Low microzooplankton grazing rates in the Arctic Ocean during a *Phaeocystis pouchetii* bloom (Summer 2007): fact or artifact of the dilution technique?

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Abstract

We studied the structure and dynamics of the microbial community of Arctic waters during July 2007 using a microzooplankton grazing dilution approach. The sampling area covered a latitudinal transect along the East Greenland Sea, and a series of stations in the high Arctic (up to 80º 50' N), west and north of the Svalbard Islands. A main feature of the area was the presence of *Phaeocystis pouchetii*, which formed dense blooms. Despite the considerable biomass of microzooplankton (mostly large ciliates and dinoflagellates), their grazing impact on phytoplankton was significant only in 6 out of 16 experiments for total chlorophyll *a*, which resulted in 8% of the standing stock consumed on average. Overall, phytoplankton instantaneous growth rates were very low and even negative at times (range:-0.24 to 0.14; average: -0.04 for total chlorophyll), which could not be attributed to nutrient limitation nor the estimated microzooplankton grazing. We present 3 nonexclusive explanations for this fact: 1) we were facing a senescent community in which many organisms were dying either as a result of virus infections or for other natural causes, as corroborated by parallel estimates of natural cell mortality using membrane permeability probes; 2) the widespread and abundant *P. pouchetii* was probably deterring grazing and adversely affecting the entire planktonic community at the time of the study; and 3) the dilution technique failed in giving a real estimate of grazing (i.e. either non significant or positive slopes), likely consequence of trophic cascades (decline of major grazers in the more concentrated treatments) combined with saturated-feeding responses. This last point calls for special attention when intending to use the dilution technique in productive environments, where grazing may be saturated.

Keywords: Arctic Ocean; Microzooplankton; *Phaeocystis pouchetii*; Dilution technique, Food web.

Running title: Food web interactions in the Arctic Ocean

Introduction

The fast melting of Arctic ice, a consequence of global warming (Cavalieri *et al.*, 2003; Johannessen *et al.*, 2004), has profound ecological consequences on Arctic wildlife, including polar bears, walruses, and ringed seals, which are threatened by the reduction of the sea-ice cover. Not so evident are the changes that the inhabitants of the waters underneath and surrounding the Polar ice cap will suffer under progressive melting conditions. One of the key groups in marine food webs, which are potentially sensitive to these Arctic scenario changes, are the microzooplankton. Microzooplankton are of paramount importance as regulating agents of primary production (Calbet and Landry, 2004). Regrettably, their role in the high Arctic still remains uncertain and needs to be determined to fully understand and predict the consequences of the changes this ecosystem will experience in the future. For instance, the relevance of the trophic impact of this group in the Arctic Ocean food web has mostly been studied either in bays or in relatively low latitude sites (Paranjape, 1987; Gifford *et al.*, 1995; Olson and Strom, 2002; Verity *et al.*, 2002; Strom *et al.*, 2008), or it has been derived indirectly (Levinsen *et al.*, 1999; Rysgaard *et al.*, 1999; Levinsen and Nielsen, 2002). Most of these studies evidence a strong control of primary production by microzooplankton grazing. However, a recent work in high Arctic waters by Sherr *et al.* (Sherr *et al.*, 2009) questions such strong control, likely due, according to the authors, to a high top-down impact of copepods on microzooplankton (Levinsen and Nielsen, 2002; Campbell *et al*., 2009).

For this reason, we undertook a study of the microbial interactions in Arctic waters during the melting season. Our research coincided with a bloom of *P. pouchetii* (Lasternas and Agustí, in press), which will add further value to our results because, despite being a successful species in Arctic waters (Schoemann *et al.*, 2005), the very few data dealing with the impact of microzooplankton feeding on these algae have rendered contradictory conclusions. Weisse and Scheffel-Moser (Weisse and Scheffel-Moser, 1990) measured microzooplankton grazing on a *P. cf. pouchetii* bloom in the North Sea using the dilution technique (Landry and Hassett, 1982)

finding grazing loss rates from 0.037 to 0.174 h^{-1}, grazing rates increasing in the course of the bloom and exceeding phytoplankton growth rates at the end. Gifford *et al.* (Gifford *et al*., 1995), also using the dilution technique, did not detect any grazing on phytoplankton in high-latitude North Atlanctic Ocean during a *P. pouchetii* bloom, but they obtained increased microzooplankton grazing as the bloom declined. Archer *et al.* (Archer *et al.,* 2000) measured microzooplankton grazing impact under relatively low abundance of *P. pouchetii* in 3 fjords of the northern Norway, and obtained significant grazing rates on total phytoplankton and on fluorescently labeled algae of similar size to *P. pouchetii*. On the other hand, Wolfe *et al.* (2000) only found significant grazing on chlorophyll and DMSP when *P. pouchetii* cells were in poor condition in the Labrador Sea. In this regard, the ability of microzooplankton to ingest *Phaeocystis* spp. has been widely discussed in the literature (see reviews by Whipple *et al*., 2005, and Nejstgaard *et al.,* 2007). It is usually accepted microzooplankton exerts their pressure mostly on single cells; however, there are evidence of *Noctiluca scintillans* (Weisse *et al*., 1994; Jakobsen and Tang, 2002) and *Gyrodinium cf. spirale* (Stelfox-Widdicombe *et al.*, 2004) are able to ingest small colonies.

Methods

This study was part of a multidisciplinary project (ATOS: POL2006-00550/CTM), and took place on board the research vessel BIO Hespérides from July 1 to 24, 2007. The cruise departed Reykjavik (Iceland), and sampling started northward along the Greenland Sea (Table 1, Fig. 1). In Arctic waters, we sampled a series of stations in the vicinity of the ice-edge, alternating between several stations free of ice and two coastal stations near the Svalbard Islands. During the study, we reached a historical minimum of Arctic ice cover (Zhang *et al.* 2008), allowing samples to be taken from

areas up to 80º 50' N. The stations lasted 24 h, starting with profiles for the measurement of salinity, temperature, and fluorescence during the early morning using a Seabird CTD911, followed by collection of water samples for the determination of chlorophyll *a* concentration (hereafter Chl *a*) with 12 l Niskin bottles fitted to a rosette during the ascending CTD casts.

At each station we conducted standard grazing dilution experiments (Landry and Hassett, 1982) to assess the microzooplankton grazing impact on primary producers and on other components of the food web (see below). This technique consists of the sequential dilution of natural water with filtered seawater to obtain a gradient of net grazing impact on phytoplankton. The water for these experiments was collected at the fluorescence maximum (Table 1) using a rosette equipped with 12-L Niskin bottles, according to the fluorescence profile. Once on deck, the water was gravity-filtered through a Pall Acropak 0.8/0.2 500 capsule (previously flushed, including tubing, with diluted HCl and thoroughly rinsed with deionised water), and then 2 replicate bottles (2.3-L acid washed polycarbonate) for each of the dilution treatments were filled with the corresponding required amount of filtered seawater. Afterwards, we added natural non-filtered seawater from the selected depth to the bottles to generate experimental water percentages of 13, 27, 50, 73, and 100%. In some stations, the presence of a dense bloom of the haptophyte *Phaeocystis pouchetii* made it impossible to efficiently filter the water through the Acropak capsules; therefore, filtered seawater originated from below the bloom depth.

To guarantee the homogeneity of the natural water poured from different Nisking bottles filled at the same depth, we used a 20-L intermediary carboy in which the water was gently mixed by its own flow. All the process was carried out under dim light conditions to avoid cell light-damage. To promote constant and saturated phytoplankton growth in the dilution series, each bottle received added nutrients (10 μM NH_4Cl and 0.7 μM Na_2HPO_4). In addition, four 100% (i.e., not diluted) bottles were prepared without nutrients to assess the natural growth of the algae. Two of these latter bottles were sacrificed for initial samples. Because we did not screen the water used for the dilution series to avoid damaging delicate microzooplankton, the experimental suspension may contain some mesozooplankton. We examined the bottles by eye to observe the presence of large copepods, and the very few times this

occurred did not result in inconsistent results in the dilution series.

All bottles were incubated on deck in a large (600 L), dark incubator with open-circuit water running from a 5-m depth at a temperature about the same as *in situ*. The natural sunlight was dimmed with appropriate dark plastic mesh to mimic the light intensity at the fluorescence maximum. We gently mixed the bottles by repeatedly turning them upside down and moving them around the incubator at least three times per day. After 27-32 h, we finished the incubations and took samples for quantification of total and > 5-µm Chl *a* concentration. To get further insight into the actual trophic interactions during dilution experiments and to detect possible artifacts (Dolan *et al.*, 2000; Agis *et al.*, 2007; Modigh and Franzè, 2009), we additionally took samples for the determination of nano- and microplankton from the 2 initials and in one of the replicates per dilution level, and preserved them with Acidic Lugol's solution (2% final concentration). To avoid damaging the delicate cells we first added the fixative and then gently siphoning the water sample directly into the sample bottle

For total Chl *a*, we filtered 50-250 ml of water (depending on station and dilution level) under low vacuum pressure (< 100 mm Hg) through Whatmann glass fiber filters (GF/F, 25 mm diameter). For the > 5-µm fraction, we filtered 100-300-ml samples through 5-µm pore-size polycarbonate Osmonics Inc. filters (25 mm diameter). After filtration, the filters were stored frozen at -20°C until fluorometrical analysis of acetone extracts, with and without acidification (Parsons *et al.*, 1984) on a Turner Designs Fluorometer.

Lugol-preserved samples were processed in the laboratory. We concentrated the most diluted treatments (13%, 25% and 50%) by first settling the entire bottle for 72 h and gently siphoning off 50-75% of the supernatant water without re-suspending the sample. Then, for all the samples, 100 ml of the concentrate was settled in Utermöhl chambers for at least 48 h prior and counted under the microscope. The whole chamber, or a fraction of it for the smallest and more abundant organisms, was counted under an inverted microscope (XSB-1A) at 100, 250, and 400X magnification, depending on the group. Fifty to one-hundred cells per group were sized, adjusted to their closest geometric shape, and converted into carbon using the equations of Menden-Deuer and Lessard (Menden-Deuer and Lessard, 2000). We did not use any correction factor to compensate for ciliate losses due to fixation, as

previously suggested by Broglio *et al.* (Broglio *et al.*, 2004), and Calbet and Saiz (Calbet and Saiz, 2005), because recent research has revealed that these sorts of corrections should apply to many other planktonic groups, not only ciliates (Zarauz and Irigoien, 2008), and universal factors have not been developed yet. Because plankton were preserved with acidic Lugol's solution, no distinction between strict heterotrophs and auto/mixotrophs was done for flagellates, ciliates, and some dinoflagellates. However, the groups identified to species level were classified trophically according to the literature.

Instantaneous growth rates in dilution grazing experiments were derived from net growth in the unamended bottles (no nutrients added) and were corrected for mortality by microzooplankton from dilution experiments when the latter was significant. All statistical tests were conducted with JMP 7.0 statistical software.

Results

Plankton biomass and distribution

We present a summary of the different fractions of Chl *a* in Table 1 and the contribution of micro- and nanoplankton to total plankton community biomass at the sampled stations in Table 2 (a detailed summary of the species composition is presented in Web Annex 1). The main feature of the data for the Arctic stations is the almost ubiquitous presence of the haptophyte *Phaeocystis pouchetii*, which formed dense blooms (Table 2, Fig. 2) and whose distribution was directly correlated with total Chl *a* ($r=0.76$, $p<0.01$), and inversely correlated with temperature ($r = 0.77$; $p < 0.05$). Following *P. pouchetii*, diatoms (mostly chain forming species > 20 μm in total length of the genera *Chaetoceros*, *Fragilariopsis*, *Nitzschia*, *Rhizosolenia*, *Thalassiothrix*, and *Thalassiosira*) were the second group in biomass relevance within phytoplankton, being dominant in the Greenland Sea and at warmer stations. Regarding micrograzers, the important contribution of large ciliates (mostly Tintinnida, *Strombidium* spp., and the mixotroph *Laboea* spp.) was noticeable in the Greenland Sea and Svalbard Islands coastal station (Table 2). Actually, ciliates were the overall major contributors to microzooplankton during the study and were substantial components of the total plankton biomass (ca. 25%), whereas

heterotrophic dinoflagellates (considering 50% of unidentified dinoflagellates as heterotrophic; Lessard and Swift, 1985; Sherr and Sherr, 2007) represented, on average, 12% of the total carbon biomass (Table 2). In relation to the relevance of heterotrophs, it is interesting to note that the quotient between heterotrophic and autotrophic carbon (indicative of the trophic characteristics of the system) was > 1 in the Greenland Sea and Svalbard Islands coastal stations (Fig. 3).

Despite the high heterotrophic biomass, we did not find significant correlations between any of the considered size fractions of Chl *a*, large heterotrophic dinoflagellates (e.g., *Gyrodinium* spp., *Protoperidinium* spp., and *Katodinium* spp.), and ciliates. However, large mixotrophic dinoflagellates (*Dinophysis* spp., *Ceratium* spp., *Gonyaulax* spp., and *Amphidinium* spp.) were positively correlated with diatoms ($r = 0.85$; $p < 0.05$). Correlations between groups do not necessarily mean causality, but they can indicate that an association exists. Likewise, unidentified > 20-µm dinoflagellates were also positively correlated with *P. pouchetti* biomass ($r = 0.72$; $p < 0.05$). However, when excluding from the analysis station 43 (the station with the maximum biomass of *P. pouchetii*) this relationship is strongly affected and it becomes non-significant.

We calculated the C:Chl *a* ratios using the Chl *a* data and the autotrophic biomass (in carbon), obtained by cell counting and conversion to carbon using literature equations (Table 2). For unidentified dinoflagellates and we assumed 50% of the organisms were phototrophs. Because our microscope counting technique, based on Lugol-preserved samples, does not allow for good resolution on the lower size-fractions, we can better estimate the C:Chl *a* ratio using only > 5-µm Chl *a*. The values averaged 18 for the entire data-set, and ranged from 4 to 47.

Dilution grazing experiments

Table 3 shows the outcome of the dilution grazing experiments based on the different size-fractions of Chl *a*. Unanticipated results from these experiments include the following: i) the little, or even at times negative, phytoplankton instantaneous growth (range: -0.24 to 0.14; average -0.04 for total Chl *a*) and ii) the low microzooplankton grazing impact on primary producers. Significant microzooplankton grazing (i.e.,

significant negative slopes in the dilution experiments) were found only in 6 out of 16 experiments for total and <5-µm Chl *a*, and no significant mortality was detected in the >5-µm fraction in any of the stations. Overall, microzooplankton grazing cannot explain the observed phytoplankton negative net growth rates (especially in the >5-µm size-fractions) and must be attributed to other causes (see discussion). However, it is interesting to note the inverse relationship between the phytoplankton net growth rates and Chl *a* concentration (Fig. 4) in the Artic Ocean stations. This points towards a naturally-occurring, density-dependent mortality effect, probably linked to the presence of *Phaeocystis pouchetii*, although not significantly related to the occurrence of either this or any other planktonic group. Likewise, and related to net phytoplankton growth rates, there was no clear evidence of nutrient limitation at most of the stations (Fig. 5).

The phytoplankton and microzooplankton composition analysis in the initial and final dilution experiments evidenced a very complex food web scenario, with frequent negative growth rates both in autotrophs and heterotrophs, frequent positive slopes, and very few cases of significant microzooplankton grazing impact (Table 4). Overall, it is difficult to extract any clear interpretation or global pattern out of the dilution data for the different plankton groups. Nevertheless, we decided to present these data because negative dilution grazing results in the literature have seldom been discussed (Dolan and McKeon, 2005). As example, we show in Fig. 6 the dilution experiment plots for stations 4 and 33. It is interesting to note in these plots the positive slopes for some prey and the negative slopes (theoretically indicative of grazing) for some potential grazers of phytoplankton.

Discussion

Community composition and microzooplankton grazing on phytoplankton

A main characteristic that distinguishes our study from previous ones in Arctic waters is the peculiarity of the composition of the heterotrophic microbial community. Past works stressed the relevance of heterotrophic dinoflagellates in Arctic waters, especially when diatoms dominated the autotrophic community (Levinsen *et al.*, 1999; Rysgaard *et al.*, 1999; Sherr *et al.*, 1997; Sherr and Sherr, 2007; Sherr *et al.*,

2009). We found a population rich in ciliates, which accounted for ca. 2/3 of the total microzooplankton biomass (assuming 50% of the unidentified dinoflagellates were heterotrophs). This high contribution was especially evident in the Greenland Sea, where they completely dominated the microplankton. We suspect that in these aforementioned waters we were facing a very unusual situation, likely the result of temporal unbalances in the structure of the community (e.g., the end of a phytoplankton bloom), as the biomass of producers seems unable to maintain such a high abundance of grazers.

Despite the relatively high biomass of microzooplankton, the grazing rates on phytoplankton (Chl *a*) were low. Certainly, trophic relationships between predators and prey do not necessarily have to be directly related to biomass, especially in areas where annual blooms are acute. If a phytoplankton bloom is senescent, grazers, even if abundant, may not graze phytoplankton cells in poor health (end of the bloom situation). Similar inconsistencies between grazing impacts and grazers' biomass have also been reported for Antarctic waters (Caron *et al.*, 2000). Regarding the Arctic Ocean, we find contrasting results for microzooplankton grazing impact in different areas. For instance, during the summer along the western coast of Greenland, Levinsen *et al.* (Levinsen *et al.*, 1999) studied the microzooplankton grazing impact by indirect methods and concluded that if this group followed a pure autotrophic diet they could remove 362% of primary daily production. Nevertheless, the authors pointed out that cannibalism could likely reduce the actual impact of this group on phytoplankton. Also by indirect measurements, Rysgaard *et al.* (Rysgaard *et al.*, 1999) estimated that the combined grazing activity of ciliates and dinoflagellates of Young Sound (NE Greenland) would potentially remove only 14% of the annual primary production, a value that highly contrasts the previous estimate. Levinsen and Nielsen (Levinsen and Nielsen, 2002) found that potential microzooplankton grazing could account for 32-55% of the primary production in Disko Bay. These data, as well as the 40-114% primary production daily grazed in Jones Sound (Paranjape, 1987), the 37-88% in Baffin Bay (Paranjape, 1987), the 64-97% in the Barents Sea (Verity *et al.*, 2002), and the 2-293% (average 110 and 81% of phytoplankton growth rates for > 10 μm and < 10 μm phytoplankton, respectively) grazed in the southeast Bering Sea (Olson and Strom, 2002) are much higher than the values observed in our study. Recent research in the high Western Arctic Ocean (Sherr *et al.*, 2009), however,

advocates a lower control of microzooplankton grazing on primary producers (average 22±26%). The study by Sherr *et al.* (Sherr *et al.*, 2009), even if in a different area, lower average temperatures, and with a community of phytoplankton not dominated by *Phaeocystis*, but by diatoms, is the study that *a priori* seems more appropriate to contrast with ours; both were located in open waters near the ice-edge zone, and both were conducted at high latitudes using the same methodology. Our data agree with the results of the Sherr *et al.* (Sherr *et al.*, 2009) study, which does not show significant grazing in about half of the experiments at the fluorescence maximum and contains total average grazing rates of < 0.1 d^{-1}. Similarly, in the Sherr *et al.* (Sherr *et al.*, 2009) study they found low and even negative values for phytoplankton growth rates during summer. They attributed this natural mortality to low light levels (samples were collected at the base of euphotic zone) and to post-bloom conditions (protist grazing rates were low because the diatom blooms were senescent).

Despite the overall low grazing rates, some associations between the distributions of several organisms seem to be evident. For instance, mixotrophic dinoflagellates were positively correlated with diatoms. Mixotrophy is widespread among dinoflagellates, and it is not uncommon that these organisms significantly contribute to the community grazing on phytoplankton (Stoecker, 1999; Stoecker *et al.*, 2009), and particularly on diatoms (Yoo *et al*., 2009). Moreover, the role of mixotrophic dinoflagellates in the fate of primary producers is likely to have been underestimated for several reasons. First, the presence of their own chloroplasts may mask the detection of prey inside the organisms, and second, the different feeding mechanisms displayed by this group (direct engulfment, tube-feeding, and pallium-feeding; Hansen and Calado, 1999) make quite unlikely to correctly assess their contribution to total community grazing. Because *pallium*-feeding and tube-feeding do not leave evident remains of the preyed cell inside the predator and because the *pallium* and peduncles are not persistent structures, they are not easily quantified when microscopically observing preserved samples. Therefore, we are of the opinion that a predator-prey association between armoured mixotrophic dinoflagellates and diatoms is meaningful. Certainly, other microbial grazers, such as ciliates among others, can impact on diatoms (Aberle *et al.*, 2007), but we did not obtain proof of this behaviour in our study. On the other hand, the diatom-dinoflagellate relationship has

been widely suggested in the literature (e.g., Sherr and Sherr, 2007; Saito *et al.*, 2006; Calbet, 2008), although seldom quantified in natural communities (Archer *et al.*, 1996).

Finding explanations for the low microzooplankton grazing impact, but the high net mortality rates of phytoplankton:

It is difficult to ascertain whether the low grazing found in our study is a general characteristic describing the system or if it was the result of some particular conditions. It is surprising, however, we found many negative net phytoplankton growth rates (based on Chl *a* changes) not associated with microzooplankton grazing. The simplest explanation for that would be the incubation light-level was not the proper, the cells adjusting their Chl *a* contents to the new conditions. This could actually be the cause in some stations; however, the same pattern persists in cell counts in many others. Therefore, we have to find alternative hypotheses, which may be nonexclusive:

- *The natural mortality hypothesis*

Parallel to our study, Lasternas and Agustí (Lasternas and Agustí, in press) used a membrane permeability probe (Agustí and Sánchez, 2002) to estimate the natural mortality of the *P. pouchetii* community, and they observed that higher percentages of dead *P. pouchetii* cells (up to ca. 90% in some stations) were associated to both cold and less saline waters across the area studied. The mechanisms behind this mortality, not related to grazing rates, can be several. It could well be we were facing a senescent community at the end of the bloom. On the other hand, we cannot disregard viruses as playing a role in controlling the population of these and other protists and responsible for the frequent negative growth rates (Baudoux *et al.*, 2006; Jacobsen *et al.*, 2007). Unfortunately, our experimental set up was not adequate for virus-related mortality quantification.

Given the contribution of *P. pouchetii* to the total phytoplankton biomass, the natural mortality of these algae could be driving the growth rates observed on the Chl *a* basis. Corroborating this, we observed an inverse relationship between

phytoplankton instantaneous growth rates and Chl *a* distribution. Although other groups of plankton could also have presented natural mortality rates, we do not have solid evidence of so.

- *The Phaeocystis pouchetii hypothesis*

As previously mentioned, our cruise coincided with high abundances of *Phaeocystis pouchetii*, which commonly blooms in these waters in July (Schoemann *et al.*, 2005). The peculiarities of the food web dominated by these algae make it difficult to extract general conclusions. We believe that our low grazing estimates (not significant in most of the stations) could be partially a consequence of the presence of *P. pouchetii* . We observed the presence of many colonies in the samples, although we have not been able to quantify the % of free cells and colonies in the Lugol preserved samples. However, it is quite likely this species either introduced variability into the samples (colonies can be heterogeneously distributed in the samples) and precluded the establishment of significant regressions based on Chl *a*, evaded grazing when in colonial form (Hansen *et al.*, 1994; Weisse *et al.*, 1994; Tang, 2003), or chemically deterred grazers (Barnard *et al.*, 1984; Nejstgaard *et al.*, 2007; van Rijssel *et al.*, 2007). Certainly, *P. pouchetii* seems the most obvious candidate when seeking chemical deterrence of grazing. Similar to macroalgae and other phytoplankters, *Phaeocystis* spp. exude chemicals that can interfere with grazing activity (see review by van Rijssel *et al.*, 2007). Although the chemicals involved in this process have not yet been identified, it has been suggested that grazing-activated DMSP cleavage by *P. pouchetii* contributes to grazing deterrence (Wolfe *et al.*, 2000). Allelopathic interactions could also be responsible for the negative growth rates of a phytoplankton (and other protists) community, which apparently was not limited by nutrients.

Overall, we believe that the net transfer of energy to higher trophic levels in this ecosystem would be greatly diminished if the autotrophic community was dominated by *Phaeocystis*, provided that other groups not considered here (e.g., copepods) do not exert a strong impact on this algae. However, the low tolerance of *P. pouchetii* to relatively high temperatures (Schoemann *et al.*, 2005), as evidenced by the reduced presence at St. 27 where temperatures reached 7.5ºC, advocates for a limited relevance of this species under global warming scenarios. Certainly, this does not mean that other *Phaeocystis* species, such as *P. globosa*, which is adapted to warmer waters, cannot replace *P. pouchetii*, further diminishing

the trophic efficiency of the system because *P. globosa* is seldom consumed by zooplankton (see review by Nejstgaard *et al.*, 2007).

-*The dilution grazing artifact hypothesis*

Chl *a* is a rough proxy for phytoplankton because it does not capture the complexity of this group, and its use in dilution experiments has been questioned because chlorophyll content per cell may change during the incubation (McManus, 1995). Moreover, the need for a close examination of the microzooplankton community during dilution experiments to detect and correct possible artifacts has also been discussed (Dolan *et al.*, 2000; Agis *et al.*, 2007; Modigh and Franzè, 2009). To shed light on this point and to deepen our understanding of the food web interactions in Arctic waters, we further examined changes in the entire microbial community during the dilution experiments. We did not make any attempt to compare the rates obtained based on Chl *a*, with those derived from cell counts and their corresponding conversion to carbon because the uncertainties associated when depicting a trophic role (autotrophy *vs* heterotrophy) to unidentified dinoflagellates and nanoflagellates. Besides, the contribution of mixotrophic species in some stations (e.j. the mixotrophic ciliate *Laboea* sp. represented most of the planktonic biomass in station St. 3; Web Annex 1) precluded any comparison.

When opening the planktonic black box in the dilution experimental bottles we faced unanticipated results suggesting a complex and intricate food web, in which choosing the major microbial grazers of phytoplankton was not so straightforward. A clear example of a puzzling response was the occurrence of positive regression slopes between the net growth rates of certain groups against the dilution factor. Positive slopes for heterotrophs and mixotrophs, even if sometimes result of complicated ecological interactions, can be easily interpreted as growth enhancement due to increased feeding in the more concentrated treatments. However, positive slopes of phytoplankton occur when the organism considered is adversely affected by the dilution treatment. The explanation for these particular responses is not easy because they can have different non-excluding causes. For instance, they may either be the result of strong trophic cascade effects during the incubations (Calbet *et al.*, 2008), chemical grazing deterrence by the algae or other organisms, toxic effects of the filtered seawater (Landry, 1993), mixotrophs being important contributors of

phytoplankton biomass (then, favoured in less diluted conditions), or of complex cycling of nutrients between internal and external pools (Landry, 1993), because nutrients would be taken up by smaller algae more efficiently and would become limiting for larger phytoplankton. We do not believe that the last three hypotheses apply to our experiments for the following reasons: i) any toxic effect would likely be persistently evident in all the groups and stations, ii) some of the groups of phytoplankton showing positive slopes, as far as we know, were not mixotrophic (e.g., diatoms), and iii) nutrients were supplied in excess. Therefore, either (or both) trophic cascades or grazing deterrence seem to be the most reasonable explanation. If *P. pouchetii* was responsible for the positive slopes found for diatoms and other groups, its effects would not be apparent in the stations where the haptophyte was not present (stations 2 and 3). While this was the case and thus supported the feeding deterrence hypothesis, it did not fully demonstrate the hypothesis because the response was not directly related to the *P. pouchetii* concentration.

We, therefore, contemplated the trophic cascade explanation for the positive slopes found in our (and others) study. It has been argued that changes of grazer abundance during dilution grazing incubation may render artifactual results (Dolan *et al.*, 2000; Agis *et al.*, 2007; Modigh and Franzè, 2009). These changes usually involve a decrease of abundance in the most diluted treatments, result of starvation. However, our experiments showed in many occasions the opposite, the grazers diminishing in the most concentrated treatments. This can be consequence of predation from other microzooplankers, either protozoans (intraguild predation) or metazoans (e.g., copepod nauplii; not included in our sampling), during the incubations.

It is relatively easy to mathematically simulate a dilution grazing experiment involving a grazer that reduced their abundance inversely related to the dilution level along the incubation. We can actually base our example on data from one of our experiments. For instance, we can use as example the response of < 20 μm ciliates in Station 4, and the positive slope for nanoflagellates, one of their likely prey (Fig. 6). We assume nanoflagellates duplicated their abundance in 24h, and that < 20 μm ciliates were the only group grazing on them. If ciliates feeding rates were linearly related to food concentration we will most likely obtain, after the incubation period, a

negative slope for nanoflagellates when plotting net growth rates as function of dilution level, as predicted by dilutions (although it would not be a true estimate of the natural grazing rate on this group because grazers varied their concentrations in the experimental bottles). However, if feeding was saturated, we could easily mimic the results found in the experiments using a constant feeding rate of only 76 nanoflagellates consumed per ciliate per day (Fig. 7). This happens because the grazing pressure is in this case only proportional to the abundance of grazers, and we have a higher net growth rate of grazers (< 10 μm ciliates) in the more concentrated treatments. Moreover, varying the concentration of grazers, prey and the growth and grazing rates we can also obtain non significant from zero grazing estimates, which are not true rates, but artifacts of the method in very special situations. This mathematical exercise was not intended to correct our grazing rates, as suggested by Modigh and Franzè (Modigh and Franzè, 2009), because given the complexity of the food web we cannot anticipate the microzoplankton group responsible for most of the phytoplankton grazing impact. However, we can use our reasoning to prove positive slopes (and non-significant slopes) are easily result of a combination of trophic cascades during the incubations (the main grazers decreasing their abundance in the most concentrated treatments) with a saturated feeding responses. The picture complicates further if microzooplankton feeding-behaviour changes with food concentration, as described by Teixeira and Figueras (Teixeira and Figueras, 2009), and if there is nutrient limitation during the incubations. Actually, and regarding this later artifact, a severe nutrient limitation during the dilution experiments inversely proportional to the dilution level will most likely favour fake negative slopes, exaggerating the grazing activity of microzooplankton because phytoplankton instantaneous growth rates will be higher in the most diluted treatments, where more nutrients per cell are available.

In summary, the data presented here depict a planktonic Arctic community dominated by *P. pouchetii* and rich in microzooplankton, which at first sight did not seem to be exerting a strong control on a phytoplankton community in decline. However, several natural and artifactual causes may have been playing important roles in some of our experiments, precluding clear grazing estimates in this very complex food web. Even though, it may seem disappointing concluding that our rates might not be actual estimates, but bound for the lower grazing impact of the microzooplankton in the area, they advocate for the need of presenting

negative results, when these are not consequence of evident mistakes or artifacts (Dolan and McKeon, 2005). Only with a whole picture of the existing data we will be able to extract solid conclusions on the dynamics of marine systems. Maybe in the future someone will find the way of extracting information out of it. Specifically regarding dilution grazing experiments, this study calls for special caution when applying the technique, originally developed for oligotrophic areas, to rich environments were saturated feeding responses may be common. In any case, as previously suggested (Dolan *et al.*, 2000; Agis *et al.*, 2007; Modigh and Franzè, 2009), it is evident we need a detailed examination of the grazer and prey dynamics during the incubations if we want to present trustable microzooplankton grazing estimates. By presenting data on counts-based rates we will enhance our resolution and avoid artifacts associated to chlorophyll analysis. However, these sorts of data involve a considerable amount of time and are highly dependent on the taxonomic skills of the researcher.

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Acknowledgements

This research was supported by the Spanish grants POL2006-0550/CTM to C. Duarte, CTM2009-08783 to A.C., CTM2006-12344-C02-01 to M.A, CTM2007-60052 TO E.S., and grant 2005SGR-00219 from the Generalitat de Catalunya to the research group, and a PhD

1 fellowship, BES-2005-7491 to RA. The authors wished to thank the crew of the BIO
2 Hesperides and the special help of the technical staff of the UTM (D. Afonso, R. Ametller, J.
3 Llinàs J. Pozo, J. Sanz, and X. Vidal) for their invaluable logistic help. Plankton
4 composition was analyzed by Dr. M. Delgado. Three anonymous reviewers highly
5 contributed to improve an earlier version of the ms.

TABLE 1. Summary of the dates of sampling (July 2007), geographic position and area, depth of sampling (m) and *in situ* chlorophyll *a* concentration (µg l^{-1} ± SE) and temperature for the different stations sampled.

Station	Date	Latitude	Longitude	Area	Sampling depth	Total Chl *a*	> 5 µm Chl *a*	Temp. (ºC)
2	2	70º 43.19 N	17º 07.70 W	Greenland Sea	20	0.48 ± 0.02	0.15 ± 0.00	-1.2
3	3	72º 57.21 N	12º 39.19 W	Greenland Sea	25	1.31 ± 0.21	0.79 ± 0.00	0
4	4	74º 53.89 N	7º 24.50 W	Greenland Sea	32	1.41 ± 0.02	0.49 ± 0.01	0.8
5	5	77º 23.29 N	1º 40.57 W	Arctic Ocean	15	6.67 ± 0.42	2.90 ± 0.13	4
6	6	78º 00.44 N	2º 29.94 E	Arctic Ocean	23	1.75 ± 0.03	1.09 ± 0.03	4
9	7	78º 43.72 N	2º 58.51 E	Arctic Ocean	15	2.97 ± 0.07	1.48 ± 0.03	5
12	8	79º 30.83 N	7º 29.74 E	Arctic Ocean	20	5.21 ± 0.33	2.57 ± 0.07	5
15	9	80º 08.39 N	11º 19.54 E	Svalbard Coast	20	3.30 ± 0.13	1.81 ± 0.36	5
18	10	80º 26.90 N	15º 35.38 E	Svalbard Coast	35	2.67 ± 0.10	0.81 ± 0.08	5
20	12	80º 13.98 N	10º 10.97 E	Arctic Ocean	24	4.81 ± 0.20	2.31 ± 0.07	5
23	13	79º 22.16 N	6º 49.39 E	Arctic Ocean	17	8.97 ± 0.63	5.01 ± 0.08	5
27	15	79º 52.71 N	8º 36.44 E	Arctic Ocean	30	1.81 ± 0.09	1.38 ± 0.07	7.5
33	17	80º 23.46 N	12º 25.98 E	Arctic Ocean	25	1.97 ± 0.01	0.66 ± 0.00	5
39	19	80º 49.96 N	13º 12.82 E	Arctic Ocean	39	0.77 ± 0.02	0.28 ± 0.03	5
43	22	80º 25.29 N	7º 57.57 E	Arctic Ocean	20	8.97 ± 0.21	2.28 ± 0.12	-1
46	23	79º 59.15 N	3º 39.63 E	Arctic Ocean	16	4.94 ± 0.07	0.73 ± 0.06	-1.5

Table 2. In situ biomass in µgC L^{-1} of the different protist groups considered at the stations sampled. Numbers between parentheses are SE of 2 replicates. The quotient "total carbon / > 5 µm Chl *a*" are also shown.

Station	Diatoms	Nanofl	P. pouchetii	< 20 µm dinofl.	> 20 µm dinofl.	Mixo. dinofl.	Het. dinofl.	< 20 µm ciliat	> 20 µm ciliat	Total biomass	C / > 5 µm Chl a
2	1.25	0.36	0.00	0.51	0.24	0.24	0.28	4.48	34.91	42.26	14.03
	(0.07)	(0.06)	(0.00)	(0.08)	(0.03)	(0.02)	(0.07)	(0.46)	(4.88)		
3	1.55	3.12	0.00	0.84	0.22	0.12	0.08	4.74	50.53	61.18	4.75
	(0.21)	(0.05)	(0.00)	(0.02)	(0.03)	(0.01)	(0.00)	(0.33)	(4.00)		
4	1.26	0.60	0.83	0.77	0.57	0.72	0.43	12.2	20.90	38.31	7.73
	(0.15)	(0.01)	(0.12)	(0.12)	(0.01)	(0.08)	(0.08)	(0.00)	(2.31)		
5	1.77	0.51	2.81	14.1	0.26	1.76	0.47	0.66	11.49	33.83	4.75
	(0.47)	(0.12)	(0.32)	(3.40)	(0.06)	(0.28)	(0.13)	(0.19)	(3.03)		
6	1.53	3.78	12.2	10.3	0.87	1.20	0.69	3.70	6.08	40.42	20.64
	(0.05)	(0.81)	(2.30)	(4.38)	(0.23)	(0.27)	(0.13)	(0.11)	(1.66)		
9	1.79	1.02	1.59	1.93	0.34	0.72	0.15	0.40	0.83	8.77	3.88
	(0.11)	(0.09)	(0.10)	(0.32)	(0.03)	(0.06)	(0.02)	(0.11)	(0.06)		
12	0.81	1.81	5.90	1.49	0.75	0.11	0.17	1.91	0.94	13.88	3.44
	(0.03)	(0.05)	(0.82)	(0.04)	(0.11)	(0.04)	(0.01)	(0.15)	(0.27)		
15	2.97	3.82	13.0	5.56	3.09	0.99	1.53	2.45	15.09	48.50	12.80
	(0.48)	(0.12)	(1.27)	(0.14)	(0.74)	(0.05)	(0.24)	(0.33)	(6.67)		
18	0.18	4.01	1.19	6.14	5.24	0.27	3.33	2.12	59.9	82.38	11.46
	(0.02)	(1.87)	(0.71)	(0.72)	(0.28)	(0.05)	(0.67)	(0.15)	(7.61)		
20	4.24	3.43	14.8	3.99	3.89	0.98	0.57	6.59	4.52	43.02	10.39
	(0.36)	(0.22)	(2.40)	(0.07)	(0.73)	(0.08)	(0.17)	(0.42)	(0.50)		
23	67.8	3.10	24.8	5.00	1.79	3.65	0.65	5.73	7.12	119.58	20.18
	(5.19)	(0.39)	(2.63)	(0.28)	(0.53)	(1.20)	(0.11)	(0.21)	(0.81)		
27	25.1	1.36	0.09	5.49	1.91	2.55	0.51	0.81	1.74	39.59	23.22
	(1.21)	(0.10)	(0.01)	(0.34)	(0.10)	(0.23)	(0.04)	(0.24)	(0.38)		
33	6.47	2.33	7.89	3.36	2.70	1.05	0.70	4.56	5.60	34.67	29.68
	(0.38)	(0.28)	(4.22)	(0.47)	(0.53)	(0.17)	(0.05)	(0.13)	(0.63)		
39	6.73	1.61	1.79	2.10	2.50	0.25	0.34	1.45	1.02	17.78	42.16

	(1.05)	(0.15)	(0.04)	(0.08)	(0.39)	(0.03)	(0.04)	(0.25)	(0.20)		
43	6.09	1.15	49.0	1.96	9.73	1.37	1.08	2.77	4.77	77.93	27.63
	(0.78)	(0.20)	(7.40)	(0.20)	(2.02)	(0.06)	(0.16)	(0.27)	(0.73)		
46	3.67	2.52	25.7	1.65	2.33	1.39	1.02	5.94	3.43	47.61	46.53
	(0.18)	(0.06)	(9.41)	(0.39)	(0.34)	(0.21)	(0.02)	(0.57)	(0.12)		

Table 3. Dilution grazing experiments. Phytoplankton instantaneous growth rates without nutrients (μ±SE; d^{-1}) and mortality rates (m±SE; d^{-1}) for total and the 2 fractions of chlorophyll *a* at the different stations. %SS correspond to the calculated impact on the phytoplankton standing stock. Determination coefficient for the regression analysis is also provided. SE error for μ was obtained using the equation $(SE_k^2+SE_m^2)^{1/2}$ were SE_k is the SE of the average of the 100% unamended bottles and SE_m is the standard error associated to the slope of the regression equation (when significant).

Station	μ (TOTAL)	m (TOTAL)	r^2 (TOTAL)	μ (< 5 μm)	m (< 5 μm)	r^2 (< 5 μm)	μ (> 5 μm)	m (> 5 μm)	%SS
2	0.12±0.029	ns	–	0.10±0.035	ns	–	0.17±0.14	ns	0.0
3	0.07±0.11	ns	–	-0.05±0.019	ns	–	0.13±0.18	ns	0.0
4	-0.23±0.11	-0.31±0.11	0.50	-0.39±0.25	-0.44±0.19	0.45	-0.22±0.13	ns	23.9
5	-0.11±0.077	-0.13±0.039	0.59	0.00±0.21	-0.34±0.056	0.84	-0.15±0.069	ns	11.6
6	-0.05±0.010	ns	–	0.29±0.11	-0.30±0.086*	0.67	-0.03±0.050	ns	0.0
9	-0.11±0.046	ns	–	-0.33±0.026	ns	–	0.07±0.090	ns	0.0
12	0.15±0.113	-0.31±0.11*	0.73	-0.25±0.026	ns	–	-0.08±0.022	ns	28.6
15	0.09±0.030	ns	–	-0.16±0.24	ns	–	0.23±0.079	ns	0.0
18	-0.06±0.059	ns	–	-0.11±0.00	ns	–	0.19±0.036	ns	0.0
20	-0.24±0.015	ns	–	-0.29±0.031	ns	–	-0.19±0.060	ns	0.0
23	-0.24±0.00	ns	–	-0.48±0.15	ns	–	-0.09±0.07	ns	0.0
27	-0.04±0.043	ns	–	-0.04±0.16	ns	–	-0.04±0.007	ns	0.0
33	0.14±0.081	-0.24±0.073	0.57	0.00±0.11	-0.30±0.11	0.48	-0.06±0.041	ns	22.9
39	-0.04±0.022	ns	–	-0.05±0.002	ns	–	-0.03±0.061	ns	0.0
43	0.01±0.152	-0.35±0.15	0.41	-0.05±0.19	-0.46±0.19**	0.45	0.17±0.069	ns	29.7
46	0.01±0.030	-0.16±0.027	0.83	-0.02±0.43	-0.25±0.058	0.70	0.34±0.70	ns	14.9

1 ns: not significant ($p > 0.05$); * Grazing saturation: 3-point method used (Gallegos 1989); ** One outlier removed

Table 4. Dilution grazing experiments. Instantaneous growth rates without nutrients (μ; d^{-1}) and mortality rates (m; d^{-1}) for the different planktonic groups considered. Determination coefficient for the regression analysis is also provided. Positive *m* values indicate positive slope.

Plank group		St 2	St 3	St 4	St 5	St 6	St 9	St 12	St 15	St 18	St 20	St 23	St 27	St 33	St 39	St 43	St 46
Diatoms	μ	-0.10	0.83	-0.21	-0.41	0.02	0.82	1.49	0.24	-0.38	0.21	-0.03	-0.36	-0.20	-0.08	0.05	0.17
	m	ns	ns	ns	ns	ns	1.16	ns	1.08	ns	ns	ns	ns	0.68	ns	ns	0.72
	r^2						0.91		0.86					0.92	0.66		0.93
Nanoflagellates	μ	-0.08	0.69	-0.22	0.58	0.08	0.99	0.87	0.62	-0.06	-0.42	-0.06	-0.62	0.62	0.12	-0.18	-0.61
	m	ns	ns	1.19	ns	-0.33	ns	ns	1.59	ns	ns	ns	ns	1.38	0.95	0.66	1.05
	r^2			0.91		0.96			0.96					0.94	0.81	0.77	0.79
P. pouchetii	μ	nd	nd	-0.47	0.95	-3.97	1.20	0.87	-0.02	0.44	-0.37	0.04	0.11	-0.62	-0.21	-0.25	0.19
	m			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	1.19
	r^2																0.93
< 20 μm dinofl.	μ	-0.29	-0.17	-0.48	-1.39	-0.77	0.83	0.32	-0.49	-0.19	-0.52	0.01	-0.78	-0.23	-0.55	-0.35	-0.29
	m	ns	ns	1.14		0.94	0.48	1.28	ns	ns	ns	ns	ns	ns	ns	0.93	ns
	r^2			0.72		0.86	0.79	0.96								0.77	
> 20 μm dinofl.	μ	0.96	1.53	-0.79	-0.37	-0.30	0.31	1.19	-0.48	0.02	0.26	0.52	0.32	-0.23	-0.02	-0.38	-0.50
	m	-0.53	-0.71	ns	ns	ns	ns	ns	ns	ns	ns	2.08	ns	ns	ns	ns	ns
	r^2	0.78	0.88									0.88					
Mixo. dinofl.	μ	0.26	0.10	0.32	-0.14	-0.61	-0.04	0.63	-0.17	0.06	1.30	0.12	-0.75	0.21	0.24	-0.03	0.03
	m	ns	ns	-0.65	ns	ns	ns	ns	ns	ns	-0.97	ns	ns	ns	ns	0.47	ns
	r^2			0.72							0.90					0.87	
Het. dinofl.	μ	1.17	0.28	0.29	-1.04	-0.22	1.88	1.02	-0.34	-0.01	0.19	0.47	0.05	0.12	0.23	0.54	-0.06
	m	-0.58	ns	-1.39	ns	ns	ns	ns	ns	ns	ns	1.00	ns	-0.60	ns	ns	ns
	r^2	0.79		0.90								0.76		0.80			

< 20 µm ciliates	µ	-0.12	0.25	0.70	1.21	-0.61	1.03	0.95	-0.54	0.15	-0.30	0.50	0.10	-0.16	0.13	0.12	-0.16
	m	ns	ns	-0.79	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	r^2			0.69													
> 20 µm ciliates	µ	-0.08	-0.17	0.03	-0.71	0.26	-1.05	0.17	-1.71	-0.45	-0.44	-0.73	-1.26	-0.14	0.06	-0.20	-1.07
	m	ns	ns	-0.70	ns	ns	ns	ns	0.55	ns	ns	ns	ns	ns	ns	ns	ns
	r^2			0.74					0.84								

ns: not significant regression equation ($p > 0.05$); nd: not determined because not enough cells;

Figure legends

Figure 1. Map of the surveyed area indicating the sampling stations.

Figure 2. *Phaeocystis pouchetii* biomass as related to latitude.

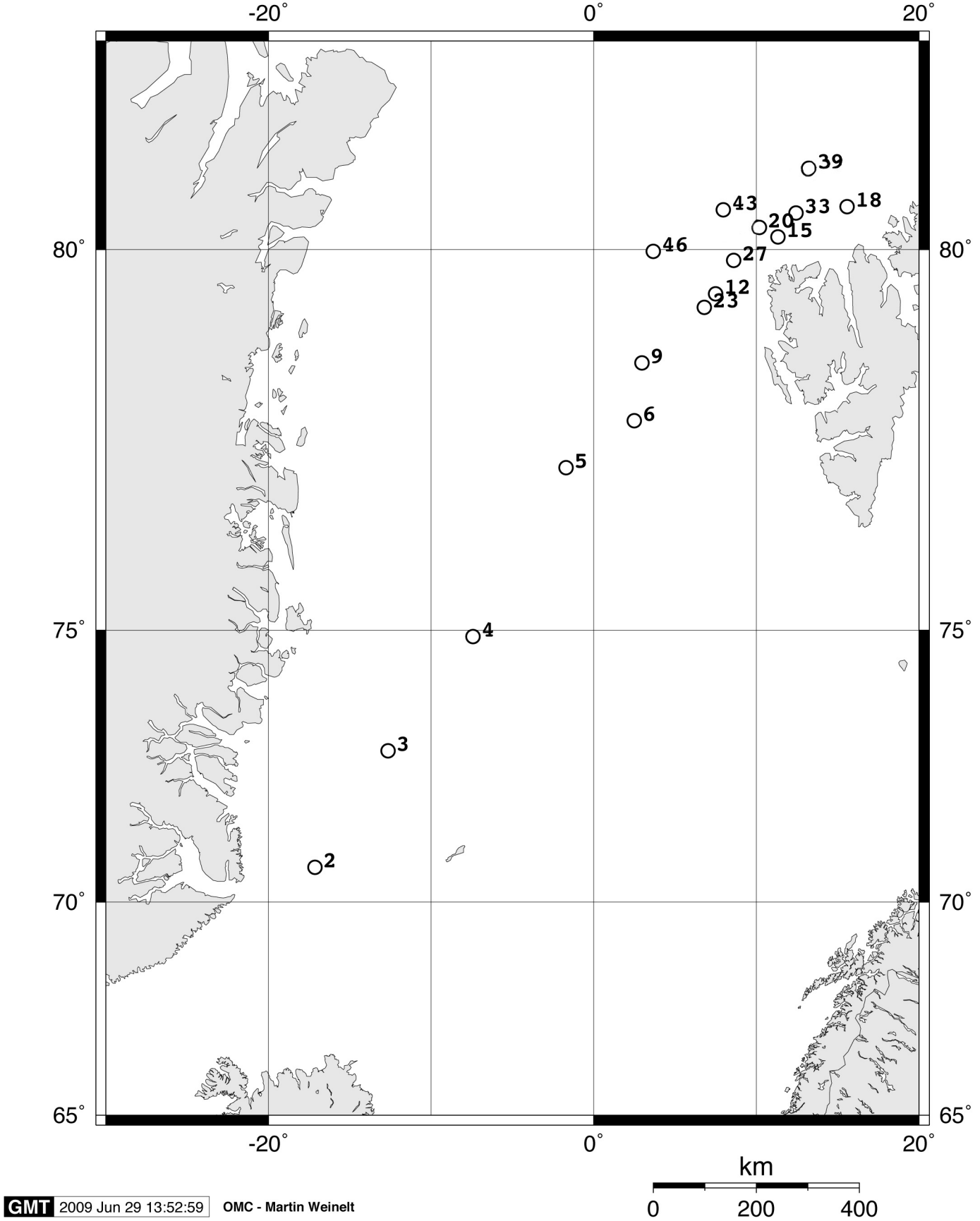
Figure 3. The quotient heterotrophic carbon / autotrophic carbon as a functio latitude. Greenland Sea and Coastal stations are indicated, the rest of stations correspond to Arctic Ocean open waters.

Figure 4. Relationship between instantaneous phytoplankton growth rates in unamended (without added nutrients) bottles (d^{-1}) and chlorophyll *a* concentr Chl *a* l^{-1}).

Figure 5. Comparison of instantaneous phytoplankton growth rates (from tot analysis) in bottles amended (with added nutrients) and unamended. The discontinuous line represents the 1:1.

Figure 6. Example of grazing dilution plots for the microbial components of planktonic community of stations 4 (left) and 33 (right).

Figure 7. Simulated outcome of a dilution grazing experiment using the abun and growth rates of < 20 μm ciliates in St. 4 as grazers and the abundance of nanoflagellates as prey. See text for further details.


-20°
0°
20°
80°
75°
70°
65°
39
43
33
18
20
15
46
27
12
23
9
6
5
4
3
2
km
0
200
400
GMT 2009 Jun 29 13:52:59
OMC - Martin Weinelt

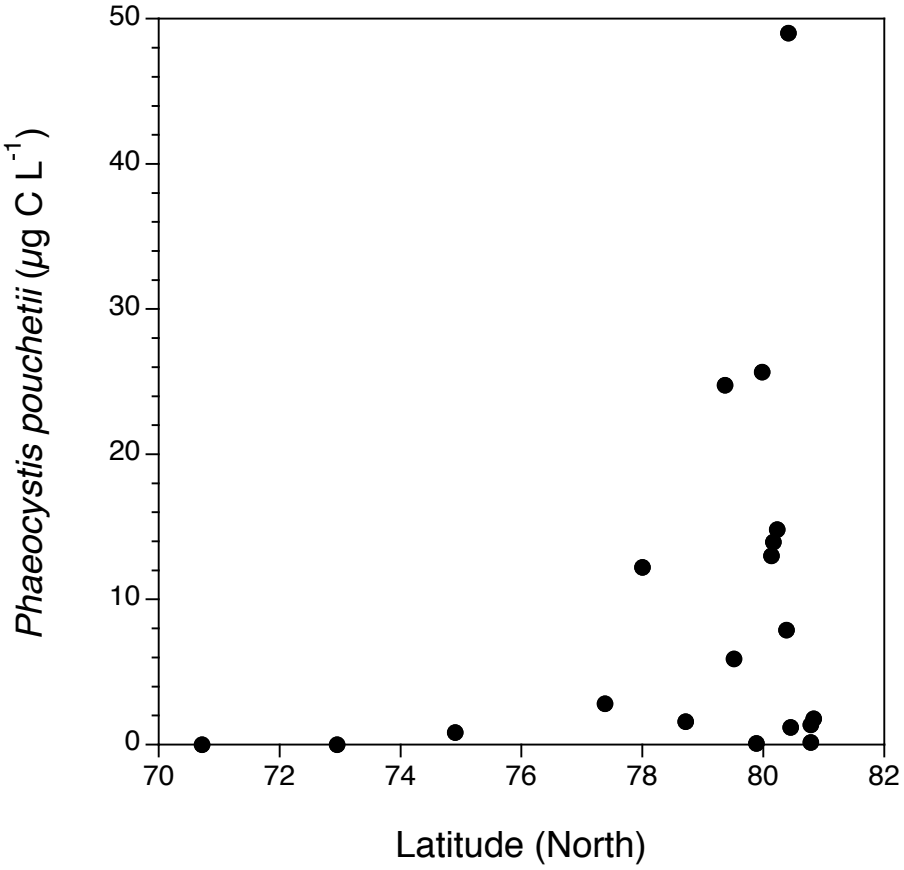

50
40
30
20
10
0
70
72
74
76
78
80
82
Phaeocystis pouchetii (µg C L-1)
Latitude (North)

Fig. 2

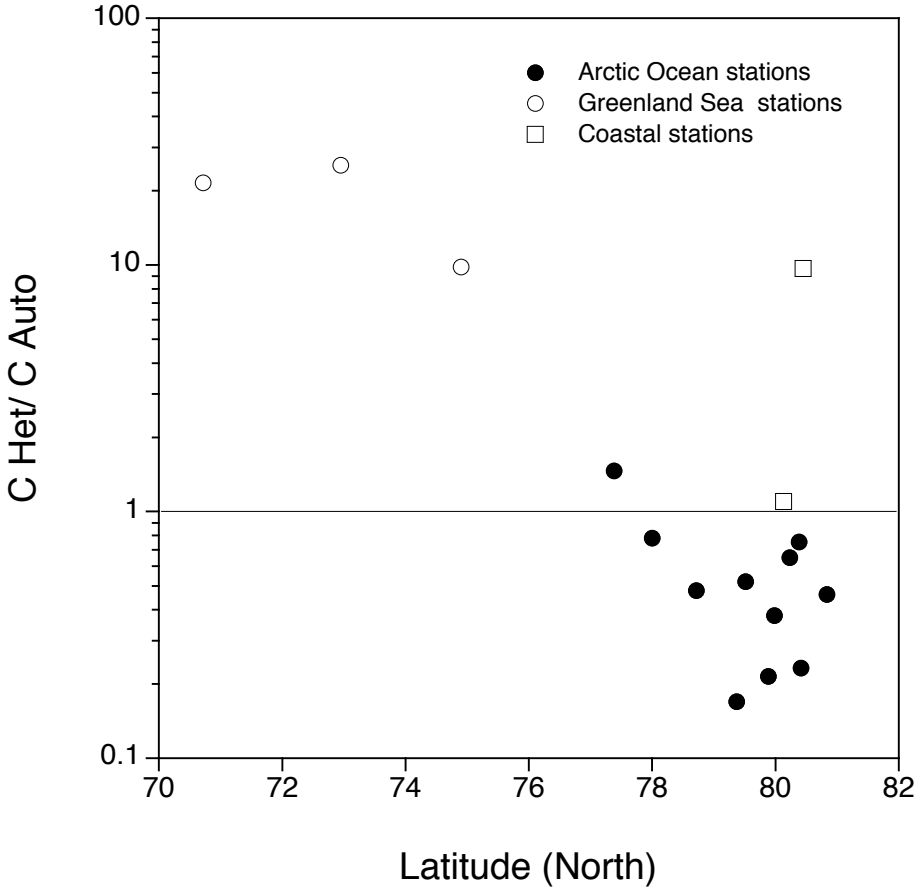

100
10
1
0.1
C Het/ C Auto
70
72
74
76
78
80
82
Latitude (North)
Arctic Ocean stations
Greenland Sea stations
Coastal stations

Fig. 3

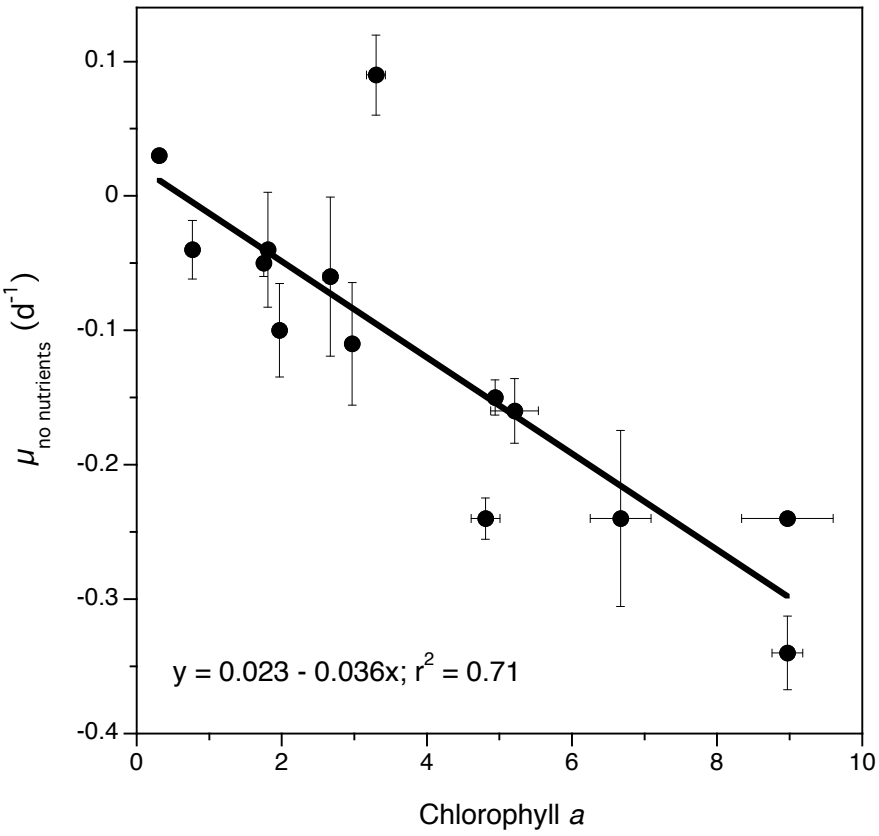

0.1
0
-0.1
-0.2
-0.3
-0.4
0
2
4
6
8
10
$\mu_{no\ nutrients}$ (d^{-1})
Chlorophyll *a*
y = 0.023 - 0.036x; r^2 = 0.71

Fig. 4

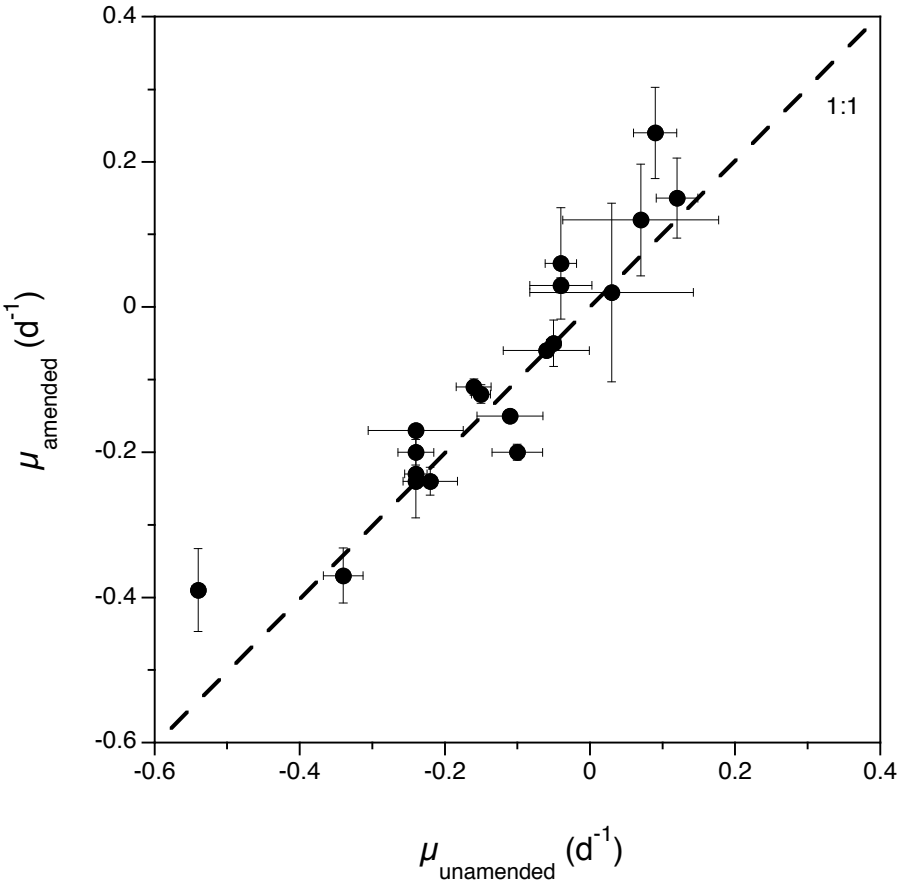


Fig. 5

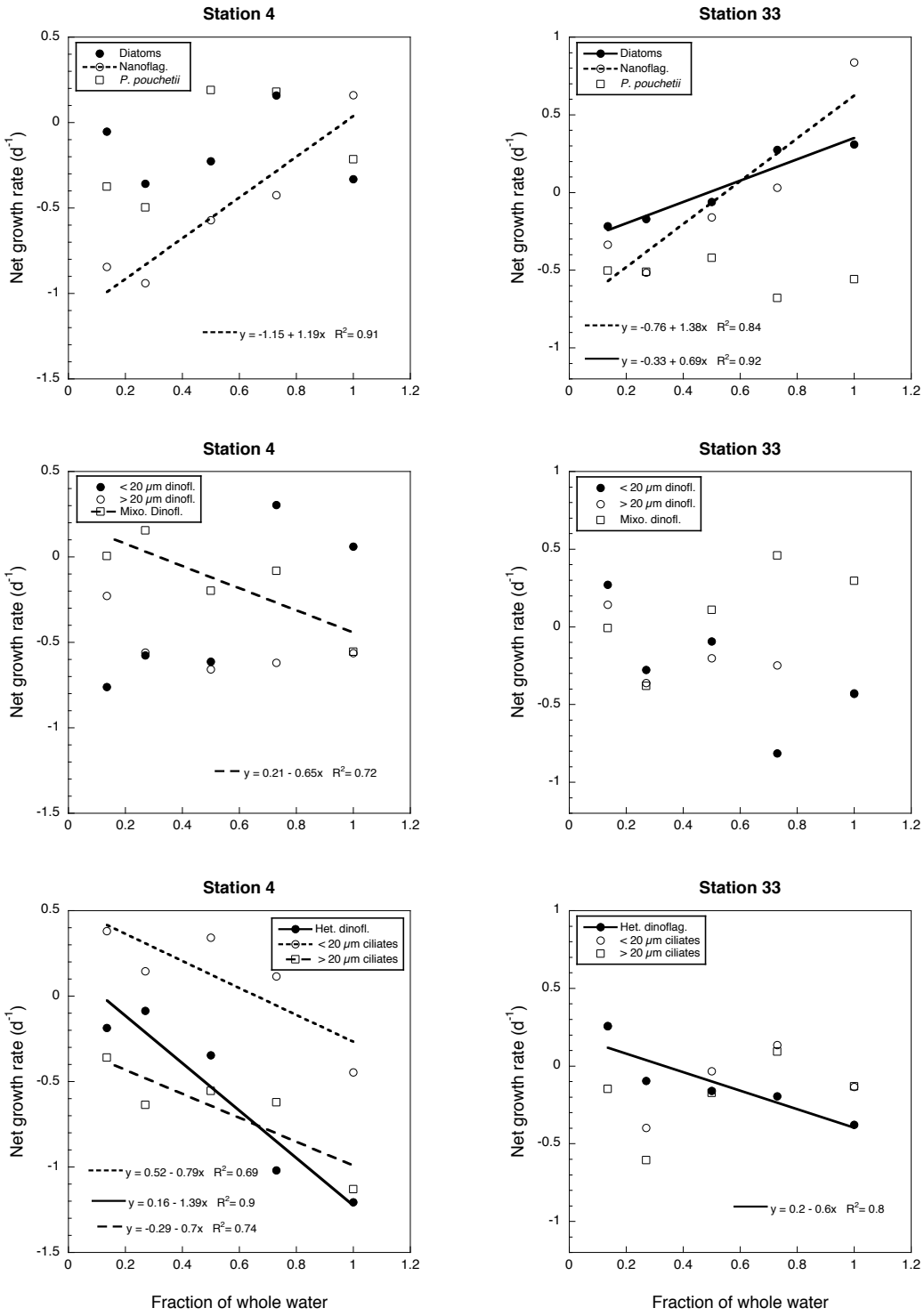

Station 4
Diatoms
Nanoflag.
P. pouchetii
y = -1.15 + 1.19x R²= 0.91
Station 33
Diatoms
Nanoflag.
P. pouchetii
y = -0.76 + 1.38x R²= 0.84
y = -0.33 + 0.69x R²= 0.92
Station 4
< 20 µm dinofl.
> 20 µm dinofl.
Mixo. Dinofl.
y = 0.21 - 0.65x R²= 0.72
Station 33
< 20 µm dinofl.
> 20 µm dinofl.
Mixo. dinofl.
Station 4
Het. dinofl.
< 20 µm ciliates
> 20 µm ciliates
y = 0.52 - 0.79x R²= 0.69
y = 0.16 - 1.39x R²= 0.9
y = -0.29 - 0.7x R²= 0.74
Station 33
Het. dinoflag.
< 20 µm ciliates
> 20 µm ciliates
y = 0.2 - 0.6x R²= 0.8
Net growth rate (d⁻¹)
Fraction of whole water

Fig. 6

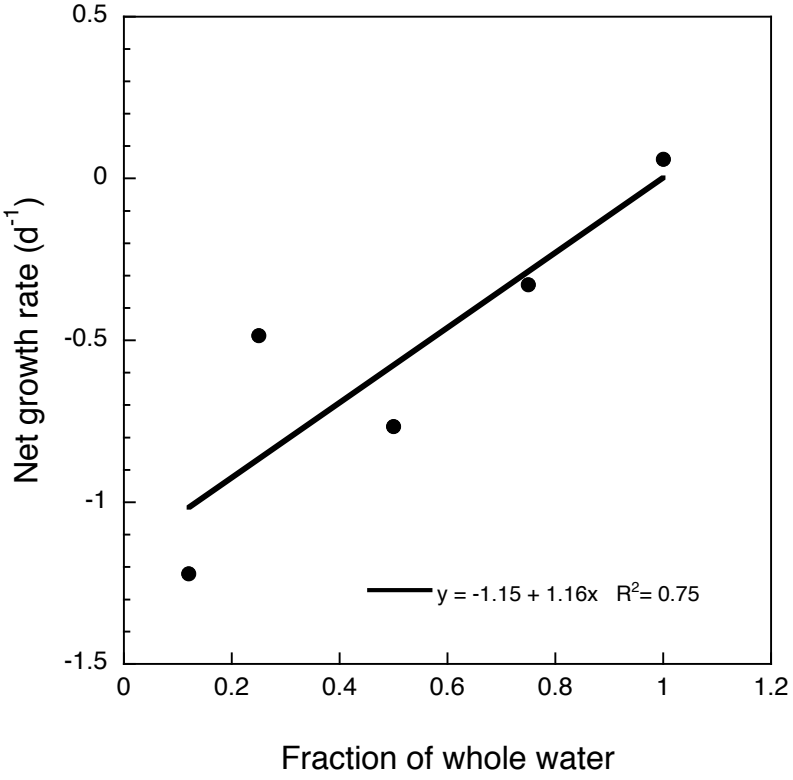

0.5
0
-0.5
-1
-1.5
0
0.2
0.4
0.6
0.8
1
1.2
Net growth rate (d-1)
Fraction of whole water
y = -1.15 + 1.16x R2= 0.75

Fig. 7