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Microzooplanktonic grazing in the western Wadden Sea during the early spring bloom of 2013

Abstract

Grazing by microzooplankton is an important factor during spring bloom formation both for phytoplankton and for higher trophic levels. We try to quantify the strength of microzooplanktonic grazing with the dilution method, both with the traditional measure of chlorophyll α and flowcytometry. While in open ocean systems chlorophyll can be used for estimating grazing pressure, in coastal sytems (where diatoms dominate spring blooms which are less grazed by microzooplankton) discrepancies may be found. We found strong grazing on small phytoplankton but non on larger species using flowcytometry. With the chlorophyll analyses little grazing pressure was found. It can be concluded that using cholorphyll α when measuring microzooplanktonic grazing might lead to an underestimation of actual grazing.

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Introduction

Early spring phytoplankton blooms are an important key in the highly productive coastal seas such as the Wadden Sea. During winter, adequate nutrients but light limitation prevents phytoplankton growth (Beusekom & Jonge, 2002). The start of spring sparks a rapid development of phytoplankton-zooplankton succession that drives the food web in these coastal regions. Not many studies investigated the strength of microzooplanktonic grazing in this area. Furthermore, the more recent studies found no indication of grazing on phytoplankton at the beginning of spring bloom development (Loebl, van Beusekom, & Philippart, 2012).

An earlier study performed in the Marsdiep found that microzooplankton grazing is responsible for the development of blooms of larger phytoplankton species (Riegman, Kuipers, Noordeloos, & Witte, 1993) Microzooplankton species like ciliates are known to exhibit high growth rates and can therefore rapidly follow phytoplankton production. Due to the large grazing impact of microzooplankton on the smaller phytoplankton species and the subsequent rerelease of nutrients, the larger species of diatoms can develop rapidly. Furthermore, these diatoms are less likely to be consumed by microzooplankton. This was partly confirmed when no grazing was observed during a phytoplankton bloom dominated by an invasive species of diatom.

The dilution technique used in most studies for determining microzooplanktonic grazing was primarily designed for use in open ocean systems, where phytoplankton is relatively small and can therefore easily consumed by microzooplankton (Landry & Hassett, 1982). Calculating plankton biomass was typically done by chlorophyll α measurement (eg. Loebl et al, 2012; Loebl & Van Beusekom, 2008). But in the Western Wadden Sea a considerable proportion of plankton biomass comprises of the large diatoms that are not easily consumed by microzooplankton. This has the tendency to obscure the signal when measuring chlorophyll α. Even when limited grazing on small phytoplankton is present, it is difficult to observe through the bulk of diatoms.

In this study we intended to estimate microzooplanktonic grazing on different sizes of phytoplankton during spring bloom development. Instead of primarily using chlorophyll α as a measure for phytoplankton production, flow cytometry analysis was used to strengthen the assay. Furthermore, the phytoplankton production is compared to the natural abundance of ciliates. We hypothesized that most grazing will be observed on the smaller species of phytoplankton and that the highest microzooplankton abundance will be found when the smaller phytoplankton species are at their peak biomass.

Materials and Method

Experimental setup

Microzooplanktonic grazing and phytoplankton net growth rates were determined in the Western Wadden Sea between 26 February and 13 May 2013 (see table 1-3 for exact dates). A total of 11 experiments were performed using the dilution technique for estimating microzooplanktonic grazing (Landry & Hassett, 1982). Sampling was performed at high tide on the NIOZ jetty (Figure 1) located in the Marsdiep inlet. Surface water was siphoned in 3 separate 10 liter plastic bottle of which one was gravity filtered through 142 mm Whatmann GF/F filters. Unfiltered seawater was diluted with filtered seawater in 0.5 L PET bottles to obtain dilution levels of approximately 25%, 50%, 75% and 100%. After 5 experiments, dilution levels of 5% and 15% were added to facilitate accurate measurements of net growth and grazing pressure in the case of non-linear responses (Teixeira & Figueiras, 2009). All air was removed from the PET bottles and the bottles were sealed with parafilm. The bottles were incubated for 24h on a rotating wheel at simulated conditions approximating *in situ* day length and seawater temperature. Light intensity during the experiment was ~350 µmol s^{-1} m^{-2} at the closest to the lightsource and the rotational speed was 1 rpm. Each dilution was incubated in triplicate. Samples for T_0 analyses (including ciliate analyses) were initially siphoned in the same 0.5L PET bottles as used in incubation to correct for handling stress. Initial concentrations of Chlorophyll α and cells ml^{-1} (through flow cytometry) in the diluted bottles were calculated from undiluted seawater sampled at T_0.

Figure 1: The western Wadden Sea and the sampling site at the NIOZ jetty

Analyses

Samples for flow cytometric analyses (3.5 ml.) were fixed with 1% glutaraldehyde (final concentration). The samples were flash frozen in liquid nitrogen and stored at -80 °C until analysis. Samples were analyzed with a BD Accuri C6. The standard volume of measurements was 200 µl. This volume was raised to 500 µl or 700 µl for samples acquired from the high dilution bottles. Data analyses were performed using the provided Cytowin software.

The remaining volume of the incubation bottles (approx. 500 ml) was vacuum filtered over 20 mm Whatmann GF/F filters for determination of Chlorophyll α. Filters were flash frozen in liquid nitrogen and stored at -80 °C until further processing. Filters were freeze dried for 24 h. Chlorophyll α was extracted in 90% acetone at 4 °C in 16-18 hours. Chl *a* concentration was determined using a spectrophotometer (Hitachi U-3010).

For the enumeration of the ciliate abundance, 0.5 L of seawater was gently poured into a jar with acid Lugol's iodine (end concentration 2%) and sedimented at 4 °C in a disturbance and light free environment for a minimum of 5 days. The samples were then concentrated by siphoning off the upper part of the sample. The remaining sample was stored at 4 °C. Microscopic enumerations were performed with Zeiss Axiovert 200 inverted microscope at 200x or 400x magnification. The Lugol's iodine in a subsample was neutralized with sodium thiosulfate and stained with Bengal rose to aid identification. The ciliates were grouped according to Petz & Foissner (1992) and counts were extrapolated to the natural abundance. Due to time constraints the enumeration of ciliate abundance was started from 12 march.

Calculating net growth and grazing pressure

The phytoplankton growth rate and microzooplankonic grazing pressure were calculated according to Landry & Hassett (1982). The phytoplankton growth was defined as

$$C_t = C_0 e^{\mu t} \quad ,$$

where C_t is phytoplankton abundance/biomass at time *t*, C_0 is phytoplankton abundance/biomass at time *0*, and µ the apparent phytoplankton growth rate (d^{-1}). From this it can be inferred that

$$\mu = \frac{1}{t} \ln \frac{C_t}{C_0} \quad .$$

Furthermore, it was assumed that

$$\mu = i - g \quad ,$$

where *i* is the net phytoplankton growth rate and *g* is the microzooplanktonic grazing rate. Since phytoplankton growth is exponential and microzooplanktonic grazing is dependent on the microzooplanktonic abundance, both the net phytoplanktonic growth rate and the

microzooplanktonic grazing rate could be calculated by linear regression using the various dilution fractions. All calculations were performed using R (R Development Core team, 2008).

Results

Flow cytometry

A large increase in the *in situ* abundance of the small sized phytoplankton cluster was observed from the beginning of April (Figure 2 A,B).The abundance peaked at 16th of April and started to decline from the 25th of April to almost absent at the end of the time series. Significant grazing was observed all through the increase and decline of the small phytoplankton cluster. Grazing rates varied between 0.25 (d^{-1}) up to 0.47 (d^{-1}) (Table 1).

Table 1: Grazing (g) and net growth rates (i) for small phytoplankton per week based on flowcytometry

Date	Natural Abundance	*g*	*i*	r^2	*p-value*
26-feb	2691.67	0.123	0.217	0.881	0.061
5-mrt	3070.00	0.016	0.320	0.038	0.804
12-mrt	3540.00	0.279	0.474	0.678	0.177
19-mrt	3608.33	0.471	0.487	0.859	0.073
28-mrt	4315.00	0.113	0.300	0.194	0.559
3-apr	5398.33	0.291	0.291	0.928	0.002*
9-apr	8028.33	0.18	0.335	0.781	0.019*
16-apr	8220.00	0.358	0.518	0.829	0.012*
25-apr	6236.67	0.258	0.529	0.898	0.004*
2-mei	2665.00	0.234	0.181	0.875	0.006*
13-mei	821.67	0.366	-0.005	0.821	0.013*

The in situ abundance of the large sized phytoplankton cluster peaked at 28th of March and slowly declined until 9th of April (Figure 2 C, D). A smaller second peak was observed on 2nd of May. During these periods no significant grazing was observed. However, strong grazing occurred on 19th of March with grazing rates exceeding 1.0 (d^{-1}) (g=1.016, p=0.040) (Table 2).

Table 2: Grazing (g) and net growth rates (i) for large phytoplankton per week based on flowcytometry

Date	Natural Abundance	*g*	*i*	r^2	*p-value*
26-feb	3183.33	0.411	0.009	0.890	0.057
5-mrt	1078.33	-0.295	0.273	0.084	0.711
12-mrt	4596.67	0.624	0.580	0.577	0.240
19-mrt	5251.67	1,016	0.744	0.921	0.040*
28-mrt	18243.33	0.121	0.578	0.043	0.794
3-apr	12945.00	0.173	0.029	0.610	0.067
9-apr	7583.33	0.506	0.300	0.381	0.192
16-apr	8765.00	0.578	0.227	0.539	0.097
25-apr	3503.33	0.095	0.708	0.039	0.708
2-mei	11166.67	-0.018	0.542	0.001	0.955

13-mei	6180.00	-0.129	0.170	0.047	0.681

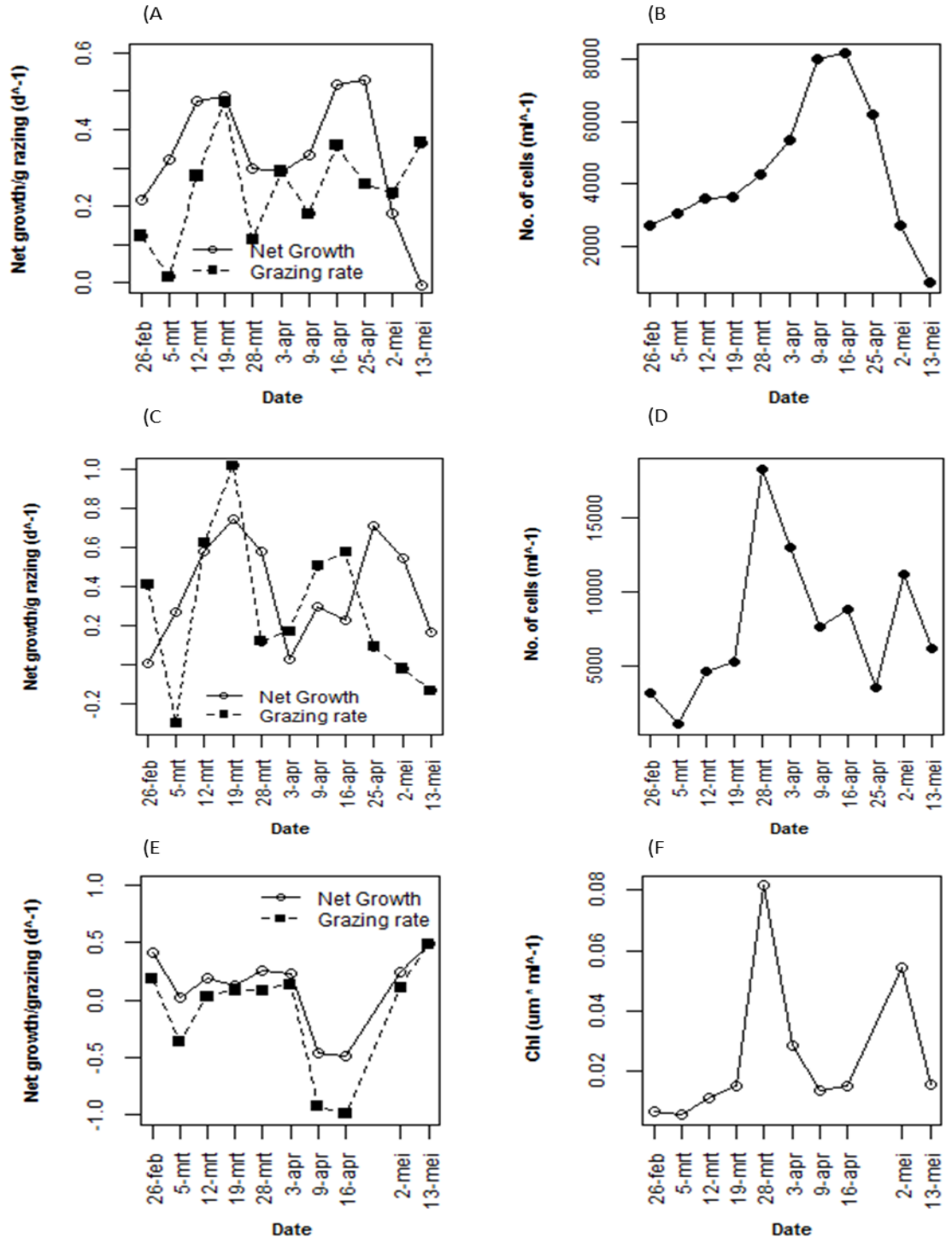

Figure 2: Net growth rate and Grazing rate (A, C, E) and natural abundance or biomass (B,D,E) per week of small phytoplankton (A,B), large phytoplankton (C,D) and Chlorophyll based measurements (E,F).

Chlorophyll α

In situ Chlorophyll α peaks coincide with the peaks in large size phytoplankton at 28th of March and 2nd of May (81.75 µg L^{-1} and 54.51 µg L^{-1} respectively) (Figure.2). Chlorophyll α data and grazing rates for 25th of April are not included due to a mechanical error in the spectrometer rendering the results unreliable. Significant grazing was observed from 3rd of April onwards, with the exception of the 16th of April. Growth rates ranged from 0.108 d^{-1} ($p<0.001$) to 0.486 d^{-1} ($p<0.001$) and was highest on the 13th of May (Table 3). The observed grazing rate for the 9th and 16th of April were negative as the relation between dilution fraction and apparent growth was positive.

Table 3: Grazing (*g*) and net growth rates (*i*) for phytoplankton per week based on chlorophyll α measurements (ug/ml)

Date	Natural Concentration	*g*	*i*	r^2	*p-value*
26-feb	6.66E-03	0.183	0.414	0.7	0.163
5-mrt	5.48E-03	-0.365	0.018	0.534	0.269
12-mrt	1.13E-02	0.031	0.189	0.205	0.547
19-mrt	1.49E-02	0.087	0.126	0.249	0.501
28-mrt	8.18E-02	0.082	0.253	0.246	0.504
3-apr	2.87E-02	0.135	0.224	0.726	0.031*
9-apr	1.39E-02	-0.926	-0.466	0.803	0.039*
16-apr	1.51E-02	-0.99	-0.484	0.707	0.074
25-apr	-	-	-	-	-
2-mei	5.45E-02	0.108	0.247	0.943	0.001*
13-mei	1.54E-02	0.486	0.49	0.939	0.001*

Ciliate abundance

Most abundant groups of Ciliates were the Naked Choreotricha (13607 ± 886 cells L^{-1} on 13th May) and to a lesser extent the Oligotrichia (8136 ± 1382 cells L^{-1} on 9th of April). Other groups were generally present in lower numbers. A large increase in the Olichotrichia abundance was observed on 3rd April and a slight decrease from 16th April onwards to 2224 ± 586 cells L^{-1} at the end of the time series. The abundance of Naked Choreotricha increased gradually over the time series until a large increase was observed on 2nd May and peaked and nearly doubled in abundance again on the 13th May.

Table 4: Ciliate abundance (cells L^{-1}) per week for different groups. (Hapt.= haptoria, Suct=suctoria, Holo=holotrichia, Peri=peritrichia,, Oligo=oligotrichia, Naked= Naked choreotricha, Tintin= tintinids, Hypo=hypotrichia, Other= unidentified ciliates)

Date	Hapt	Suct	Holo	Peri	Oligo	Naked	Tintin	Hypo	Other
26-feb	-	-	-	-	-	-	-	-	-
5-mrt	-	-	-	-	-	-	-	-	-
12-mrt	25	0	540	23	688	1006	631	0	244
19-mrt	0	0	215	26	1736	635	116	50	235
28-mrt	133	0	444	0	3226	1224	0	791	0
3-apr	140	0	514	0	7731	1502	72	281	0
9-apr	153	0	290	0	8137	1914	36	40	0
16-apr	46	46	138	0	7497	2654	143	97	0
25-apr	134	108	1832	27	4802	3211	464	27	0
2-mei	342	1651	263	0	3785	7799	36	142	0
13-mei	260	989	1784	0	2225	13607	76	0	0

Discussion

In general, our data support the theory of size differential control of the phytoplankton community by microzooplankton. Almost no grazing was observed on larger phytoplankton while grazing rates on smaller phytoplankton was present when the natural abundance increased. The grazing rates of smaller phytoplankton seems to correspond with the global average. Furthermore, the increase in abundance of the Oligotrichia ciliates appeared to correspond with the increase of the abundance of smaller phytoplankton as well as the observation of microzooplanktonic grazing.

Microzooplanktonic grazing on smaller phytoplankton began earlier in the season when compared to previous studies performed in the German Bight (Loebl & Van Beusekom, 2008). In that study, grazing was not observed until after the diatom bloom. One cause could be that this study utilized chlorophyll α as a measure for phytoplankton biomass. Coastal systems are known to have a significantly higher ratio of larger phytoplankton species when compared to open ocean systems and can make up large proportion of the biomass as measured by chlorophyll α. In the Marsdiep area up to 80% of chlorophyll α is found in phytoplankton larger than 8 mu (Riegman et al., 1993). If these larger species are not consumed by microzooplankton this could result in discrepancies in regard to observed grazing pressure. Furthermore, compared to Riegman *et al* (1993) larger growth rates were observed for larger phytoplankton, but similar grazing pressure when compared to smaller phytoplankton. This indicates that grouping of size fractions in one bulk chlorophyll α analysis might underestimate true microzooplanktonic grazing during spring blooms. For summer and autumn grazing estimates in coastal areas and

in open ocean where diatom production is lower it can however be a good estimate (eg. Burkill *et al.*, 1987).

The use of chlorophyll α as a biomass measure is being recently disputed (Lenhart et al., 2010). C:Chl levels are influenced by phytoplankton species composition, irradiance, temperature and nutrient concentration and it is therefore difficult to obtain a precise biomass estimate from chlorophyll α measurements alone (Alvarez-Fernandez & Riegman, 2014). Diatoms have relatively low C:Chl ratio and tend to obscure the other, smaller species, while these can still have a significant contribution to overall biomass. This is especially important when grazing experiments are performed to gain insight into how much energy is transferred to higher trophic levels.

The analyses of the larger zooplankton clusters in the flow cytometry analysis support this hypothesis. While limited grazing based on chlorophyll α measurement is observed in the western Wadden Sea when grazing on small phytoplankton was present, no grazing on the larger phytoplankton was observed. Furthermore, the natural abundance of larger phytoplankton and chlorophyll α concentration appear to correspond. The natural abundance of larger phytoplankton throughout this study was considerably higher which could have large effects on the chlorophyll α measurements.

The large spike in chlorophyll α concentrations at the beginning of the season that coincided with the peak in large phytoplankton cluster is counter-intuitive to the theory of size progression of phytoplankton during bloom development (Riegman et al., 1993). The peak occurred much earlier than the peak of smaller phytoplankton abundance. However this peak did not occur in simultaneous analysis (HPLC) of biomass of parallel sampled seawater (unpublished data). Due to our methodology this study has not corrected for a patchy distribution of phytoplankton. Even in turbulent open ocean patch formation can occur (Mitchell *et al.*, 2008). The abundance of phytoplankton in patches can be several times larger than the surrounding water (McManus *et al.* 2013). In this scenario we might have sampled an unusually high concentration of phytoplankton, leading to a large increase in both chlorophyll α and phytoplankton abundance based on flow cytometry.

Some non-linear feeding responses were observed during the start of the experiments (see appendix, figure A1). Teixeira & Figueiras (2009) state that these observations can be due to reaching maximum grazing rates already at higher diluted steps. When the abundance of microzooplankton is low compared to the abundance of zooplankton, grazing rates are no longer linear in relation to the apparent growth rates. This looks to be case in our experiments as well. This could imply that the grazing rates in the experiments earlier in the season are higher than measured. We however did not correct for this.

The rise in abundance of the *Oligotrichia* ciliate population appears to follow the increase in smaller phytoplankton cells. Both the phytoplankton and *Oligotrichia* population peak at the same time and both crash at the end of the season. The Naked *Choreotrichia* population however does not follow a clear trend in phytoplankton development. This population starts its

expansion when most phytoplankton is disappearing from the system, and peaks at the end of the time series when phytoplankton abundance is at its lowest point. This would imply that this ciliate population has a different food source than the phytoplankton observed in this study. Naked *Choreotrich* ciliates are known to be able to photosynthesize (e.g. Montagnes, 1996). The decline of the phytoplankton community could have removed the competitive disadvantage this ciliate population had, which could have caused the explosion.

General conclusions

Bulk chlorophyll α analysis might not be suitable for Landry and Hasset's technique of grazing estimation during spring bloom formation in coastal areas. Although Riegman *et al.* used chlorophyll α as well, the phytoplankton community was separated by size. While this gives a clearer image of the smaller fraction of the community, filtering has its own disadvantages. When large amounts of larger and chain forming diatoms are present in the samples, filters can get clogged and smaller phytoplankton species might not pass the filter. This could ultimately affect the observed biomass of smaller phytoplankton. Flow cytometry does not have this problem and could assist in a more accurate estimate. However flow cytometry will not help give insight into the biomass transfer to microzooplankton. Some studies (eg. Grattepanche *et al.*, 2011) use direct estimates of carbon, but these are very time consuming and rely heavily on estimates. Depending on the goal of research and time constraints the proper technique of analysis should be chosen.

Microzooplanktonic grazing is a large flux of carbon in the system and is therefore an important process in both phytoplankton loss assessment (especially for smaller phytoplankton species) and trophic interaction analysis. However, there does not appear to be a significant grazing effect on the larger phytoplankton species and might therefore not affect the sedimentation rate of carbon that is mainly determined by diatoms. This support earlier research that found no microzooplanktonic grazing in diatom dominated blooms (Loebl *et al.* 2012). The results of this study should ultimately be considered when investigating trophic structure through stable isotope analysis, because the addition of an intermediate step between the classical phytoplankton-zooplankton interactions could introduce a larger distance between these two trophic levels.

Recommendations for further research

As previously mentioned this study had no correction for a patchy distribution. By separating the initial water samples in triplicates it would be possible to correct for patchiness. However this would also mean that number of incubation bottles needs to be tripled. This would result in a significant increase in handling time that would affect the starting conditions.

The flow cytometry analysis was not calibrated on size. While it is possible to get some size information by cluster analysis, it is not possible to accurately describe the size difference of the phytoplankton species being consumed. Size calibrating the FCM analysis would also result in a more accurate comparison of phytoplankton abundance over time.

In this study we mainly looked at the ciliate population but there are other grazers present that could potentially be of large effect on the phytoplankton loss and should be included in further studies.

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Appendix

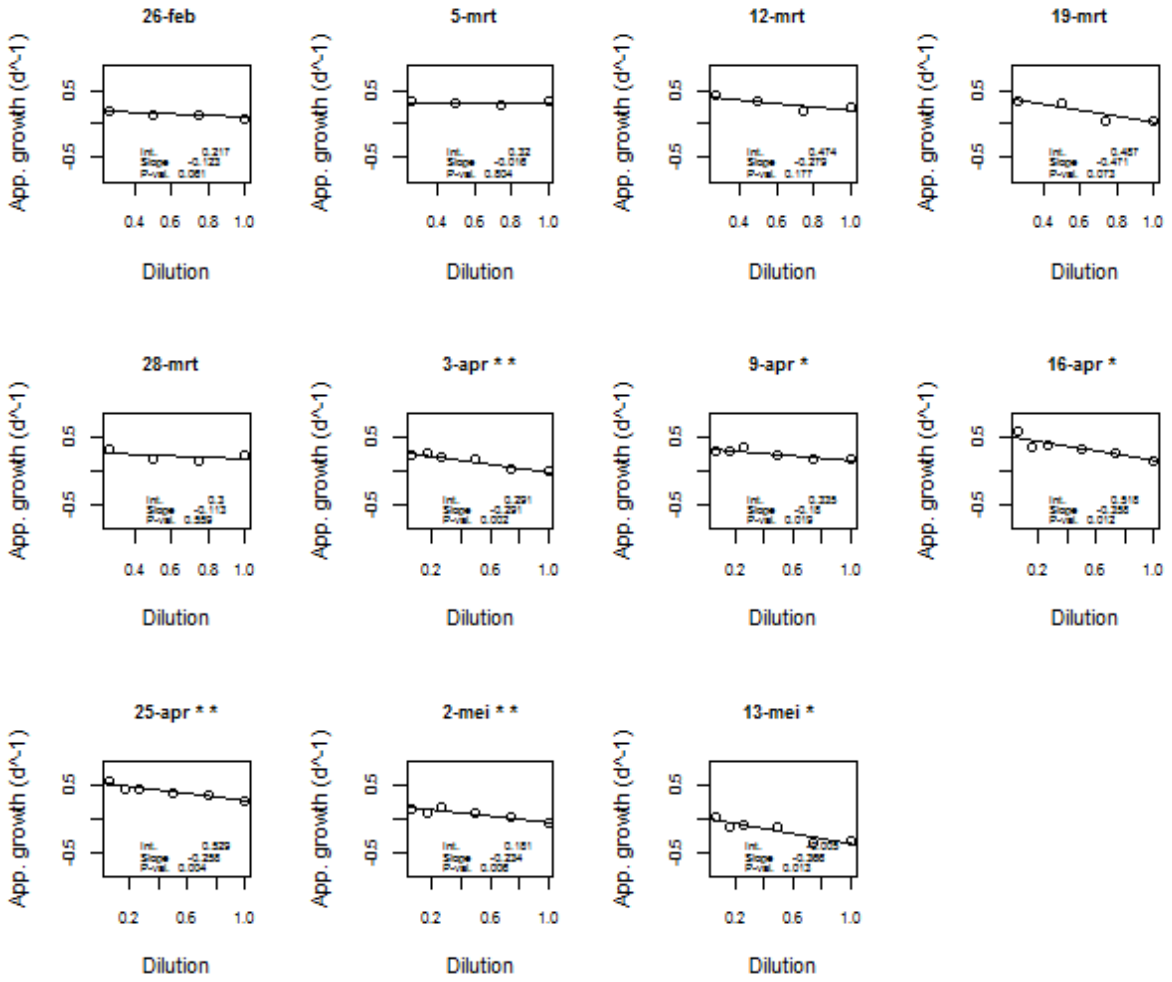

Figure A1: Apparent growth of small phytoplankton based on flow cytometry over dilution fraction per date after 24 h. incubation. Linear regression is plotted.

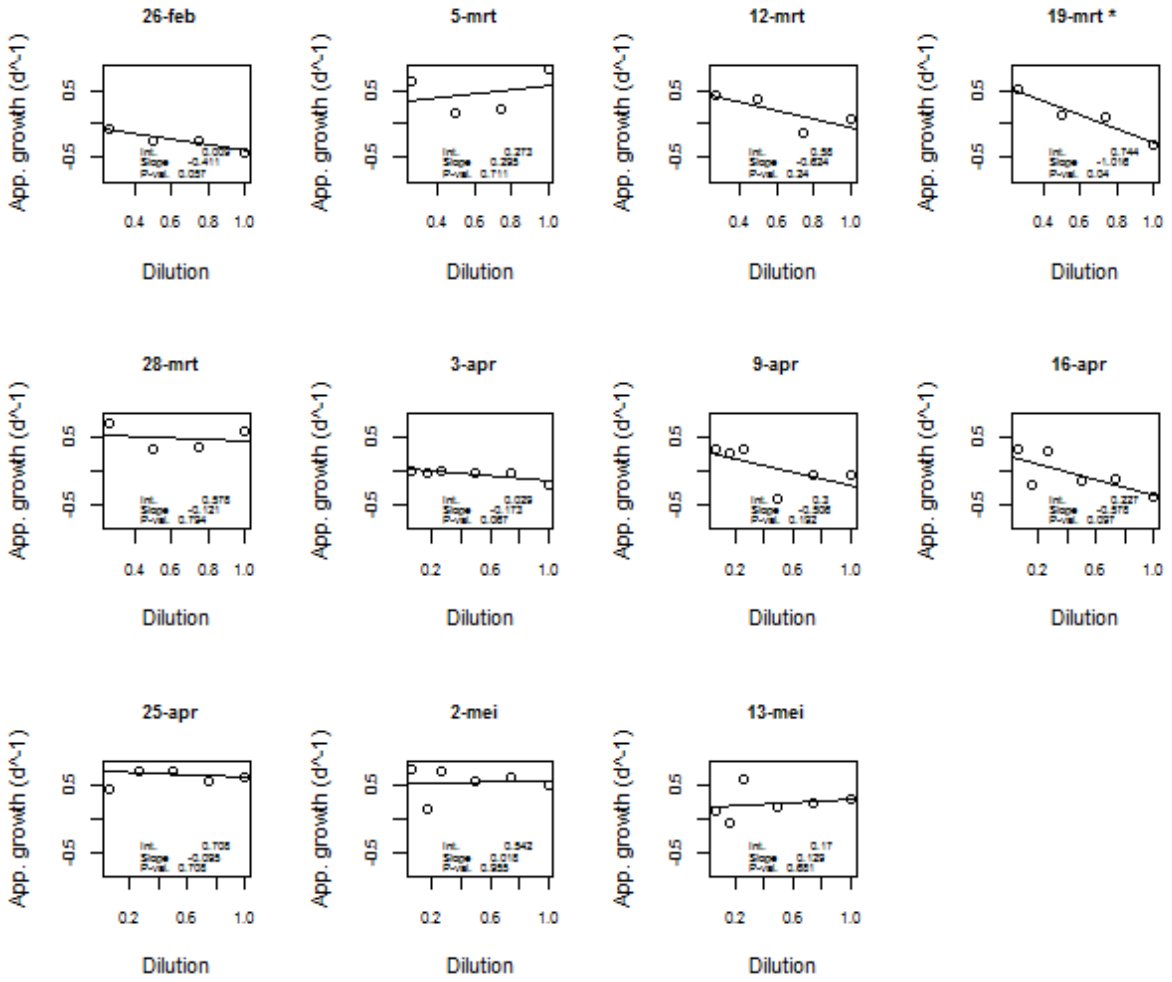

Figure A2: Apparent growth of large phytoplankton based on flow cytometry over dilution fraction per date after 24 h. incubation. Linear regression is plotted.

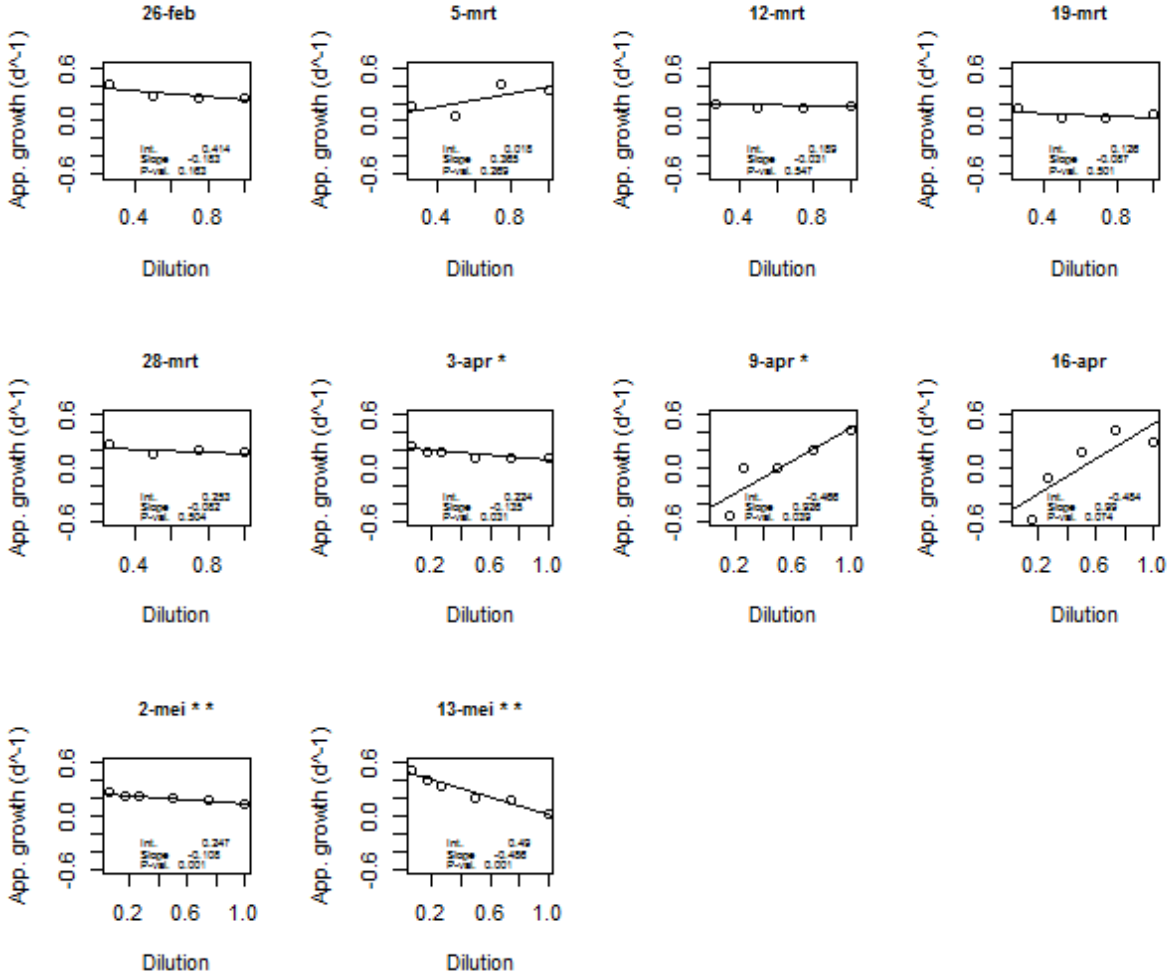

Figure A3: Apparent growth of all phytoplankton based on chlorophyll measurements over dilution fraction per date after 24 h. incubation. Linear regression is plotted.