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Microzooplankton, the missing link in Finnish plankton monitoring programs

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Finnish plankton monitoring is divided into phytoplankton and mesozooplankton sampling. Using the phytoplankton protocol, we included all organisms identified in samples from the Baltic Sea during spring (n = 125). The plankton was converted to carbon, and including all microscopy derived carbon (MDC), increased the carbon content by 22%, on average, compared with only phytoplankton. Particulate organic carbon (POC) and chlorophyll *a* (Chl *a*) were also measured, and the general relationship between MDC and POC was: slope = 1.04, intercept = 240 µg POC l^{-1}, R^2 = 0.66; for phytoplankton to Chl *a*: 0.037 g Chl *a* (g MDC)$^{-1}$, R^2 = 0.68. Our results demonstrate that a variable fraction of the plankton biomass is not recorded in the monitoring programs. Most of the unaccounted biomass was ciliates, which constituted 14.1% ± 3.7% (mean ± maximum error) of the plankton biomass. Based on the results we recommend including microzooplankton in the existing phytoplankton monitoring program.

Introduction

Marine ecosystems are facing increasing environmental pressures from human activities. At the same time, our dependence on marine resources is increasing and there is a need to follow and understand the effect of environmental change on marine food webs (Board 1990). For this purpose, different monitoring programs exist, and time series data can be used to evaluate the ecosystem structure and functioning, for example community composition, community assembly and food web assessment (Wasmund *et al.* 2011, Lehtinen *et al.* 2016, Klais *et al.* 2017). Monitoring programs may also form the basis for decisions to change management practices in order to maintain or improve the environmental conditions (Borja *et al.* 2016).

For determining the marine phytoplankton community, the traditional method is to preserve water samples and concentrate the plankton using the settling method of Utermöhl (1958), followed by identification and enumeration with an inverted microscope. Larger organisms such as mesozooplankton are concentrated by net sampling (i.e. plankton net) and are typically counted with a stereomicroscope (Hays *et al.* 2005). For phytoplankton, net samples are also used, but as a qualitative sample, examining the community composition. Water transparency (i.e. Secchi disk measurements) and chlorophyll *a* (Chl *a*) measurements are other variables with

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long monitoring tradition that is relevant for the plankton biomass concentration.

Microscopy is very time consuming and the counting procedure is a bottleneck in present day monitoring programs. New techniques have been developed, such as semi-automatic particle counting (e.g. flow-cytometry) and genetic sequencing (Aylagas *et al.* 2014, Besmer *et al.* 2014). These methods generate data at a fraction of the time and is a better option for picoplankton (< 2 µm), which is difficult to identify by microscopy without using dyes and immersion oil. However, microscopy still produces better taxonomical resolution for nano- and microplankton compared with flow-cytometry (Haraguchi *et al.* 2017). Genetic sequencing is a good tool for obtaining taxonomic data, but has limitations in terms of quantification of biomass and detection of relevant ecological data e.g. life cycle stage (Valentini *et al.* 2016), which can be critical for the plankton community development (Lee *et al.* 2018). Using the traditional microscopy method is also the best way to compare present sampling with historical data.

The design of monitoring programs has often been sub-optimal, often due to a poor coupling between monitoring, scientific development (e.g. methodology) and environmental interpretation (Board 1990). Monitoring programs should be carried out in a rigorous way in terms of spatial and temporal coverage and community composition should be coupled with complementing environmental data (De Jonge *et al.* 2006). Data harmonization and publicly available meta-data is important for promoting scientific use (Klais *et al.* 2015, Zingone *et al.* 2015). However, due to the cost of microscopy, plankton monitoring programs always need to make compromises of where and when samples are taken, to which taxonomical level identification is carried out, and which organism groups are identified and quantified.

One of the areas with best coverage of different monitoring programs is the Baltic Sea. The Baltic Sea is an almost landlocked sea, surrounded by nine countries and with approximately 80 million people living within its catchment area. The limited water exchange with the ocean and relatively large population has led to several environmental problems, and monitoring is a key tool in the management of the Baltic Sea ecosystem (HELCOM 2013a). All of the countries have their own monitoring programs of different Baltic sub-basins and the effort is coordinated by the Baltic Marine Environment Protection Commission — Helsinki Commission (HELCOM). In all of these monitoring programs there is a separate phytoplankton and zooplankton sampling. In most countries, the zooplankton monitoring focuses only on mesozooplankton, which is typically sampled with a 100 µm plankton net, whereas phytoplankton monitoring focuses on pelagic primary producers.

A group of organisms that receives less attention than phytoplankton and mesozooplankton in monitoring programs, are the smaller size classes of zooplankton, e.g. microzooplankton. Microzooplankton comprises heterotrophic and mixotrophic organisms ranging from 20 to 200 µm, and consists of phagotrophic protists such as flagellates, dinoflagellates, ciliates, acantharids, radiolarians, foraminiferans, and juveniles of mesozooplankton. The low coverage of these groups in monitoring programs is unfortunate as their role in the aquatic food web is important, in particular for linking the microbial loop with higher trophic levels (Calbet and Landry 2004). For example, ciliates are able to significantly reduce abundances of phyto- and bacterioplankton in the Baltic Sea (Mironova *et al.* 2011). Climate change is causing warmer surface waters and changes to precipitation in the Baltic Sea catchment area, which may have pronounced effects on water stratification (HELCOM 2013b). The overall projection for the future, is a decrease in the overall size of phytoplankton, increased importance of regenerated production, and top-down control of primary production (Hoegh-Guldberg and Bruno 2010). This could increase the importance of smaller heterotrophic groups, such as microzooplankton, and cause temporal and spatial shifts of zooplankton populations (Calbet 2008, Richardson 2008).

Different methods are needed for plankton monitoring as large, rare organisms cannot be reliably enumerated in the same way as small, abundant organisms. The error in abundance estimates decreases with number of counted units, i.e. the more individuals counted per volumetric unit, the more accurate is the estimate

of their abundance. Because of the large size difference, it is evident that phytoplankton and mesozooplankton cannot be enumerated using the same method. However, the smaller microzooplankton has similar size as micropytoplankton and it should, in principle, be possible to count them using the same sampling method.

Remote sensing forms the backbone of ocean biogeochemical models, and ocean color, detected by satellite imaging, is a key input for estimating e.g. phytoplankton biomass and primary production. These types of models have commonly been used to quantify interactions between climate and ocean biogeochemical cycles; however, they are not well suited for understanding how environmental changes shift ecosystem dynamics (Allen and Polimene 2011). The plankton community composition affects the biogeochemistry of the ocean, and taking into account the different functional groups present in the community, provide better understanding and improve modeling of marine material fluxes (Litchman *et al.* 2015). Linking data from plankton monitoring with biogeochemical models could be a way to better predict consequences of environmental changes on marine ecosystems, and a step in this direction would be to improve our understanding of the variability in commonly used conversion ratios between biomass proxies and carbon e.g. Chl-*a*:C ratio.

Using the Finnish monitoring program as a case study, we wanted to test what information is missed by the present phyto- and mesozooplankton monitoring. In particular, we wanted to determine the contribution of microzooplankton to the total nano- and microplankton biomass and furthermore compare the microscopy derived carbon (MDC) with particulate organic carbon (POC) and Chl *a* in order to see how well the MDC correlates with these variables.

Material and methods

Sampling was performed during four cruises on board R/V *Aranda* (2013–2016). The cruises were conducted in April and May, and covered large parts of the Baltic Sea (Fig. 1). In total 125 stations were visited and the water was taken from 3 m depth with the CTD (Conductivity Temperature Depth) sampling rosette using Niskin bottles (n = 120) or from the flow-through system on the ship (n = 5). Most of the sampling stations were in the open sea and all stations within one nautical mile (nm) from the nearest shore were characterized as coastal.

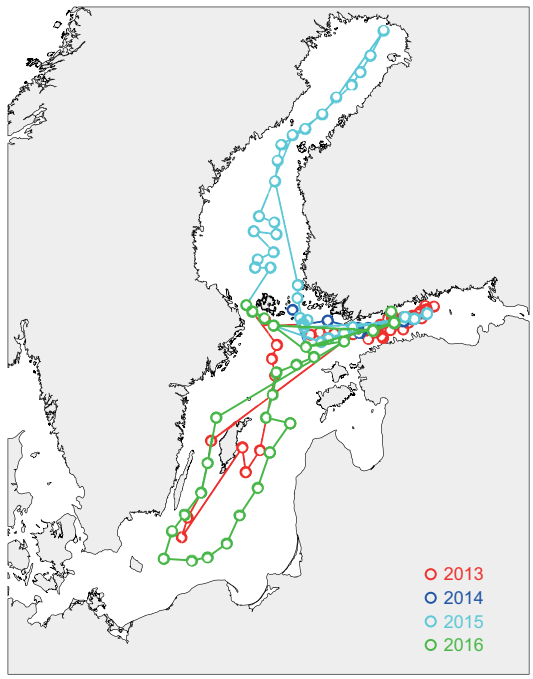

Fig. 1. Sampling stations (n = 125) during four cruises (2013–2016) on board R/V *Aranda* in the Baltic Sea.

Sub-samples were taken for plankton enumeration and for determination of Chl *a* and POC. For Chl *a* and POC samples, a volume of 50–200 ml (depending on the concentration) was filtered onto GF/F filters. For the POC these had been pre-treated by acid washing (10% HCl) and combustion (4 h at 450 °C). POC was determined on a mass spectrometer (Europa Scientific ANCA-MS 20-20, Europa Scientific Ltd.). Chl *a* was extracted with ethanol (Jespersen and Christoffersen 1987) and the Chl *a* concentration was determined on a fluorescence spectrophotometer (Agilent Cary Eclipse) calibrated against known Chl *a* standards (Sigma-Aldrich).

For microscopy, 200 ml samples were preserved with acidic Lugol's solution (0.5% v/v) at each station and stored in darkness at 4 °C until the microscopic enumeration of plankton. Prior to microscopy, samples were acclimated

to room temperature and prepared according to Utermöhl (1958). An appropriate volume (10–50 ml), depending on the Chl *a* concentration, was used for sedimentation of cells into the counting chamber (Hydro-Bios) after mixing the samples gently. Samples with high biomass (> 10 µg Chl *a* l^{-1}) were diluted with artificial sea water (Tropical Marine Salt mixed with ultrapure water and then 0.2 µm filtered), with the sample salinity, to a dilution factor of 2. Samples were enumerated under an inverted microscope (Leitz DM IRB, Leica) and a defined area of the counting chamber was considered at three different magnifications (125×, 250×, 500×) for enumeration of different size classes. Following the recommendation of the Finnish monitoring program (organized by the Finnish Environment Institute, SYKE), a minimum of 60 counting grids evenly distributed across the counting chamber were considered for each magnification, which corresponds to 11.1% of the sedimentation area at 125×, 2.8% at 250×, and 1.3% at 500×. The counting software EnvPhyto was used and the data stored directly into the Hertta database (SYKE). Calculations of abundance, biovolume and carbon biomass for the species that are part of the phytoplankton monitoring program were done automatically by the software according to Olenina *et al.* (2006), the biovolume list of HELCOM Phytoplankton Expert Group (PEG) (http://helcom.fi/helcom-at-work/projects/phytoplankton) and Menden-Deuer and Lessard (2000).

In the counting software, following the HELCOM PEG taxon and biovolume list, there is a predetermined set of species that can be entered. It is mainly pelagic phytoplankton with some exceptions including mixotrophic organisms (e.g. the ciliate *Mesodinium rubrum*) and a selection of heterotrophic organisms, such as some dinoflagellates, silicoflagellates, and some heterotrophic nanoflagellates. The heterotrophic organisms are not officially part of the phytoplankton monitoring program that focuses on pelagic primary producers, but have historically been included and is still today recorded in the counts. There are, however, a number of organisms that can be identified that are not recorded. For example plankton resting stages, microzooplankton, such as ciliates, and benthic diatoms. In the present investigation, all of these organisms were counted and their biovolume and carbon content was calculated using the same conversion factors as described above. For microzooplankton, we used biovolume equations from Olenina *et al.* (2006) and conversion factors from Auf dem Venne (1994) and Putt and Stoecker (1989). The counting was done in parallel with the phytoplankton monitoring counting protocol mentioned above. We excluded mesozooplankton (e.g. copepods and rotifers) as these are covered by the present Finnish mesozooplankton monitoring and their abundances cannot be accurately determined with the sampling-and microscopic method used for the presented study. For simplicity, we included nanozooplankton (< 20 µm) in the microzooplankton group, which we use throughout the rest of the text.

Graphs and linear regressions were done in Sigma Plot 13 (SYSTAT) fitting the equation:

$$f(x) = a + bx \quad (1)$$

to the data where a is the intercept with the *y*-axis and b the slope of the linear regression. Comparisons of two different slopes were done with the null hypothesis that the slopes are equal:

$$b_1 - b_2 = 0 \quad (2)$$

to test the probability of the null hypothesis being true, the test statistic (*Z*) can then be written:

$$Z = \frac{\left(b_1 - b_2\right)}{\sqrt{\left(S_{b_1}^2 + S_{b_2}^2\right)}} \quad (3)$$

where *S* is the coefficient variance associated with the first and second group, respectively (Paternoster *et al.* 1998). In order to estimate the error of the counts we used the equation presented in Willén (1976):

$$\mathrm{MaxErr} = \frac{\pm 200\%}{\sqrt{n}} \quad (4)$$

where MaxErr is the maximum error and n is the number of counted units of a group or species. The error of ciliate biomass estimation was calculated using this percentage.

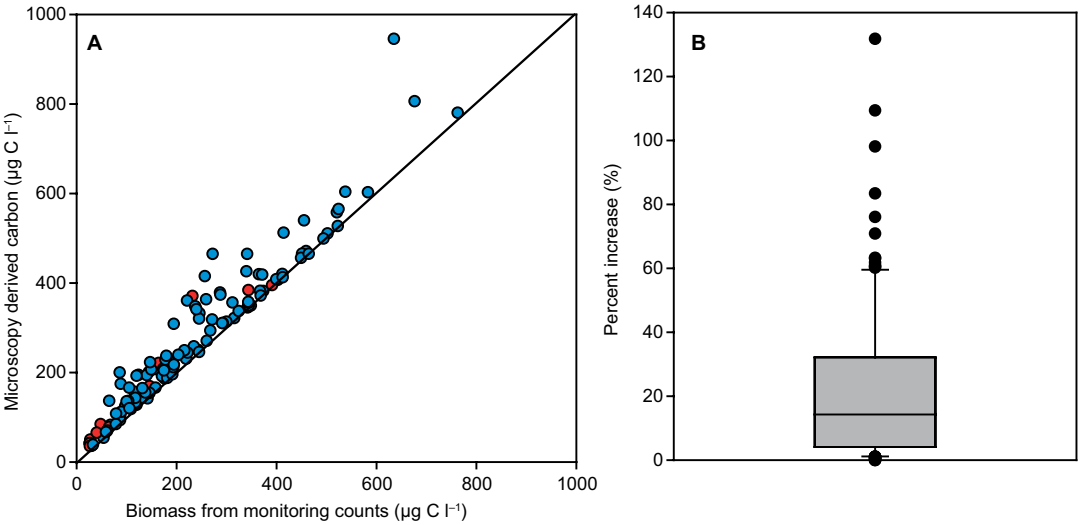

Fig. 2. (**A**) The spring-bloom data from phytoplankton monitoring counts plotted against the total microscopy derived carbon and (**B**) a boxplot of the increase of carbon content when including all identified organisms. Blue dots represent open sea sampling stations, red dots coast near (< 1 nm) sampling stations. In the box plot, the box represents the 25–75 percentiles, the horizontal line the median and whiskers the 10–90 percentiles (n = 125).

Results

There was a clear effect on the estimated plankton carbon biomass when including the organisms not counted in the Finnish plankton monitoring program (Fig. 2). The mean increase in carbon content was 22% (median 14.2%), and at 10% of the stations, the carbon content was ≥ 60% higher in the total counts compared with the phytoplankton monitoring counts. There was no apparent difference in the results of the coast-near stations compared with open water stations (Fig. 2).

The largest group of organisms not present in the plankton monitoring data was ciliates, which constituted an average of 78% (including both < 100 and > 100 µm size fractions) of the unaccounted biomass (Fig. 3). The most abundant ciliates were *Lohmaniella oviformis* and different tintinnids in terms of abundance and biomass, respectively. Resting stages of diatoms and dinoflagellates constituted 2.1% and 2.6%, respectively. The remaining 17.6% included a plethora of other biological components e.g. individual species not included in the phytoplankton monitoring such as benthic diatoms and other plankton resting stages. The ciliate biomass was highly variable but in some stations constituting > 50% of the total nano- and microplankton biomass (Fig. 3). The average maximum counting error (Eq. 4), considering the highly variable ciliate counts for all 125 stations was ±63.2% (Fig 4). When pooling all of the 125 stations, the ciliate biomass was 14.1% ± 3.7%, of the biomass recorded in the phytoplankton monitoring.

There was a strong positive correlation between the microscopy derived carbon (MDC) and POC determined by filtration (Fig. 5). Both the phytoplankton monitoring counts and total microscopy counts produced similar slopes close to parity with the POC data. The coefficient of determination was slightly better for the total microscopy counts compared with the monitoring counts, R^2 = 0.68 and 0.66, respectively, but there was no statistical evidence suggesting that the slopes were different (Eq. 3; p = 0.4). The slope and intercept was 1.04 and 239 µg C l^{-1} for monitoring counts MDC; 0.97 and 220 µg C l^{-1} for total counts MDC (both with $p < 0.0001$). The autotrophic carbon biomass to Chl *a* ratio was 0.037 (Fig. 6), determined by linear regression (R^2 = 0.68, $p < 0.0001$).

Discussion

In half of the stations there was relatively little

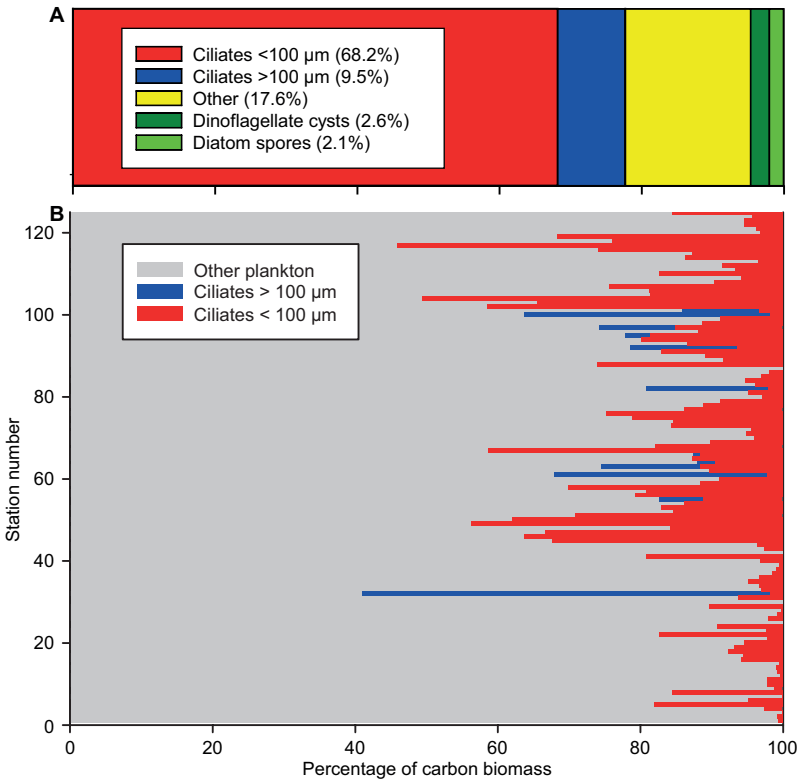

Fig. 3. (**A**)The average (n = 125) proportion of different groups of unaccounted plankton that is not included in the Finnish phytoplankton monitoring counting protocol, and (**B**) the contribution of heterotrophic ciliates (excluding *Mesodinium rubrum*) to the total plankton biomass (phytoplankton monitoring plus extra counts) at the different stations. The ciliates > 100 µm are in principle included in the zooplankton monitoring that samples with a 100 µm mesh net, but ciliates < 100 are not included in the phytoplankton monitoring and is consequently not recorded at all in regular monitoring programs.

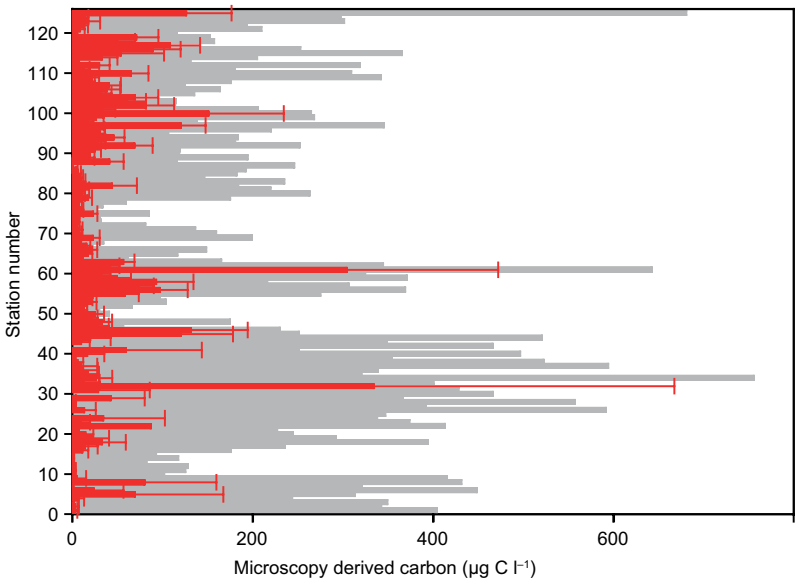

Fig. 4. The total microscopy derived plankton biomass minus ciliates (grey) and ciliate biomass (red) at the different sampling stations (n = 125). The error bars represents the maximum error according to Eq. 4, based on the number of counted units.

(< 15%) biomass that was unaccounted for in the regular phytoplankton monitoring protocol, however, at 10% of the stations there was a clear difference with up to two-fold increase in the biomass estimate (60%–130% increase). This implies a clearly biased plankton biomass estimate for these stations when microzooplankton biomass is not recorded. Our data originated

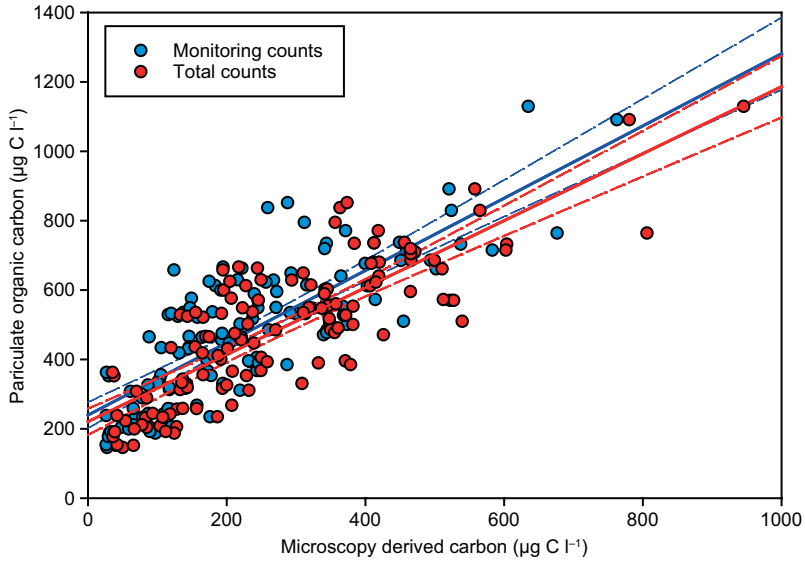

Fig. 5. The phytoplankton monitoring counts (blue) and total microscopy derived carbon (MDC, red) plotted against the particulate organic carbon (POC) determined by filtration (n = 124). The difference between these is presented in Fig. 2. The solid lines represent the linear regression of monitoring counts (red) and MDC (blue) and dashed lines the 95% confidence intervals. Monitoring counts: slope = 1.04, R^2 = 0.66, $p < 0.0001$; MDC: slope = 0.97, R^2 = 0.68, $p < 0.0001$.

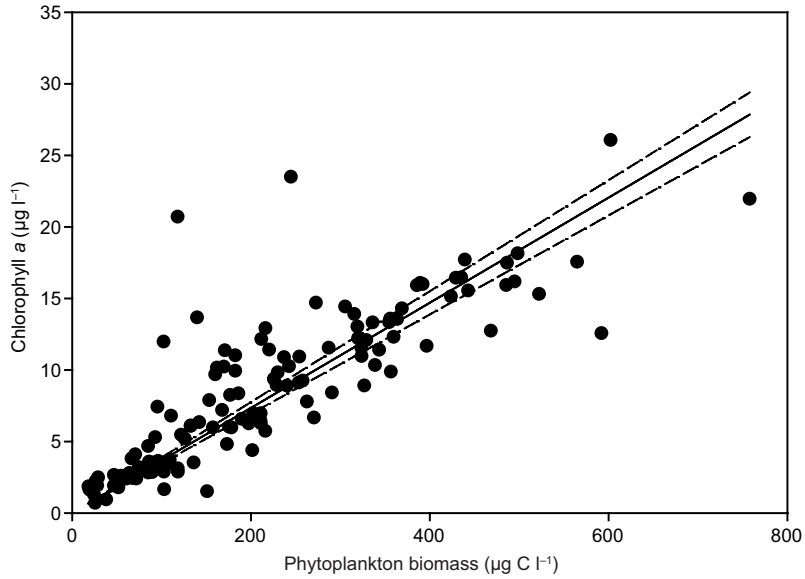

Fig. 6. Carbon content defined as autotrophic in the Finnish phytoplankton monitoring program plotted against the chlorophyll *a* (Chl *a*) concentration. The total share of autotrophs in the unaccounted biomass data was negligible (Fig. 3) and was not included here. The solid line represents the linear regression passing origin (slope = 0.037, R^2 = 0.68, $p < 0.0001$) and dashed lines the 95% confidence interval.

from the spring, a season dominated by spring bloom phytoplankton and limited grazing pressure (Lignell *et al.* 1993). We therefore expect that the share of unaccounted biomass is even higher during summer with a more developed grazing community and higher abundances of microzooplankton (Mironova *et al.* 2011).

The main fraction of the unaccounted biomass was heterotrophic ciliates. These are at present not included in the Finnish phytoplankton or mesozooplankton monitoring. That is unfortunate as this group of grazers is clearly an integral part of the pelagic food web, and important to follow during ongoing changes to the marine ecosystem (Calbet 2008). It is not only in the Finnish plankton monitoring programs where microzooplankton is missed, and the reason for this is likely related to the traditional separation between phyto- and zooplankton and the use of different methods for their sampling and quantification. However, the Finnish phytoplankton monitoring is at present not strictly monitor-

ing autotrophs, as e.g. heterotrophic flagellates (albeit not officially part of the monitoring program) as well as the mixotrophic ciliate *M. rubrum* are recorded. We enumerated the full nano- and microplankton community using the same method. There are consequently no technical difficulties in recording microzooplankton while enumerating phytoplankton and this could be implemented into the existing phytoplankton monitoring program with relatively small efforts, e.g. not requiring a new sampling regime. This would fill the gap between phytoplankton and mesozooplankton monitoring and increase the coverage of the planktonic community composition.

In order to implement microzooplankton counts into the monitoring program, it requires proper training of personnel and efforts to evaluate the counting procedures and its effect on the counting accuracy. When counting plankton in a defined volume, there will be a large error in the biomass estimate for large (e.g. > 100 µm) and rare organisms. This is the reason why mesozooplankton cannot be counted reliably using phytoplankton counting methods, as one or two counted units is not sufficient to make an accurate abundance estimate. Integrating the uncertainty of the biomass estimate as a function of counted organisms per volume unit could be a way of dealing with this problem (Miller *et al.* 2011). Our counted ciliates ranged from 1 to > 100 organisms per sample, which corresponds to maximum errors of ±200 and < 20%, respectively. For almost half of our stations (n = 125), the error was ≤ 40% and for 40% of the stations it was > 50%. For our purpose, having a rough estimate of the microzooplankton biomass was better than having none at all. Within the scope of starting microzooplankton monitoring, some measures should be taken in order to improve counting accuracy, compared with our counting method. For example, by using higher sedimentation volumes, which would increase the number of counted units. This is to some extent done in the Finnish phytoplankton monitoring, where the sedimentation volume providing optimal cell density is tested prior to counting. Different size classes could be counted at defined magnifications, e.g. larger cells at 125× and very small ones at 500×. As ciliates are motile, the processing of an integrated sample (e.g. 0–10 m, as done for the Finnish phytoplankton monitoring) rather than sampling only one water depth, as we did, will likely be a better approach for monitoring purposes. We would suggest counting > 50 microzooplankton individuals for all abundant species to achieve an abundance error of less than ±30%, which would be an appropriate compromise of counting effort and accuracy

Identifying the microzooplankton community all the way to species level is difficult to impossible, but getting counts with estimates on the biomass of larger entities such as ciliates will have great scientific value for e.g. food web assessments. For example the abundant species *Lohmaniella oviformis* can be identified relatively easy and the separation of biomass-relevant tintinnids from other heterotrophic ciliates would yield ecological relevant data on the community composition. Other techniques, such as metagenomics, are developing quickly and could be the source of more taxonomic driven studies.

Additional plankton groups that could be important but are largely missed in the present Finnish monitoring programs are picoplankton (< 2 µm) and juvenile mesozooplankton such as rotifers and early stages of copepod nauplii (≤ 100 µm). Picoplankton is problematic to count using a microscope and would require setting up a different method e.g. flow-cytometry. The problem with intermediate sized zooplankton is that they may slip through the mesozooplankton nets used for monitoring. Counting these in the phytoplankton monitoring would create the problem of low numbers of counted units, giving large uncertainty in the biomass estimation as stated above. These needs to be counted using a different protocol e.g. settling a larger volume and counting using lower magnification (e.g. stereomicroscope), or alternatively they could be sampled with a plankton net with a smaller mesh size (e.g. 50 µm) than what is currently used in mesozooplankton monitoring.

The POC was higher than the MDC, as expected, due to the fact that detritus is not included in the microscopy counts whereas POC is measuring all the carbon that remains on the GF/F filter (0.7 µm, nominal pore size). The surprising part was how well the POC and MDC match with a slope close to parity. With increas-

ing plankton carbon we had expected an increasing proportion of detritus, which would produce a slope above one. With a slope close to parity, the results suggest that the concentration of detritus in the POC pool is constant (220 to 240 µg POC l^{-1}) and its proportion of the total carbon pool is decreasing with increasing biomass in the water. This is most likely related to rapid aggregation and sinking out of dying cells and detritus from the surface water. The sampling was taking place throughout most of the Baltic Sea, before, during and after the spring bloom when the biomass in the water is at, or close to the annual maximum. Rapid settling of biomass is typical during this period (Heiskanen 1998).

The Chl-*a*:C ratio varies between different species and is also affected by environmental variables such as light, temperature and nutrient concentrations (Geider 1987, Cloern *et al.* 1995). For the diatom *Chaetoceros wighamii*, a dominant species in the Baltic Sea during spring, the Chl-*a*:C ratio varied with a factor 2 during exponential growth and decreased by almost an order of magnitude during nitrogen-limitation (Spilling *et al.* 2015). The Chl-*a*:C ratio may also be influenced by the phytoplankton community composition during the spring bloom in the Baltic Sea (Spilling *et al.* 2014). Consequently, the ratio between autotroph carbon biomass and the Chl *a* concentration can be expected to vary between different phases of the bloom. With a coefficient of determination of 0.68, the variability around the average Chl-*a*:C ratio we found (0.037, or C:Chl-*a* ratio 27) was relatively low. This was most likely because the environmental conditions were relatively uniform (e.g. low temperature), suggesting that the presented Chl-*a*:C ratio is a good conversion estimate between Chl *a* and phytoplankton carbon in the Baltic Sea surface-water during spring. This is supported by Simis *et al.* (2017) that presented an average Chl-*a*:POC ratio of 0.043 during spring, a slightly higher (15%) estimate. Including samples from different depths (light conditions) or seasons (temperature and community composition) would likely increase the variability in the Chl-*a*:C ratio, for example the Chl-*a*:POC ratio during summer in the Baltic Sea presented by Simis *et al.* (2017) was approximately 50% lower than the spring value (0.022 *vs*. 0.043).

The phytoplankton monitoring data have been used for several descriptive studies of phytoplankton community patterns (Olli *et al.* 2013) and long term changes to the community composition (Klais *et al.* 2011, Olli *et al.* 2011, Wasmund *et al.* 2011). These data may, however, have value beyond descriptive studies of community structure. Carbon is the most used 'currency' by marine biogeochemical models whereas Chl *a* is the most commonly used proxy of aquatic autotrophic biomass, e.g. obtained from satellite images. Our results suggest that Chl *a* and POC can be estimated with relatively high precision ($R^2 > 0.6$) from specific seasons, indicating that existing phytoplankton monitoring programs are valuable for modeling biogeochemistry and food web models in the Baltic Sea ecosystem, in particular when incorporating different functional groups of plankton (Lehtinen *et al.* 2016, Fransner *et al.* 2018).

Conclusion

Microzooplankton (and also nanozooplankton) is generally an undersampled group in plankton monitoring programs around the Baltic Sea. We presented data demonstrating that this group can be a considerable part of the total plankton biomass, and we recommend including this group into existing phytoplankton monitoring programs. This can be added with relatively little effort, but care should be taken to optimize the counting efforts *vs*. the uncertainty in biomass estimates (number of units counted). We presented a positive correlation between microscopy derived carbon with total POC and Chl *a*, highlighting the value of existing monitoring programs, which breaks down the biomass into different taxa/functional groups. This can be used to improve models of material budgets and fluxes in the Baltic Sea ecosystem. The value of monitoring would further increase with the inclusion of microzooplankton, providing more complete data of the planktonic food web.

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