_t}{P_0}\right) \tag{1}$$

When these three assumptions are met, linear regression analysis of net growth rates of prey against dilution factor results in an reliable estimate of grazing (g, slope) and growth of the prey population in absence of any grazing (μ,

y-axis intercept) as shown in Eq. 2 (Landry and Hassett 1982).

$$k = \mu - (g \times D) \tag{2}$$

Several studies have tested whether these assumptions are met when using the dilution method for herbivory, as reviewed in Schmoker et al. (2013), but only a few studies have tested these assumptions with bacteria as prey organisms despite increased use of the dilution method to quantify bacterivory. Tremaine and Mills (1987) addressed each of the three assumptions when using the method for bacterivory and tested the first assumption by including different concentrations of dissolved organic carbon (DOC) in the dilutions to simulate potential increase in DOC in the diluent due to shear stress on cells during filtration. They found increased growth with increasing carbon concentrations, although not statistically significant. Berninger and Wickham (2005) also tested the first assumption by including one set of replicates with amended nutrients to ensure no nutrient limitation was given. However, specific growth of bacteria may not only be affected by dissolved nutrients or organic matter in the seawater but also by presence or absence of dissolved metabolites caused by release from phytoplankton cells or adsorbance/breakdown during filtration. Here, we argue that 0.2 μm filtration during the preparation of dilutions alters concentrations of dissolved metabolites and consequently affects specific growth rate of bacteria. We suggest that cell specific rates of incorporation of radioactive precursors, such as leucine, can be used as index of specific growth as shown in Eq. 3 (Ducklow 2000).

$$\mu_{\text{specific}} = \frac{BP_t}{P_t} \tag{3}$$

where μ_{specific} is converted from leucine uptake (BP_t, biomass d^{-1} cell^{-1}) of bacterial mass (P_t, biomass cell^{-1} at the end of the experiment). Bacterial production measurement is a measure of "gross production" of biomass (Ducklow 2000; Kirchman 2001), and can serve as a relatively simple control during the dilution method to test the underlying assumption that specific growth of bacteria is not affected by dilution.

Study sites and sampling

The study was carried out during the PHAEONIGMA project cruise in May 2013 on board of R/V *Håkon Mosby*. Water for dilution experiments was collected at two different locations in the Porsangerfjord (ca. 70°32′N 26°31′E), northern Norway at the maximum fluorescence depth (20 m). The first experiment was conducted on 4–5th May 2013 in IF and the second experiment on 6–7th May in OF.

At the time of sampling there was an ongoing bloom of colonial *P. pouchetii* as assessed on board by FlowCAM imaging using a color FlowCAM (ver. VS IV) with the same settings as described in Jonasdottir et al. (2011). We estimated

cell numbers using a calibrated regression between manually counted number of cells per colony and colony grey scale area (ABD) according to Jakobsen and Carstensen (2011). Cell carbon was then estimated assuming a *Phaeocystis* cell-volume of 60 μm^3 cell^{-1} and the generic volume to carbon scaling of Menden-Deuer and Lessard (2000). *Phaeocystis* single cells (motile and nonmotile stages) and other phytoplankton were identified and enumerated by epifluorescence microscopy as described in Sazhin et al. (2007). In brief, samples were stained with primuline, fixed with 3.6% glutaraldehyde and gently filtered onto black Nucleopore filters (0.4 μm) and stored at −20°C until analysis.

Chlorophyll *a* (Chl *a*) was assessed in triplicate water samples (200–250 mL) according to Parsons et al. (1984). Water was filtered onto 47 mm 0.2 μm polycarbonate filters, and immediately frozen. Prior to measurement of fluorescence, Chl *a* was extracted in 90% acetone overnight at 4°C, and analyzed using a Turner Designs AU fluorometer.

Concentrations of dissolved inorganic nutrients (i.e., PO_4^{3-}, NO_3^- and dissolved Si), were determined by colorimetric continuous flow analysis by a Skalar San Plus autoanalyzer. Analysis procedures were done on routine basis following a standard ISO17025 accredited procedure according to the methods described by Hansen and Koroleff (1999). The precision was 0.06 μM, 0.1 μM and 0.2 μM for PO_4^{3-}, NO_3^- and dissolved Si, respectively.

Experimental set-up

Before dilution experiments, all containers, bottles, filters and tubing were soaked in 10% HCl and rinsed with ultra pure water. Water for dilution experiments was collected with Niskin (5 L) bottles attached to a CTD-rosette and transferred into 20 L containers (Nalgene). The experiments consisted of four dilutions in duplicates, in the proportions of 10%, 25%, 50%, and 70% of sample relatively to the sum of sample and filtered seawater, and triplicates of whole seawater (100%, WSW). Routinely in dilution experiments, nutrients (10 μM NO$_3$, 0.6 μM PO$_4$) were added to all dilutions, to avoid mineral nutrient limitation, except of another set of triplicates of WSW without nutrient addition, serving as control. Filtered seawater (FSW) was obtained by prefiltration through a 35 μm mesh and then gravity filtration through 0.2 μm sterile inline filter (Whatman Polycap capsule). Dilutions were prepared in 20 L Nalgene containers, and subsequently syphoned into experimental bottles (2.4 L) in a staggered way to ensure homogenous water masses between the replicates. All experimental bottles were incubated at in situ temperature (4–5°C) and light conditions (∼ 6% surface irradiance) in a large volumetric container on-deck with running seawater pumped from ca. 2.5 m depth in the bow of the ship. Initial sampling for flow cytometry and bacterial production was done from remaining water from all dilutions and WSW and after 24 h of incubation from experimental bottles.

Bacteria net growth (k, d^{-1}) was calculated for each dilution and WSW treatments as in Eq. 1. Grazing (g) and gross growth rate (μ) of bacteria were calculated by linear regression analysis of net growth rate against dilution factor. Specific growth rates of bacteria were derived as shown in Eq. 3. Single analysis of variance (ANOVA) was used to test for significant differences ($p < 0.05$) among mean values of specific growth rates in the dilutions and WSW. For linear regression, ANOVA and calculation of diversity indices R-studio (including vegan-package, Oksanen et al. (2015)) was used.

Abundance of bacteria and heterotrophic nanoflagellates

Bacteria were enumerated using a FACSCalibur flow cytometer (BD, Biosciences, Franklin Lakes, New Jersey, U.S.A.) equipped with an air-cooled laser providing 15 mW at 488 nm with standard filter set-up. Samples for bacterial counts were fixed in glutaraldehyde (0.5% final conc.) for 30 min at 4°C, frozen in liquid nitrogen and stored at −80°C until further analysis. For analysis samples were thawed and diluted 5- to 100-fold in TE buffer (Tris 10 mM, EDTA 1mM, pH 8). Samples were stained with a green fluorescent nucleic-acid dye (SYBR Green I) for 10–15 min in the dark and run in the flow cytometer with the discriminator at green fluorescence and a flow rate of 30 μL min^{-1}. Bacterial population determination was based on scatter plot observations of the side-scatter signal vs. the green fluorescence signal of SYBR Green I. The cell numbers were calculated from the instrument flow rate based on volumetric measurements. For calculation of specific growth rates bacterial concentrations were converted to biomass using a carbon content of 20 fg C cell^{-1} (Lee and Fuhrman 1987).

Abundances of HNF were determined on an Attune$^\circledR$ Focusing Flow Cytometer (Applied Biosystems by Life technologies) with a syringe-based fluidic system and a 20 mW 488 nm laser. Samples were fixed and stored in the same manner as bacterial samples and were stained with SYBR Green I for 2 h in the dark and run in the flow cytometer with discrimination on basis of green and red fluorescence, side-scatter and forward-scatter following the protocol of Zubkov et al. (2007). Volumes of 800 μL were run using a flow rate of 200 μL min^{-1}.

Bacterial production

Bacterial production (BP) was determined by incorporation of ^{3}H-leucine as described in Smith and Azam (1992). Triplicate samples were incubated with ^{3}H-leucine (60 nM final concentration) for 1 h in the dark at in situ temperature in a water bath on-deck with continuous water flow. Protein synthesis was terminated by adding trichloroacetic acid (TCA) to a final concentration of 5%. A fourth sample served as control and was fixed with TCA before isotope addition. After incubation all samples were centrifuged (10 min) and the supernatant was removed and washed with TCA. The pellets were washed twice by adding 5% TCA followed by centrifugation. After removing the supernatant, scintillation cocktail (Ecoscint) was added and samples were radio assayed in a Tri-Carb 2900TR scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, Massachusetts). Disintegrations per minute (DPMs) from killed controls were subtracted from the average of live DPM. Incorporation rates of leucine were converted to bacterial production assuming a conversion factor of 1797 as the grams of protein produced per mole of incorporated leucine and 0.86 as the weight ratio (g: g) of total C: protein in bacteria (Simon and Azam 1989).

Dissolved metabolites within seawater

Sampling for metabolic profiles was done at both stations at Chl a maximum (IF, OF, $n = 3$) and during set-up of the dilution experiments of both unfiltered (IF-WSW, OF-WSW) and 0.2 μm gravity filtered sea water (IF-FSW, OF-FSW). Until processing water was kept in carboys in the dark at 15°C. For metabolic foot printing all particulate matter from 2–3 L WSW was gently removed under vacuum (600 mbar, GF/C filter) in analytical duplicates. Dissolved metabolites were absorbed to inline installed Chromabond$^\circledR$ EASY solid phase extraction cartridges (Macherey-Nagel) which were conditioned with 4 mL methanol and rinsed with 4 mL ultra pure water prior sample loading (Barofsky et al. 2009). Loaded cartridges were rinsed again with 4 mL ultra pure water, dried with a vacuum pump, and gravity eluted with 2 mL methanol and 2 mL methanol: tetrahydrofuran (1: 1 v/v; Chromasolv$^\circledR$ Plus, Sigma Aldrich; HiPerSolv, VWR). Samples were stored at −20°C until transport on ice to Jena, Germany, and at −80°C until analysis approximately 1 yr later. After thawing, 5 μL 4 mM aqueous ribitol were added as internal standard and an aliquot of 1.5 mL per sample was dried under vacuum. Derivatization was done by adding 50 μL methoxyamine solution (20 mg mL^{-1} methoxyamine hydrochloride in pyridine, Chromasolv$^\circledR$ Plus, Sigma Aldrich), incubation at 60°C for 1 h and room temperature for 11 h, and subsequent addition of 50 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide for 1 h at 40°C. Immediate GC-MS analysis and further data processing were performed as described by Vidoudez and Pohnert (2012). The DB-5ms column had a length of 30 m attached to a 4.6 m pre-column, source temperature was set to 250°C, and the split to 1. Chromatogram deconvolution was performed using AMDIS 2.71 with a smoothing window of five scans and peak integration using MET-IDEA 2.08 with a lower mass limit of 50. Artifacts found also in solvent controls were excluded using Excel 2010. The effect of 0.2 μm gravity filtration and station differences were investigated with a canonical analysis of principal coordinates (CAP) and the strongest treatment correlated peaks were putatively identified with the spectral library NIST 2011.

Assessment

Different conditions in IF and OF: background information

At the time of sampling, phytoplankton community of Porsangerfjord was dominated by colonial cells of *P. pouchetii*, accounting for 82% (IF) and 94% (OF) of the total carbon of phytoplankton at Chl a maximum (20 m). Chl a

Table 2. Overview of biological parameters and nutrient concentrations at IF and OF at Chl *a* max (20 m).

	IF	OF
Chl *a* (μg L^{-1})	6.3 ± 1.5	6.0 ± 1.7
Bacteria (mL^{-1})	7.4×10^5	2.0×10^5
HNF (mL^{-1})	615	1249
$\mu_{specific}$ (d^{-1})	1.0	0.6
Dissolved Si (μM)	0.22	0.81
NO$_3^-$ (μM)	0.00	2.10
PO$_4^{3-}$ (μM)	0.05	0.23

concentrations were similar at both stations (6.3 ± 1.5 μg Chl *a* L^{-1} in IF, 6.0 ± 1.7 μg Chl *a* L^{-1} in OF, Table 2). However, microscopy revealed higher diversity of phytoplankton community (based on carbon estimates) in IF than in OF (1.24 Shannon index in IF and 0.91 in OF). The diatom *Thalassiosira* spp. accounted for the major difference in phytoplankton assemblage of those two stations and was only present in IF and not in OF. Nutrient concentrations of dissolved Si, NO$_3^-$, and PO$_4^{3-}$ at 20 m depth were low and close to detection limit in IF but higher in OF (Table 2). Together these findings indicate that phytoplankton community at IF was at the reminiscence of a diatom bloom whereas in OF there was a monospecific *P. pouchetii* bloom.

Bacteria and HNF varied in terms of abundance in these different bloom situations (Table 2). In IF bacteria concentration at 20 m depth was 7.4×10^5 mL^{-1} and HNF abundance was 615 mL^{-1}. In OF, bacteria concentration was 3.7 × lower (2.0×10^5 mL^{-1}) and HNF abundance 2 × higher (1249 mL^{-1}). Bacterial specific growth was 1.0 d^{-1} in IF and 0.6 d^{-1} in OF. Microzooplankton abundances were low at both stations (1 mL^{-1}) and consisted of ciliates only.

Dilution experiments

Following the method of Landry and Hassett (1982) the regression line provided an estimate of gross growth rate of bacteria of 0.58 d^{-1} at IF and 0.75 d^{-1} at OF. Estimates of grazing mortality were also higher in OF (0.19 d^{-1}) than IF (0.06 d^{-1}, Fig. 1). Bacterial growth in controls of WSW was not significantly different to WSW with nutrient amendment and therefore is not shown ($p > 0.05$, Table 3).

Apparent growth rates were not significantly related to dilution factor in any of the two experiments ($p > 0.05$, Table 3) and linear regression line yielded low R^2 values ($R^2 = 0.13$ in IF, $R^2 = 0.29$ in OF) a commonly observed problem when using dilutions for bacterivory (Berninger and Wickham 2005) and herbivory (Calbet and Saiz 2013).

Specific growth at the start of the experiment (Fig. 2a) was not affected by dilution and was higher in IF (1.1 ± 0.2 d^{-1}) than in OF (0.7 ± 0.2 d^{-1}). However, at the end of the experiment (24 h) specific growth increased by a factor of 9 in dilutions of 10% and 25% compared with WSW during

1
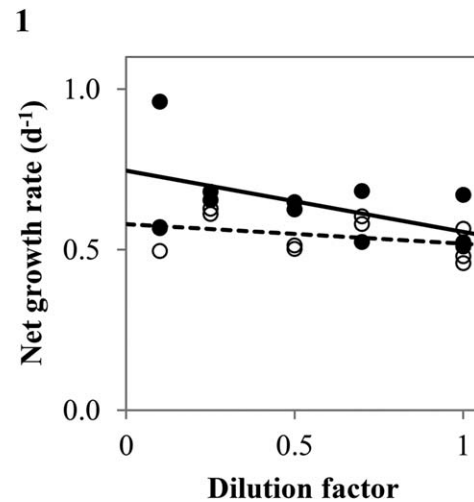
Fig. 1. Net growth rates of bacteria (d^{-1}) derived from initial and final (24 h) cell counts from IF and OF. Grazing and gross growth rates were higher in OF ($\mu_{gross} = 0.75$ d^{-1}, $g = 0.19$ d^{-1}, $R^2 = 0.29$) than in IF ($\mu_{gross} = 0.58$ d^{-1}, $g = 0.06$ d^{-1}, $R^2 = 0.13$). Filled circles and solid line symbolize samples from OF, open circles and dashed line from IF.

**Table 3.** *p*-values of statistical analysis (ANOVA and regression) of dilution experiments.

ANOVA, *p*-values	IF	OF
Regression total bacteria abundance	0.264	0.090
WSW (+nutrients), WSW (−nutrient)	0.211	0.869
Regression LNA bacteria abundance	0.939	0.013
Regression HNA bacteria abundance	0.292	0.010

the experiment in OF, corresponding to a maximum of 13.2 d^{-1} in the 10% dilution (Fig. 2b). In dilutions of IF specific growth rates remained at the same level as at the start of the experiment (1.2 ± 0.2 d^{-1}).

It should be noted that bacterial production measurements by ^{3}H-leucine uptake is controversial due to the conversions to carbon content of a "typical" bacterial cell (Ducklow 1993). In spite of this controversy we assume a constant conversionfactor for all dilutions and WSW to show how dilution affects bacterial carbon incorporation. Another issue is that leucine might act as fertilizer for bacterial growth, as it is labile and especially in oligotrophic conditions stimulating growth. For this study, we consider this a negligible problem, since incubation is only 1 h, and if leucine acted as a carbon source for C-limited bacteria, it would have had the same effect in all bottles and not change the overall result. Moreover, enrichment bioassays performed with water from both stations (Fig. 1 in Supporting Information) show that C addition in form of glucose did not increase bacterial production significantly for any of them, suggesting that C was in fact not limiting.
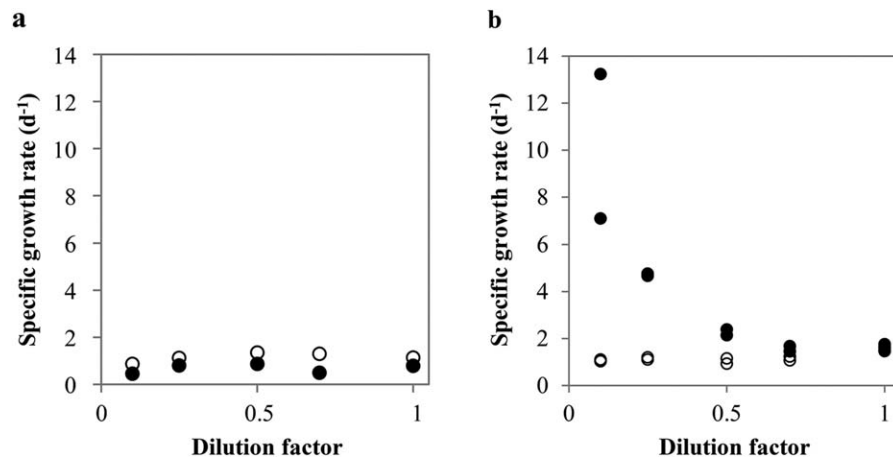
Fig. 2. Specific growth rates of bacteria (d^{-1}) at the start (**a**) and at the end (24 h, **b**) of the experiment in dilutions and WSW. Open circles display samples from IF, filled circles from OF.
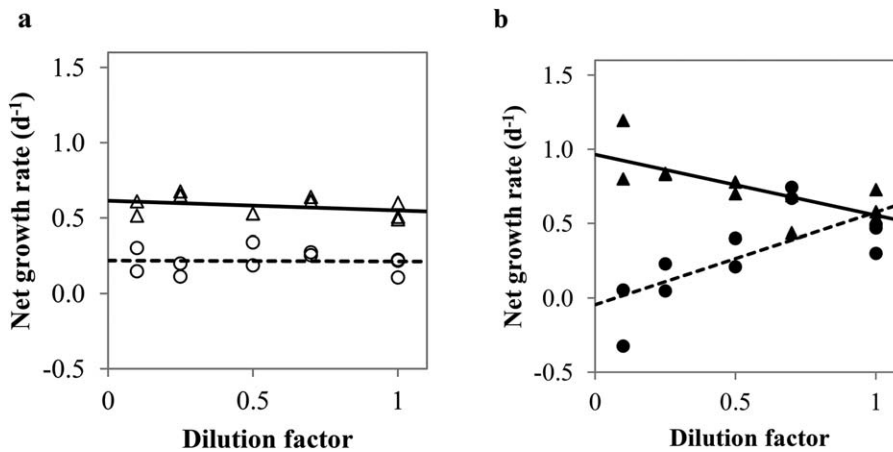

Fig. 3. Net growth rates (d^{-1}) of HNA (triangles and solid lines) and LNA (circles and dashed lines) bacteria in IF (**a**) and OF (**b**). In IF, HNA, and LNA bacteria had similar net growth in all dilutions and WSW, whereas in OF, HNA, and LNA had opposite trends.

How the differences in specific growth rates in OF affect the net growth rates of bacteria we can only speculate at this stage. One possible inference could be that bacteria in dilutions of OF did not only increase in numbers during incubation, but also in biomass and/or additionally changed community composition. Interestingly, flow cytometer plots indicated a shift in bacterial community or activity. Scatter plots of samples from WSW at the start of the dilution experiments showed two distinct populations of bacteria (Fig. 2 in Supporting Information), corresponding to different (high and low) nucleic-acid staining properties, called HNA and LNA bacteria (Li et al. 1995; Gasol et al. 1999). During 24 h of incubation, HNA and LNA bacteria developed differently at the two stations. To illustrate this change, we repeated the calculations for HNA and LNA bacteria separately (Eq. 1) and performed linear regression analysis against dilution factor. We did not aim to derive growth or

grazing rates from these calculations but to demonstrate the difference in HNA and LNA bacteria in dilutions compared with WSW. For bacteria from IF (Fig. 3a), the regression lines are more or less parallel to each other for both populations and similar to the one derived from total community counts. In OF (Fig. 3b), the discrimination into HNA and LNA bacteria revealed that HNA and LNA bacteria developed differently in dilutions. Linear regression of HNA and LNA bacteria against dilution factor was significant ($p < 0.05$) for OF but not for IF (Table 3).

Gasol et al. (1999) found a similar increase in abundances of HNA bacteria in size fractionation experiments when grazers were absent (0.8 μm filtered water). They suggested that HNA bacteria are large and active cells preferable grazed on and as a consequence of reduced grazing pressure HNA bacteria increased. We find one case where HNA and LNA developed similar in the dilution experiment (IF) and one case
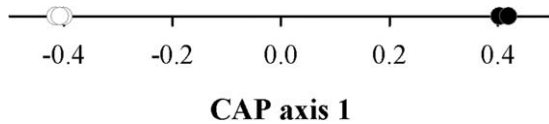
Fig. 4. Score plot of a canonical analysis of principal coordinates (CAP) showing a clear separation of seawater samples from the Chl *a* maximum at IF (open circles) compared with OF (filled circles) based on relative abundances of dissolved metabolites (*n* = 3). [Eigenvalues 0.99986, squared correlations 0.99972, misclassification error 66.67%].

Fig. 5. Score plot of a canonical analysis of principal coordinates (CAP) showing a clear separation of WSW samples (circles) compared with 0.2 *μ*m gravity filtered seawater samples (triangles) based on the relative abundances of dissolved metabolites in IF (open symbols) and OF (filled symbols) (*n* = 2). [Eigenvalues 0.9421, squared correlations 0.88755, misclassification error 25%].
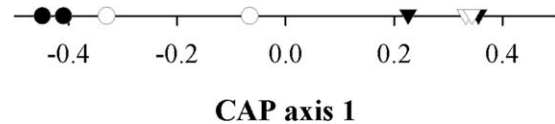
where they showed opposite net growth along a gradient of dilutions with filtered seawater (OF). Filtered seawater is not only predator free but also contains a different level of metabolites released from phytoplankton cells during filtration or reduced during filtration compared with unfiltered seawater. Therefore, we cannot determine whether HNA bacteria in dilutions of OF grow better due to decreased grazing or due to change in concentrations of metabolites. We did not examine community composition and we cannot tell whether HNA and LNA are different phylogenetic groups (Zubkov et al. 2001; Vila-Costa et al. 2012) or whether this discrimination is due to different levels of bacterial activity from the same taxa (Li et al. 1995). However, the increase of specific growth rate in OF corresponds well with the increase of HNA bacteria numbers in OF and most importantly constitutes a violation of one of the basic assumptions of the dilution method (Landry and Hassett 1982).

Whereas we found increased growth rates of bacteria in dilutions with FSW in OF, Pasulka et al. (2015) recently described decreased specific growth of bacteria in dilutions prepared for estimating viral lysis (30 kDA filtrate) and grazing on picophytoplankton. Pasulka and colleagues were not aiming for retrieving rates for bacterivory from their experiments but suggest that altered growth of bacteria in dilutions have an impact on the other communities like picophytoplankton and consequently the grazing rates. Findings of Pasulka et al. (2015) provide additional support of the main objective of this study, which is to show, that dilution method needs modifications and cautionary use when applying it for bacterivory estimates.

Dynamics within grazer community during incubation are important to consider during dilution experiments because, e.g., if there is a trophic cascade within grazers during incubations, grazing rates become biased (Calbet and Saiz 2013). In our study, ciliate abundances were similar and low (1 mL^{-1}) at both stations, and although HNF concentrations were different in IF and OF, (Table 2) their growth rates during the experiments (Fig. 3 in Supporting Information) were similar in dilutions and WSW suggesting that there was neither a strong trophic cascade nor an effect of dissolved metabolites on HNFs.

Filtration altered sea water chemistry

The profiles of metabolites in seawater (exometabolome) of IF and OF can be separated with a CAP when using IF/ OF as groups (Fig. 4). By subtraction of chromatographic profiles of control samples it was made sure that only metabolites in the seawater and no contaminations contributed to this difference. This result is in line with the other parameters previously used to indicate different bloom status despite similar Chl *a* concentrations at the two stations (Table 2).

For dilution experiments at both sampling sites significant differences in the metabolic profile of the seawater were observed when comparing FSW and WSW (Fig. 5). Thus, filtration has a clear impact on the metabolic composition of seawater masking the initial differences of metabolic profiles of seawater of IF and OF (Fig. 4).
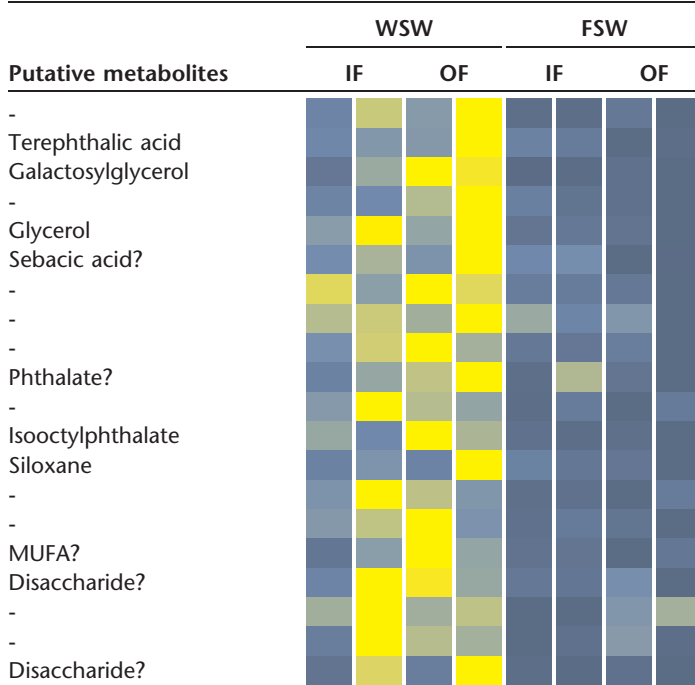
The GC-MS metabolic profiling approach allows to tentatively assign structures to compounds that cause the separation into distinct groups. The 0.2 *μ*m gravity filtration reduced some contaminations (phthalates, sebacic acid, siloxanes) but also saccharides (galactosyl-glycerol, disaccharides; Table 4a). This can be due to an adsorption effect on the filter or depletion due to a lack of stability of metabolites during filtration. Further, we also detected several metabolites that were more abundant after filtration (Table 4b), possibly released due to stress and disruption of cells during filtration. Unfortunately most metabolites remain unknown, however, some fatty acids and dodecanol could be tentatively assigned by spectral library search.
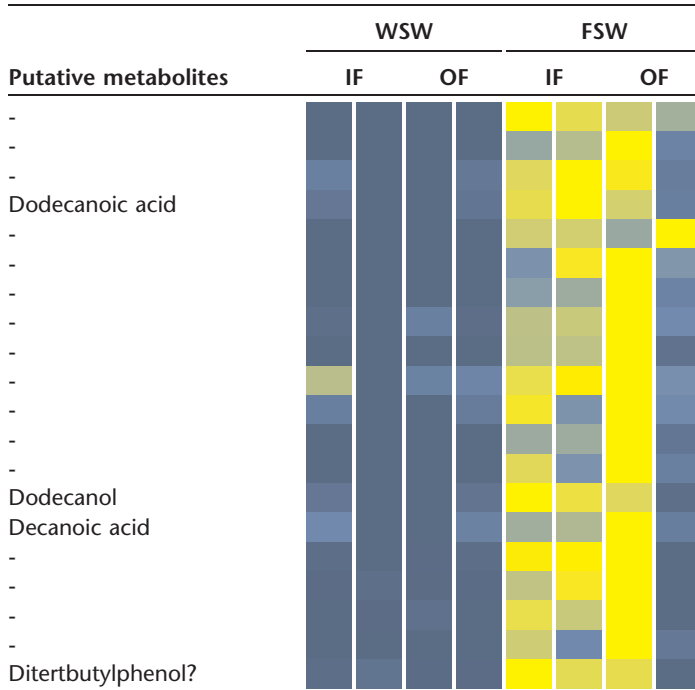
Discussion

The main objective of this study was to provide a relatively simple adjustment of the dilution method to test the first basic assumption of Landry and Hassett (1982) by measuring bacterial production additional to abundance. We provide results of two dilution experiments used to derive bacterivory rates where in one case (IF) specific growth rates were not affected by dilution and another case (OF) where these were significantly affected. Chemical analysis revealed differences in metabolic profiles of metabolites in seawater from the two stations as well as a change in metabolic profiles caused by the filtration. Bacteria growth might be affected by release of allelochemicals but also by the adsorption of metabolites, such as saccharides due to filtration. For

Table 4. Heat map of dissolved seawater metabolites with highest correlation to the CAP axis separating WSW and FSW as groups for both the inner (IF) and outer (OF) Porsangerfjord station. Yellow: more abundant, blue less abundant.

a) Putative metabolites which decreased or depleted during filtration.

	WSW		FSW	
Putative metabolites	IF	OF	IF	OF
-				
Terephthalic acid				
Galactosylglycerol				
-				
Glycerol				
Sebacic acid?				
-				
-				
-				
Phthalate?				
-				
Isooctylphthalate				
Siloxane				
-				
-				
MUFA?				
Disaccharide?				
-				
-				
Disaccharide?				

b) Which are only present or increased after filtration.

	WSW		FSW	
Putative metabolites	IF	OF	IF	OF
-				
-				
-				
Dodecanoic acid				
-				
-				
-				
-				
-				
-				
-				
-				
-				
Dodecanol				
Decanoic acid				
-				
-				
-				
-				
Ditertbutylphenol?				

MUFA, monounsaturated fatty acid.
"-", unknown metabolite.
"?", reverse match in comparison with NIST library < 800.

an in-depth evaluation a structure determination of the relevant metabolites causing the differences would be required but this was clearly beyond the scope of this work.

Obviously, our study is limited to dilution experiments during a *P. pouchetii* bloom and further studies under different conditions are needed. Nevertheless, *Phaeocystis* sp. is an important primary producer as it seasonally forms dense blooms at higher latitudes and may dominate the pelagic biomass at certain periods (reviewed by Verity et al. 2007). Moreover, change of metabolic profiles when filtering seawater for dilution experiments is not only an issue during blooms of *Phaeocystis* but has also been shown during other phytoplankton blooms such as *Skeletonema marinoi* (Stoecker et al. 2015).

Analysis of metabolic profiles before and after filtration during the set-up of dilution experiments is desirable but not always feasible due to high workload and costs. However, the suggested modification of including BP measurements as a proxy of specific growth when using dilution method for bacterivory helps to unravel uncertainties coming along with the method and to understand whether derived bacterivory rates are reliable or not. In terms of additional costs/efforts the suggested modification is minor, also, only little volume of the incubation bottles is needed for BP measurements (<10 mL).

For previous studies of bacterivory using dilution method based on flow cytometric analysis, one could reanalyze the scatter plots and check whether two different populations (HNA, LNA) are distinguishable. Further, if they developed differently during incubation in dilutions and WSW the assumption can be drawn that either bacterial community or bacteria specific growth was affected by filtration and bacterivory rates are biased.

Comments and recommendations

We recommend including measurements of BP to the protocol of the dilution method for bacterial grazing to test and validate the effect of dilution with 0.2 μm filtered water on bacteria specific growth rates. By that, one can identify the circumstances under which the dilution technique provides reliable estimates for bacterivory and minimize the uncertainty the impact filtration might have on bacterial growth. In case of different specific growth rates in dilutions and WSW the derived grazing rates are inaccurate. If future studies show that in the majority of dilution experiments specific growth rates differ between WSW and dilutions, alternative methods not using a filtration step should be preferred. At the same time, improvement of the filtration protocol, by combining biological and chemical measurements, is a necessity for further developing the dilution method toward more accurate estimates of bacterivory rates.

References

Agis, M., A. Granda, and J. R. Dolan. 2007. A cautionary note: Examples of possible microbial community dynamics in dilution grazing experiments. J. Exp. Mar. Biol. Ecol. **341**: 176–183. doi:10.1016/j.jembe.2006.09.002

Alderkamp, A. C., A. G. J. Buma, and M. Van Rijssel. 2007. The carbohydrates of *Phaeocystis* and their degradation in the microbial food web. Biogeochemistry **83**: 99–118. doi:10.1007/s10533-007-9078-2

Anderson, M. R., and R. B. Rivkin. 2001. Seasonal patterns in grazing mortality of bacterioplankton in polar oceans: A bipolar comparison. Aquat. Microb. Ecol. **25**: 195–206. doi:10.3354/ame025195

Barofsky, A., C. Vidoudez, and G. Pohnert. 2009. Metabolic profiling reveals growth stage variability in diatom exudates. Limnol. Oceanogr.: Methods **7**: 382–390. doi:10.4319/lom.2009.7.382

Berninger, U.-G., and S. Wickham. 2005. Response of the microbial food web to manipulation of nutrients and grazers in the oligotrophic Gulf of Aqaba and northern Red Sea. Mar. Biol. **147**: 1017–1032. doi:10.1007/s00227-005-1565-1

Calbet, A., and E. Saiz. 2013. Effects of trophic cascades in dilution grazing experiments: From artificial saturated feeding responses to positive slopes. J. Plankton Res. **35**: 1183–1191. doi:10.1093/plankt/fbt067

Dolan, J. R., and K. Simek. 1999. Diel periodicity in *Synechococcus* populations and grazing by heterotrophic nanoflagellates: Analysis of food vacuole contents. Limnol. Oceanogr. **44**: 1565–1570. doi:10.4319/lo.1999.44.6.1565

Ducklow, H. W. 1993. Bacterioplankton distributions and production in the northwestern Indian Ocean and Gulf of Oman, September 1986. Deep-Sea Res. Part II Top. Stud. Oceanogr. **40**: 753–771. doi:10.1016/0967-0645(93)90056-S

Ducklow, H. W. 2000. Bacterial production and biomass in the oceans. *In* D. L. Kirchman [ed.], Microbial ecology of the oceans. John Wiley and Sons.

Evans, C., S. D. Archer, S. Jacquet, and W. H. Wilson. 2003. Direct estimates of the contribution of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp. population. Aquat. Microb. Ecol. **30**: 207–219. doi:10.3354/ame030207

Ferguson, R. L., E. N. Buckley, and A. V. Palumbo. 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47**: 49–55.

Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman, and A. Hagstrom. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl. Environ. Microbiol. **65**: 4475–4483.

Hansen, H. P., and F. Koroleff. 1999. Determination of nutrients, p. 159–228. *In* K. Grasshoff [ed.], Methods of seawater analysis. Wiley-VCH Verlag GmbH.

Jakobsen, H. H., and J. Carstensen. 2011. FlowCAM: Sizing cells and understanding the impact of size distributions on biovolume of planktonic community structure. Aquat. Microb. Ecol. **65**: 75–87. doi:10.3354/ame01539

Jonasdottir, S. H., and others. 2011. Extensive cross-disciplinary analysis of biological and chemical control of *Calanus finmarchicus* reproduction during an aldehyde forming diatom bloom in mesocosms. Mar. Biol. **158**: 1943–1963. doi:10.1007/s00227-011-1705-8

Kirchman, D. 2001. Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments, p. 227–238. *In* J. H. Paul [ed.], Methods in microbiology. Academic Press.

Landry, M. R. 1994. Methods and controls for measuring the grazing impact of planktonic protists. Mar. Microb. Food Webs **8**: 37–57.

Landry, M. R., and R. P. Hassett. 1982. Estimating the grazing impact of marine micro-zooplankton. Mar. Biol. **67**: 283–288. doi:10.1007/BF00397668

Landry, M. R., J. Kirshtein, and J. Constantinou. 1995. A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental tests in the central equatorial Pacific. Mar. Ecol. Prog. Ser. **120**: 53–63. doi:10.3354/meps120053

Lee, S., and J. A. Fuhrman. 1987. Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl. Environ. Microbiol. **53**: 1298–1303.

Lessard, E. J., and E. Swift. 1985. Species-specific grazing rates of heterotrophic dinoflagellates in oceanic waters, measured with a dual-label radioisotope technique. Mar. Biol. **87**: 289–296. doi:10.1007/BF00397808

Li, W. K. W., J. F. Jellett, and P. M. Dickie. 1995. DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. Limnol. Oceanogr. **40**: 1485–1495. doi:10.4319/lo.1995.40.8.1485

Menden-Deuer, S., and E. J. Lessard. 2000. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. Limnol. Oceanogr. **45**: 569–579. doi:10.4319/lo.2000.45.3.0569

Moigis, A. G. 2006. The clearance rate of microzooplankton as the key element for describing estimated non-linear dilution plots demonstrated by a model. Mar. Biol. **149**: 743–762. doi:10.1007/s00227-005-0202-3

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'Hara, L. G. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner 2015. vegan: Community Ecology Package. R package version 2.3-0. http://CRAN.R-project.org/package=vegan.

Parsons, T. R., Y. Maita, and C. M. Lalli. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press.

Pasulka, A. L., T. J. Samo, and M. R. Landry. 2015. Grazer and viral impacts on microbial growth and mortality in

the southern California Current Ecosystem. J. Plankton Res. **37**: 320–336. doi:10.1093/plankt/fbv011

Pearce, I., A. T. Davidson, P. G. Thomson, S. Wright, and R. Van Den Enden. 2010. Marine microbial ecology off East Antarctica (30-80 degrees E): Rates of bacterial and phytoplankton growth and grazing by heterotrophic protists. Deep-Sea Res. Part II Top. Stud. Oceanogr. **57**: 849–862. doi:10.1016/j.dsr2.2008.04.039

Pearce, I., A. T. Davidson, P. G. Thomson, S. Wright, and R. Van Den Enden. 2011. Marine microbial ecology in the sub-Antarctic Zone: Rates of bacterial and phytoplankton growth and grazing by heterotrophic protists. Deep-Sea Res. Part II Top. Stud. Oceanogr. **58**: 2248–2259. doi: 10.1016/j.dsr2.2011.05.030

Proctor, L. M., and J. A. Fuhrman. 1990. Viral mortality of marine bacteria and cyanobacteria. Nature **343**: 60–62. doi:10.1038/343060a0

Sazhin, A., L. F. Artigas, J. Nejstgaard, and M. Frischer. 2007. The colonization of two *Phaeocystis* species (Prymnesiophyceae) by pennate diatoms and other protists: A significant contribution to colony biomass, p. 137–145. *In* M. A. van Leeuwe, J. Stefels, S. Belviso, C. Lancelot, P. G. Verity and W. W. C. Gieskes [eds.], Phaeocystis, major link in the biogeochemical cycling of climate-relevant elements. Springer Netherlands.

Schmoker, C., S. Hernández-León, and A. Calbet. 2013. Microzooplankton grazing in the oceans: Impacts, data variability, knowledge gaps and future directions. J. Plankton Res. **35**: 691–706. doi:10.1093/plankt/fbt023

Sherr, B. F., E. B. Sherr, and R. D. Fallon. 1987. Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bacterivory. Appl. Environ. Microbiol. **53**: 958–965.

Sherr, E. B., and B. F. Sherr. 1994. Bacterivory and herbivory: Key roles of phagotrophic protists in pelagic food webs. Microb. Ecol. **28**: 223–235. doi:10.1007/BF00166812

Simon, M., and F. Azam. 1989. Protein content and protein synthesis rates of planktonic marine bacteria. Mar. Ecol. Prog. Ser. **51**: 201–213. doi:10.3354/meps051201

Smith, D. C., and F. Azam. 1992. A simple, economical method for measuring bacterial protein synthesis rates in seawater using 3H-leucine. Mar. Microb. Food Webs **6**: 107–114.

Stoecker, D. K., and others. 2015. Underestimation of microzooplankton grazing in dilution experiments due to inhibition of phytoplankton growth. Limnol. Oceanogr. **60**: 1426–1438. doi:10.1002/lno.10106

Suttle, C. A. 2007. Marine viruses—major players in the global ecosystem. Nat. Rev. Microbiol. **5**: 801–812. doi: 10.1038/nrmicro1750

Tremaine, S. C., and A. L. Mills. 1987. Tests of the critical assumptions of the dilution method for estimating bacteriovory by microeucaryotes. Appl. Environ. Microbiol. **53**: 2914–2921.

Vaque, D., J. M. Gasol, and C. Marrase. 1994. Protists grazing rates: the significance of methodology and ecological factors. Mar. Ecol. Prog. Ser. **109**: 263–274. doi:10.3354/meps109263

Verity, P. G., C. P. Brussaard, J. C. Nejstgaard, M. A. Van Leeuwe, C. Lancelot, and L. K. Medlin. 2007. Current understanding of *Phaeocystis* ecology and biogeochemistry, and perspectives for future research. Biogeochemistry **83**: 311–330. doi:10.1007/s10533-007-9090-6

Vidoudez, C., and G. Pohnert. 2012. Comparative metabolomics of the diatom *Skeletonema marinoi* in different growth phases. Metabolomics **8**: 654–669. doi:10.1007/s11306-011-0356-6

Vila-Costa, M., J. M. Gasol, S. Sharma, and M. A. Moran. 2012. Community analysis of high- and low-nucleic acid-containing bacteria in NW Mediterranean coastal waters using 16S rDNA pyrosequencing. Environ. Microbiol. **14**: 1390–1402. doi:10.1111/j.1462-2920.2012.02720.x

Wright, R. T., and R. B. Coffin. 1984. Measuring microzooplankton grazing on planktonic marine bacteria by its impact on bacterial production. Microb. Ecol. **10**: 137–149. doi:10.1007/BF02011421

Zubkov, M. V., B. M. Fuchs, P. H. Burkill, and R. Amann. 2001. Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. Appl. Environ. Microbiol. **67**: 5210–5218. doi:10.1128/AEM.67.11.5210-5218.2001

Zubkov, M., P. H. Burkill, and J. N. Topping. 2007. Flow cytometric enumeration of DNA-stained oceanic planktonic protists. J. Plankton Res. **29**: 79–86. doi:10.1093/plankt/fbl059