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Original Article

Optimising sampling frequency for monitoring heterotrophic protists in a marine ecosystem

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Heterotrophic protists are essential components of the marine ecosystem, yet they are often excluded from monitoring programmes. With limited resources, monitoring strategies need to be optimised considering both scientific knowledge and available resources. In doing so, it is crucial to understand how sampling frequency affects the value of the data. We analysed 11 years of weekly heterotrophic protist time-series data from Station L4 in the Western English Channel to explore how different sampling intervals impact data quality. In the L4 dataset, comprising 55 protist taxa, the reduction of sampling frequency from weekly to four times a year at specific seasons decreased the number of taxa encountered by 38% for ciliates and 29% for heterotrophic dinoflagellates while the mean annual biomass or its mean variation were not affected. Furthermore, when samples were taken only four times a year, biomass peaks of the ten most important taxa were often missed. The primary motivator for this study was furthering the development of the heterotrophic protist monitoring in temperate and subarctic marine areas, e.g. the Baltic Sea. Based on our findings, we give recommendations on sampling frequency to optimise the value of heterotrophic protist monitoring.

Keywords: Baltic sea, biological monitoring, ciliates, choanoflagellates, heterotrophic dinoflagellates, Protists, sampling interval

Introduction

Plankton communities are sensitive to environmental and other human induced-perturbations in the oceans and as such are good indicators of change. For example, climate change may lead to major changes in the marine food webs (Edwards *et al.*, 2013), specifically triggered by the changes in microplankton communities (Calbet *et al.*, 2014). Monitoring data are therefore essential in order to analyse and understand causes, responses, and long-term changes in freshwater and marine ecosystems (e.g. Widdicombe *et al.*, 2010; Hällfors *et al.*, 2013; Suikkanen *et al.*, 2013). In addition, such data provide information on the potential spreading of introduced or invasive species (Lehtiniemi *et al.*, 2015).

Primary producing phytoplankton form the basis of pelagic food webs, and their abundance, biomass and species composition affect directly or indirectly the growth, reproduction and survival of primary consumers such as heterotrophic nano- and microplankton and mesozooplankton. Heterotrophic microplankton consists of both unicellular (e.g. ciliates, heterotrophic dinoflagellates, radiolarians, foraminiferans and other amoebae) and multicellular organisms (e.g. rotifers, copepod nauplii, and meroplanktonic larvae of benthic animals) between 20 and 200 μm in size, while heterotrophic nanoplankton are protists 2–20 μm in size (mainly nanoflagellates, but also including small-sized ciliates and dinoflagellates) (Sieburth *et al.*, 1978; Sherr *et al.*, 1997). These nano- and micro-sized heterotrophs form an important functional link be-

tween phytoplankton and mesozooplankton (200–2000 μm) (e.g. Calbet and Saiz, 2005). While the major protistan heterotrophic microplankton groups, i.e. ciliates and heterotrophic dinoflagellates, mainly comprise species belonging in the microplankton size category, individuals smaller than 20 μm do occur. In addition to ciliates and heterotrophic dinoflagellates, we also investigated choanoflagellates, which were enumerated from the same samples. Including this easily identified nanosized heterotrophic group offered an opportunity to compare the results among different groups of heterotrophic organisms operating within the microbial loop.

The classic marine food chain, phytoplankton-zooplankton-fish, was amended by the microbial loop concept almost 40 years ago (Azam *et al.*, 1983). This concept differs from the traditional grazing food chain (phytoplankton-copepods-fish) by introducing a step where dissolved organic matter acts as substrate for planktonic bacteria. In the microbial loop, energy is also transferred via autotrophic picoplankton and nanoplankton to heterotrophic microplankton, which could be a major link between small-sized phytoplankton and mesozooplankton (Azam *et al.* 1983). Heterotrophic protists, as essential grazers of picoplankton including bacteria, were soon recognized to be important members of the planktonic food webs and major players in marine ecosystems (Porter *et al.*, 1985; Sherr and Sherr, 2008). Subsequent research established the quantitative importance of the microbial loop (e.g. Pierce and Turner, 1992; Kivi, 1996), and today we know there are marine systems which almost exclusively are based on the microbial loop (Zeldis and Décima, 2020). In addition to the central role of heterotrophic microplankton in the microbial loop, it has also been estimated that up to 60–70% of phytoplankton production can be consumed by heterotrophic microplankton daily (Calbet and Landry, 2004). In both oligotrophic and eutrophic pelagic systems, heterotrophic protists are often quantitatively more important as grazers of phytoplankton than mesozooplankton (Johansson *et al.*, 2004; Sherr and Sherr, 2007; Calbet, 2008). The significance of heterotrophic dinoflagellates has been unequivocally demonstrated (Smetacek, 1981; Hansen, 1991; Sherr and Sherr, 2007). Like dinoflagellates, planktonic ciliates are also known to be important micrograzers in various aquatic systems (Johansson and Coats, 2002). Ciliates are also a favoured diet for mesozooplankton and even fish larvae (Kivi, 1996). That ciliates and dinoflagellates are part of the diets of larval fish, such as herring and other clupeids, has been recognized for some time; but it is still often overlooked (Stoecker and Pierson, 2019).

Heterotrophic protists show great spatial and temporal fluctuations. In temperate and subarctic areas, two heterotrophic protist biomass peaks often occur; the first in spring, associated with the phytoplankton spring bloom period, and the second in late summer or autumn (Kivi, 1986; Montagnes *et al.*, 1988; Müller *et al.*, 1991; Setälä, 2004; Mironova *et al.*, 2014 and references therein). However, regional differences exist, and in some areas the highest heterotrophic protist abundances occur during summer (Dolan and Coats, 1990). Seasonal changes in the ciliate and heterotrophic dinoflagellate communities may be rapid, and distinct seasonal species can be distinguished (Montagnes *et al.*, 1988; Johansson *et al.*, 2004; Hällfors 2013). Variation and fluctuations in heterotrophic protist communities are regulated by several environmental factors such as temperature, prey availability and grazing pressure by higher trophic levels, especially crustacean zooplankton (e.g. Hansen, 1991; Kivi, 1996; Sherr *et al.*, 1997; Grinienė *et al.*, 2019). Due to the rapidly fluctuating nature of the heterotrophic protist communities, their monitoring is fraught with challenges. This, however, does not explain the scarcity of established monitoring programmes, since phytoplankton communities are equally (if not even more) dynamic in character.

A plankton time-series survey was carried out in 2018 by the ICES working group for zooplankton ecology (WGZE) to determine to what extent heterotrophic microplankton is included in plankton monitoring programmes in the ICES area (North Atlantic and adjacent seas: Mediterranean Sea and Baltic Sea). The results revealed that out of a total of 57 long-term sampling stations, heterotrophic microplankton was regularly (i.e. at least once a month) monitored at 40% of the stations, but at 34% of the stations no heterotrophic microplankton monitoring took place. At the remaining 26% of stations, heterotrophic microplankton were monitored on a less than once a month basis.

Taking the Baltic Sea as an example, extending over some 1300 km between the 54°N and 66°N latitudes in northern Europe, it is the largest brackish water body in the world. It is a severely eutrophied inland sea surrounded by nine countries, and having a drainage area with a population of approximately 90 million, the Baltic Sea is threatened by anthropogenic pressures from both land and sea. The first plankton monitoring campaigns in the Baltic Sea were undertaken in the early 1900s within the framework of investigations coordinated by the International Council for the Exploration of the Sea (ICES) (Kyle, 1910; Hällfors *et al.*, 2013). As concerns modern monitoring, many of the contracting parties of the Baltic Sea regional sea convention, i.e. the Baltic Marine Environment Protection Commission HELCOM (HELCOM, 1993), have been conducting national monitoring for several decades, and have worked together towards harmonizing methods, developing the station network and accurate species identification since 1979, when the joint monitoring (HELCOM COMBINE programme) of physical, chemical and biological parameters began. Although significant in species number, abundance and biomass (Hällfors, 2004; Mironova *et al.*, 2014), heterotrophic protists are not covered as a separate entity in the marine monitoring guidance for the Baltic Sea (HELCOM, 2017). However, it has been recommended that heterotrophic protists should in the future be included in the monitoring scheme in the Baltic Sea (Suikkanen *et al.*, 2013; Lipsewers and Spilling, 2018).

In the present study, we explore the effect of different sampling frequencies on the quality and usefulness of heterotrophic protist monitoring data. For this purpose, we utilized monitoring data from station L4, a long-term sampling station in the Western English Channel. This is one of the few monitoring campaigns, which includes ciliates, and is certainly one with relevant data for our purposes.

Material and methods

Sampling, sample analysis, and data preparation

The year-round dataset of protist abundance and biomass from 2005 to 2015 sampled at the long-term monitoring station L4 (50° 15.00′N, 4° 13.02′W), in the Western English Channel off Plymouth, United Kingdom, was chosen for the data analysis (Figures 1–2). Station L4 (depth 50 m) is situated in temperate coastal waters where seasonal changes in the abiotic environment affect plankton communities. The water is mixed during autumn and winter months while a weak stratification of the water column develops during spring and summer. Samples were collected on a weekly basis, weather permitting (Widdicombe *et al.*, 2010), from a depth of

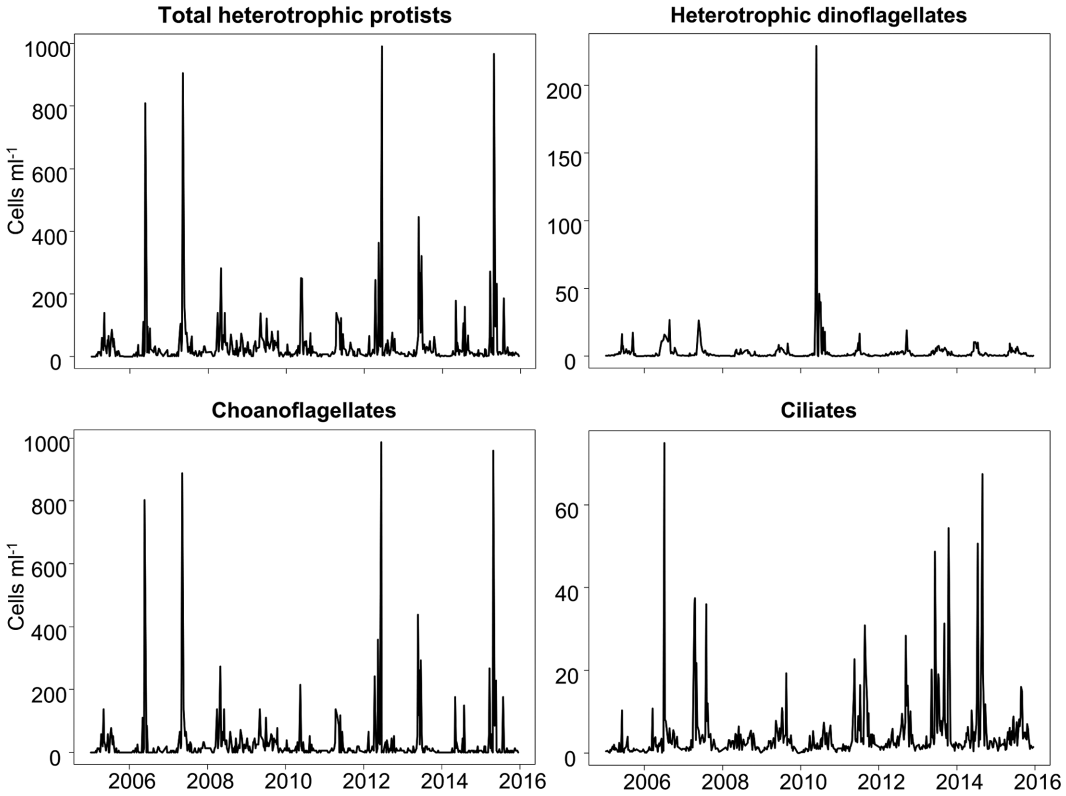

Figure 1. Abundance (cells mL^{-1}) of total heterotrophic protists included in the study, heterotrophic dinoflagellates, choanoflagellates and ciliates over the study period 2005–2015 with the original weekly sampling frequency at station L4. Note different scales on Y-axis.

10 m using a 10 L Niskin bottle. A 200 mL subsample was immediately fixed with 2% (final concentration) acid Lugol's iodine solution (Throndsen, 1978) and returned to Plymouth Marine Laboratory. Species-specific protist abundance was quantified using the Utermöhl counting technique (Utermöhl, 1958) following the British Standard (BS EN 15 204, 2006) (Widdicombe *et al.*, 2010). The same Utermöhl chamber volume (50 mL) was always used, but depending on cell density, cells from either the whole or half chamber were enumerated. In cases where certain taxa occurred in bloom-forming quantities, either half a chamber, transects or fields of view were counted to achieve a minimum count of 50 cells. In an attempt not to miss sparsely occurring taxa, large-sized and rare taxa were always counted from the whole chamber area. Mean cell measurements of heterotrophic protist taxa were used to calculate cell biovolume assuming appropriate geometric shapes (e.g. Olenina *et al.*, 2006). Choanoflagellates were identified by their distinct collar-like ring of microvilli; many species also possess a characteristic lorica composed of silica rods. Their average dimensions were approximately 10 μm in length and 4 μm in width.

In analysing long-term data it is important to consider possible artefacts caused by changes in taxonomy, species concepts, different analysts, as well as the improved skills of individual analysts with time. To ensure the consistency of the data set, the taxa were scrutinized case by case, and taxa which were deemed to be inconsistently counted or identified were either excluded or pooled with another taxon. Further, as our aim was to examine the sampling required for protistan grazers, we excluded the ciliates and dinoflagellates which potentially do not follow typical grazer-prey dynamics (D'Alelio *et al.*, 2016). Hence, we excluded all chloroplast-bearing dinoflagellates, leaving only the obligate heterotrophs. Of the ciliates, we eliminated the mixotrophic species *Mesodinium rubrum* (obligate phototroph; Moeller *et al.* 2011) and the oligotrichs *Laboea strobila*, and *Tontonia ovalis* which heavily rely on their functional chloroplasts for supplying energy although this may lead to underestimates in the ciliate abundance and biomass data. We included the ciliate taxa which were identified to higher than species level, under the assumption that they have a role as grazers. The use of iodine-based Lugol's solution stains the protoplasm rendering it difficult to distinguish the presence or absence of chloroplasts in both ciliates and dinoflagellates; it is therefore possible that among the specimens not identified to a sufficiently detailed level (which varies with taxon) some chloroplast-bearing specimens may have been included. In all, a total of 55 taxa (30 ciliates, 24 heterotrophic dinoflagellates and choanoflagellates as a group) were included (Table 1).

Statistical analysis

The aim of the statistical analysis was to determine the optimal sampling frequency for reliably estimating the annual heterotrophic protist diversity, biomass and its seasonal fluctuations, compared to the original, weekly sampling, while minimizing sampling and analysis efforts. This was done by examining the effect of different sampling frequencies on the estimates of mean annual biomass and its variability, as well as on the number of observed taxa. In addition, we examined how often the annual biomass peaks of the ten most important taxa (based on mean biomass) coincided with the least frequent sampling, to see how a reduction of sampling frequency affects observation of annual succession.

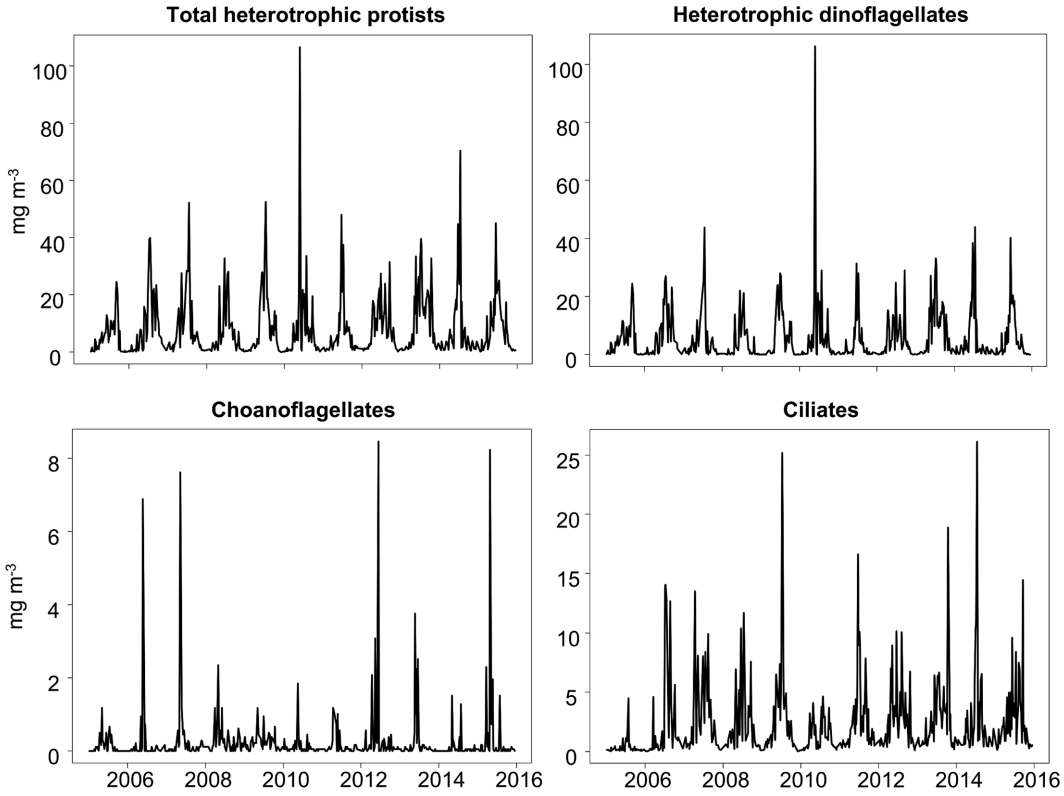

Figure 2. Biomass (mg m^{-3}) of total heterotrophic protists included in the study, heterotrophic dinoflagellates, choanoflagellates and ciliates over the study period 2005–2015 with the original weekly sampling frequency at station L4. Note different scales on Y-axis.

Four different sampling frequencies were examined: (1) once a week, (2) twice a month, (3) once a month, and (4) four times a year (in January, April, June, and August). These months were selected to represent the low wintertime (January) and higher spring (April) and summer (June, August) biomasses of heterotrophic protists. Instead of including January in the sampling period, another option to add a low biomass period to the data would be to assume no biomass during the winter season. This was tested, and the mean total annual biomass results of actual sampling versus forced zero biomass in January were almost identical. Thus, only the real sampling data are presented. The analyses were performed using the biomass and the number of observed taxa of the following four groups: total heterotrophic protists included in the study, i.e. the following three groups together: heterotrophic dinoflagellates and ciliates in the microzooplankton size class, and choanoflagellates in the nanoplankton size class. Mean total annual biomass was estimated using the area under curve (AUC), calculated based on all samples taken each year. For the sampling frequency with four annual observations, the AUC was essentially computed by "wrapping" the ends of the sampling period together, i.e. using the first observation from January to connect the end and the start of the sampling periods. Annual interquartile range (IQR) was used to describe the extent of variation in the data, because the biomasses were not normally distributed. The differences in AUC as well as in the number of observed taxa in the samples between the original data (sampling once a week) *vs*. twice a month, once a month and four times a year were statistically analysed with the Wilcoxon test.

For the analyses, the sampling frequency "twice a month" was simulated as two separate samples (observations from odd and even weeks) from the original dataset with the approximately weekly sampling interval. Similarly, the sampling frequency "once a month" was simulated as four separate samples, each containing the observations from an evenly distributed sampling with four-week intervals, starting from week 1, 2, 3, and 4, respectively. The sampling frequency "four times a year" included observations from January (weeks 3, 4), April (weeks 15, 16, 17), June (weeks 23, 24, 25, 26), and August (weeks 32, 33, 34, 35). Within these months, observations from weeks with less than nine samples in the entire dataset were excluded. The samples were composed of all possible combinations of the selected weeks, altogether 96 different combinations, all of which were analyzed.

The results are expressed as an average of the results for the separate samples for each sampling frequency: One sample consists of data for the 11 consecutive years. Weekly averages were first calculated by averaging over all years and then over all possible samples. For example, for the bi-monthly sampling, the weeks were first averaged over all 11 years, yielding two series of weekly averages, one for each sample. The final average was calculated by averaging these two samples on the weekly level. Thus, the results are expressed as means of means. For graphical purposes, the weeks were also averaged in order to get a representative horizontal axis.

Rarefaction curves were used to study how the number of samples affects the number of observed taxa. Rarefaction is a method for estimating species richness for a certain number of analysed samples. We used the bootstrapping method (from R package vegan; Oksanen *et al.* 2019) to estimate yearly rarefaction curves. Central tendencies of variables in two different groups were compared using the Wilcoxon test.

Table 1. The heterotrophic protist taxa included in this study (heterotrophic dinoflagellates, choanoflagellates and ciliates). The species composition was determined with the accuracy permitted by inverted light microscopy (Utermöhl technique) of samples preserved with acid Lugol's solution, except choanoflagellates, which were identified to group level only. The dinoflagellate nomenclature follows Guiry and Guiry (2020) unless otherwise indicated; the choanoflagellate and ciliate nomenclature are according to WoRMS Editorial Board (2020).

Heterotrophic dinoflagellates	**Taxon**	**Ciliates**	**Taxon**
Dinophysiales	*Phalacroma rotundatum*	Heterotrichea	*Peritromus*
Amphidiniales	*Amphidinium crassum*		*Helicostomella*
	Amphidinium sphenoides		*Leegaardiella sol*
Gymnodiniales	*Cochlodinium/Nematodinium* (1) (2)		*Leegaardiella*
	Erythropsidinium		*Lohmanniella oviformis*
	Gymnodinium (2)		*Parafavella*
	Gymnodinium (small, < ca. 20 μm) (2)		*Proplectella*
	Gyrodinium spirale (3)		*Salpingella*
	Gyrodinium (large, > ca. 50 μm) (2)		*Strombidinopsis*
	Polykrikos schwartzii		*Strobilidium*
	Warnowia		*Tintinnopsis*
Tovelliales	*Katodinium* (2)		Tintinnid (small, < ca. 20 μm)
Torodiniales	*Kapelodinium vestifici* (4)		Unidentified ciliate
Noctilucales	*Kofoidinium lebourae*		Unidentified tintinnid
	Pronoctiluca pelagica	Oligotrichea -Oligotrichida	*Strombidium* (large, > ca. 50 μm)
Peridiniales	*Diplopsalis* group (5)		*Strombidium* (medium, ca. 20–50 μm)
	Protoperidinium bipes		*Strombidium* (small, < ca. 20 μm)
	Protoperidinium brevipes		*Tontonia*
	Protoperidinium curtipes	Prostomatea	*Balanion*
	Protoperidinium depressum		*Tiarina*
	Protoperidinium divergens		Prorodontida
	Protoperidinium pyriforme	Oligohymenophorea	*Uronema*
	Protoperidinium steinii		*Vorticella/Carchesium*
	Protoperidinium (6)	Litostomatea—Haptorida	*Didinium*
Other heterotrophic flagellates	Choanoflagellatea (7)	Litostomatea—Cyclotrichida	*Askenasia stellaris*
			Rhabdoaskenasia

(1) In Lugol's preserved samples, distinguishing of the genera *Cochlodinium* and *Nematodinium* is not always unambiguous, hence these taxa were considered together.
(2) Within monitoring, it is practical to classify the gymnodinioid dinoflagellates that cannot be identified to species level according to the traditional genus concept based on the relative position and displacement of the cingulum (for traditional genus delimitations see e.g. Hoppenrath *et al.* 2009). Members of the dinoflagellate genera *Cochlodinium*, *Gymnodinium*, *Gyrodinium*, *Katodinium* and *Nematodinium* include both chloroplast-bearing and heterotrophic species. Iodine-based Lugol's solution stains the protoplasm, rendering it difficult to distinguish the presence or absence of chloroplasts; hence it is possible that some chloroplast-bearing specimens have been included among these heterotrophic dinoflagellates.
(3) *Gyrodinium spirale* and *G. britannicum* Kofoid & Swezy are very similar species, differentiated only by the number of striae on the cell surface (Hoppenrath *et al.*, 2009); a feature which is not always easily distinguished in Lugol's preserved samples. While refraining from taking a stand on whether variation in striation is a valid species-specific characteristic [cf. *Sclerodinium* (*Gyrodinium*) *calyptoglyphe* and *S. striatum*; Hoppenrath *et al.* (2009)]; specimens of the *G. britannicum*-type may have been identified as *G. spirale* in the present study.
(4) Nomenclature according to Boutrup *et al.* (2016).
(5) *Diplopsalis* group *sensu* Hoppenrath *et al.* (2009).
(6) *Protoperidinium* spp. comprises unidentified *Protoperidinium* specimens as well as the possibly inconsistently determined species *P. obtusum*, *P. oceanicum* and *P. ovatum*.

Analyses were conducted using R 3.6.0 and 4.0.2 (R Core Team, 2019–2020; RStudio Team, 2018–2020).

Results

In total, the dataset included 55 taxa, and 52 of these occurred at least once using the least frequent four times a year sampling interval during the 11-year study period. However, the mean annual number of observed heterotrophic protist taxa decreased markedly when sampling frequency was reduced (Table 2, Figure 3).

The annual number of observed taxa in the total heterotrophic protists group decreased from a mean of 43 taxa observed in the weekly sampling to a mean of 29 taxa observed when sampling four times per year (Table 2). Thus, the number of observed taxa per year decreased by 33% compared to the original sampling frequency, and the decrease was statistically significant (Wilcoxon test: $p < 0.001$). The decrease in annual number of observed taxa was significant also compared to once a month sampling (14%; Wilcoxon test: $p < 0.001$) and to sampling frequency of twice a month (7%; Wilcoxon test: $p = 0.049$). Similarly, the mean number of observed heterotrophic dinoflagellate taxa decreased significantly from 21 to 15 (29% decrease, Wilcoxon test: $p < 0.001$) and the mean number of observed ciliate taxa from 21 to 13 (38% decrease, Wilcoxon test: $p < 0.001$), when the sampling frequency was decreased from once a week to four times a year. The decrease in the mean number of observed heterotrophic dinoflagellate taxa and ob-

Table 2. Results of the statistical analyses. The significant differences (Wilcoxon test) are shown with ∗ compared to the original sampling frequency (once a week).

Variable	Sampling frequency	Total heterotrophic protists	Heterotrophic dinoflagellates	Choanoflagellates	Ciliates
Mean annual number of observed taxa	Once a week	43	21	1	21
	Twice a month	41 (−5%)	20 (−5%)	1	20 (−5%)
	Once a month	38 (−11%)∗	19 (−10%)∗	1	17 (−19%)∗
	Four times a year	29 (−33%)∗	15 (−29%)∗	1	12 (−43%)∗
Mean total annual biomass (AUC) mg m^{-3}	Once a week	404.0	275.8	12.9	112.1
	Twice a month	406.5 (+0.6%)	278.3 (+0.9%)	11.6 (−10%)	112.2 (+0%)
	Once a month	411.3 (+2%)	276.3 (+0.1%)	11.5 (−10%)	114.7 (+2%)
	Four times a year	405.1 (+0.3%)	276.1 (+0.1%)	11.5 (−10%)	117.5 (+5%)
Mean variation of annual biomass (IQR) mg m^{-3}	Once a week	9.65	6.85	0.15	2.30
	Twice a month	9.9 (+2%)	6.9 (+0%)	0.2 (+13%)	2.5 (+10%)
	Once a month	8.7 (−10%)	6.4 (−6.8%)	0.2 (+7%)	2.2 (−6%)
	Four times a year	9.5 (−1%)	6.9 (+1%)	0.3 (+80%)	2.3 (−1%)

Values in parentheses indicate the percentage change compared to the original sampling frequency. AUC refers to the estimated biomass using the area under the curve; annual IQR shows the variability in the data. Choanoflagellates were identified to group level only.

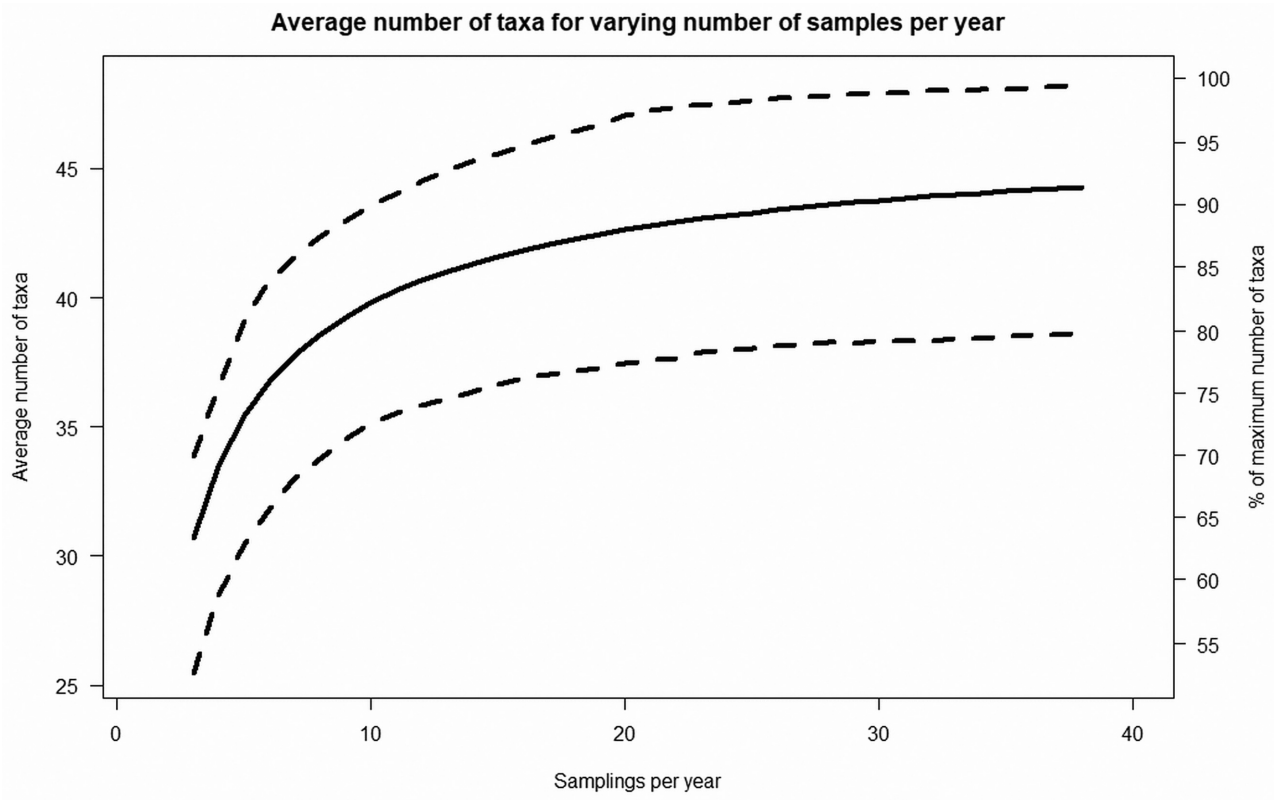

Figure 3. The average number of taxa observed *vs.* the number of samples taken per year. The average of 11 yearly rarefaction estimates (solid line). The dashed lines represent the minimum and maximum values of the yearly rarefaction estimates. The graph is truncated at 38 weeks, because this is the minimum number of samples taken in any one year.

served ciliate taxa was significant also compared to once a month sampling (10 and 19%, respectively; Wilcoxon test for both groups: $p < 0.001$). All choanoflagellates were counted as one group, and thus the effect of sampling frequency on the number of observed taxa in that group could not be estimated.

The timing of the biomass peaks varied between years and for the 10 dominant taxa peaks spread over 6 to 11 weeks, depending on taxon, during the study period (Figure 4). The least frequent monitoring scheme, i.e. four times a year, easily missed biomass peaks of the 10 most important taxa (Figure 4). By taking samples four times a year, the biomass peaks of these 10 taxa were captured 3 to 6 times during the studied 11 years, depending on the taxon. Of the months included in the four times a year sampling scheme (January, April, June, and August), the best month to sample was June. This resulted in observing some of the biomass peaks of 9 out of the 10 dominant taxa. Similarly, when the annual succession of individual groups is

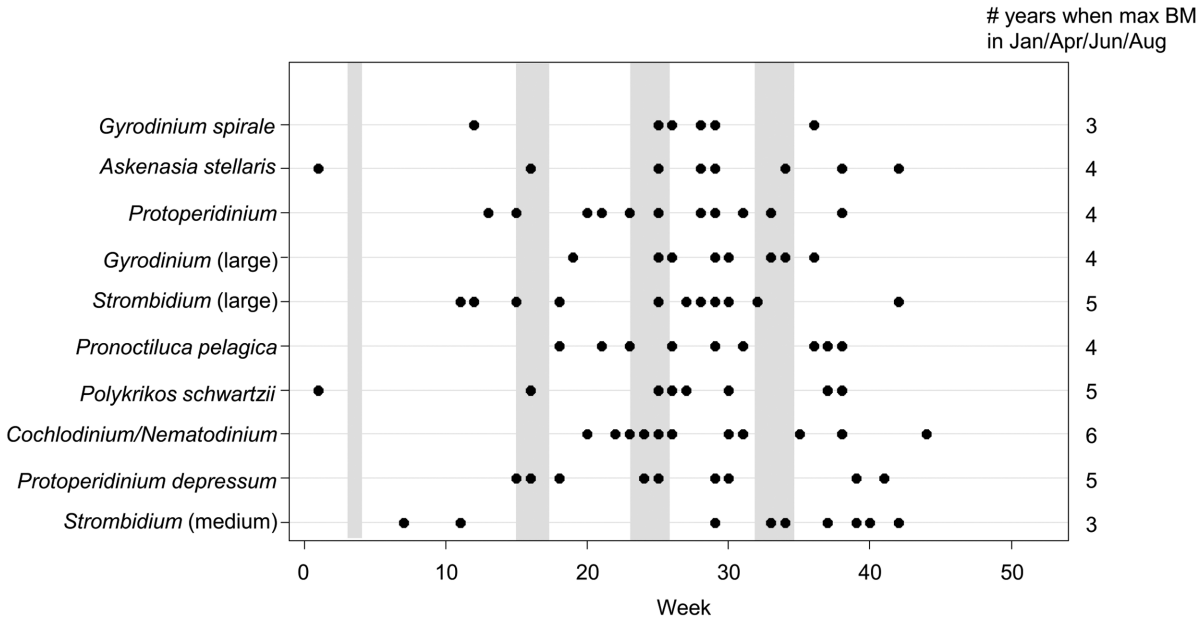

Figure 4. Timing of annual biomass maxima of the 10 most important taxa (based on mean biomass 2005–2015) over the 11-year study period at station L4, and number of years when the annual maximum biomass occurred in the simulated January, April, June or August sampling periods. One dot may denote one or several maxima. The grey areas denote the weeks analysed for the sampling frequency of four times a year.

considered, reducing sampling frequency to monthly sampling already misses most of the maxima, and results in much smoother succession (Figure 5). As an example, we examined the maximum biomass period, August 2005 and 2006. We found that dividing the total number of samplings of the previous year into two consecutive years did not change the biomass estimate significantly. Considering the number of observed taxa, as already noted in rarefactions, the number of samples per year should be over 8–10 to observe most of the taxa (Figure 3).

The mean total annual biomass (AUC) of the four investigated groups, i.e. total heterotrophic protists, heterotrophic dinoflagellates, choanoflagellates or ciliates, was not affected significantly when sampling frequency was decreased from once a week to four times a year (Wilcoxon test: $p > 0.05$; Table 2, Figure 5). The overall change varied between +3% (ciliates) and −11% (choanoflagellates). With a sampling frequency of once a month, the decrease was up to 17% compared to the original weekly sampling in choanoflagellates. However, the total annual biomass of choanoflagellates was very small compared to the biomass of the other groups examined. It is reasonable to assume that this is explained by their small cell size, falling actually in the (lower end of the) nanoplankton range (2–20 μm). The maximum reduction of the mean total annual biomass in all other groups was 4% for ciliates with the sampling frequency of twice a month.

There were no clear trends in the mean variation of annual biomass (IQR) with regard to sampling frequency (Table 2). Decreasing the sampling frequency from once a week to twice a month increased the IQR in all groups, the increase ranging from 5% (total heterotrophic protists) to 20% (choanoflagellates). The reduction of sampling frequency to once a month increased the IQR in all other groups (5–20%) except the total heterotrophic protists, where the IQR decreased by 2% compared to the original weekly sampling. When sampling four times a year, the IQR increased in heterotrophic dinoflagellates (1%) and choanoflagellates (80%), but it decreased in total heterotrophic protists (8%) and ciliates (1%). The great change in the IQR of choanoflagellates is again connected to the very small total annual biomasses of the group.

Discussion

The requirements for monitoring data stem from its potential use in multiple functions. Monitoring data are essential for understanding long-term changes in ecosystems, analysing cause and effect relationships for finding indicators of change in the environmental status (Hays *et al.*, 2005), and in constructing food webs and energy flow models. The applicability of monitoring data is based on reliable estimates of community composition and population dynamics of indicative taxa. Monitoring in the marine environment faces a number of challenges depending on the monitored system and target organisms. Continuous monitoring however allows for timely reactions when changes occur. Detecting early-warning signs allows more time to find less costly measures compared to reacting later to avoid large ecosystem changes (Hutchings and Myers, 1994). Automated monitoring methods may help in many cases, and earth observations are rapidly developing, but in the case of microscopic plankton communities, the bottlenecks are in the labour-intensive sampling and in particular in species identification and enumeration (Sieracki *et al.*, 1998). Due to the great functional diversity and ecological importance of protists in food webs (Hansen 1991; Kivi, 1996; Johansson *et al.*, 2004; Fileman *et al.*, 2011), information on their seasonal succession and long-term community changes along with other monitored communities, such as phytoplankton and mesozooplankton, is needed.

Sampling frequency and timing

Our results show that sampling frequency has marked effects on both the number of observed taxa and the annual succession observed. By decreasing the sampling frequency from weekly sam-

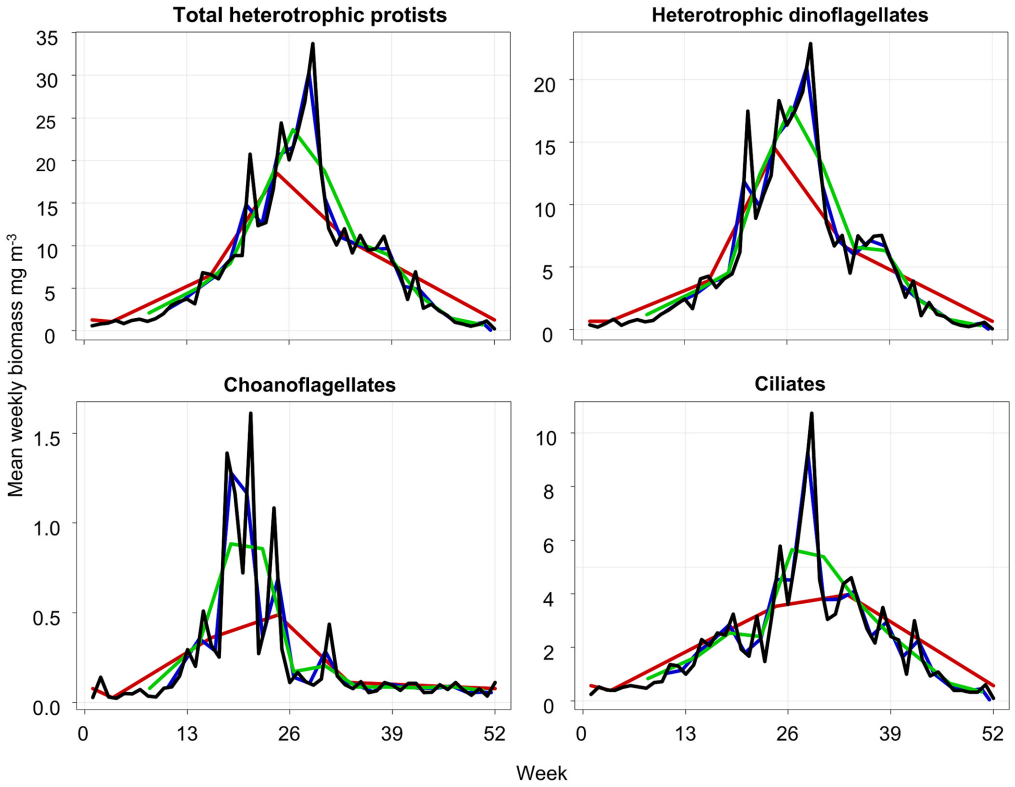

Figure 5. Mean weekly biomasses (mg m^{-3}) of total heterotrophic protists, heterotrophic dinoflagellates, choanoflagellates and ciliates over the study period using different sampling frequencies: black = once a week, blue = twice a month, green = once a month, red = four times a year.

pling to four times a year, >30% of the annual mean number of observed taxa were lost, and biomass peaks were missed. This has important implications for monitoring strategies, where compromises between available resources and the usability and accuracy of data must be considered. Even if sampling once a month affected the estimated annual mean biomass values only modestly, this sampling strategy flattened individual peaks. The linear interpolation over non-sampled periods smooths biomass peaks and thus gives slightly underestimated values. This causes problems in estimating food web interactions since biomass production (and grazing) during periods of intense growth (= peaks) is underestimated (growth between t_0 and t_{peak}), as is also the amount of carbon transfer to the next trophic level (decline between t_{peak} and t_{trough}). Furthermore, the timing of peaks may be incorrect. Simultaneously, a considerable part of individual species information is compromised and the confidence, when using the data in environmental status assessments, reduced.

Resources often limit the year-round monitoring and sampling frequency that is required to detect variability of autotrophic and heterotrophic microplankton abundance, biomass and species composition and to cover various functional groups (Porter *et al.*, 1985). Thus, there is a need to evaluate which time or times of the year to prioritize for sampling effort. In Europe, the Marine Strategy Framework Directive (MSFD) of the European Commission (European Commission, 2008) requires the use of indicators in order to determine the environmental status of marine waters (European Commission, 2016). The development and implementation of indicators for the heterotrophic microplankton community requires sufficient data of appropriate temporal frequency and timing.

The optimal monitoring of heterotrophic protists would cover the most intensive growing period but would also provide baseline samples from the period when population biomass is low. Heterotrophic protist data is needed to develop an adequate food web indicator to provide a better understanding of the present climate warming and other human-related changes on carbon flow via heterotrophic protists from primary producers to higher trophic levels. The data from station L4 in the Western English Channel demonstrated that to track the population fluctuations properly at least twice a month sampling during the main growth season is needed, keeping in mind, however, that flexibility may be needed due to possible changes in phenology caused by the ongoing climate change (cf. Horn *et al.*, 2016). This sampling frequency would enable the detection of rapid changes in the community and biomass. Additional sampling during winter, e.g. in January, is necessary to assess the population minimum biomass for ecosystem models. However, even once a month sampling for heterotrophic protists would be a welcome addition to present plankton monitoring in areas where it is totally lacking; while peaks are easily missed, the overall course of the biomass and biodiversity dynamics are relatively well captured. In addition, continuing regular monitoring for decades (even if the annual sampling frequency is suboptimal) will eventually produce data for long-term community change analyses, as long as the sampling is timely. Hence, if the sampling frequency were to be less frequent, e.g. four times a year, it would be important to know the beginning and end dates of biomass growth from seasonal studies. Furthermore, if the frequency of sampling cannot be increased, seasonal adjustments would decrease the variation in the mean value of annual monitoring data and probably most effectively

reduce bias and increase precision of the assessments (Carstensen 2007).

Heterotrophic microplankton has been included in quantitative food web models as a separate trophic compartment. However, as functional diversity can be high (Weisse, 2017), complex planktonic food web models including up to tens of functional nodes (representing auto-, mixo- and heterotrophs) have been developed to integrate the trophic diversity present in plankton. The model developed by D'Alelio *et al.* (2016) showed that the responses of metazoan and protozoan consumers can lead to widely differing trophic transfer efficiencies. Their model consisted of 22 hetero- and mixotrophic microplankton groups. Generating even simple food web models including heterotrophic microplankton would require information on their community structure and abundance in relation to their generation time, which can be a day. This means that a biomass maximum can be reached rapidly as the population can increase several fold in a week in optimal growing conditions. This clearly sets pressure on the sampling frequency of the monitoring programme.

It has been shown for mesozooplankton that temporal variability in communities is larger than spatial variability (Klais *et al.*, 2016), meaning that limited resources should be focused on sampling frequently at fewer stations, rather than infrequently at a higher number of stations. The same applies to the heterotrophic protist community, where fluctuations are even more rapid (e.g. Smetacek, 1981). The temporal sampling frequency may vary greatly from region to region. This leads to temporal gaps in certain sea areas and strongly influences the overall results (e.g. annual mean biomass or the observed number of taxa). To compensate for the limited number of research vessel-based samples, utilizing existing automatic flow-through sampling technology, so called ferrybox platforms onboard commercial vessels (aptly named ships-of-opportunity, or SOOPs), would increase the spatiotemporal coverage of samples and data in the open sea areas markedly e.g. in sea areas like the Baltic Sea (Rantajärvi and Leppänen, 1994). Such high-frequency data have proved to be valuable in registering fast dynamics of phytoplankton in spring (Lips *et al.*, 2014) and summer (Kanoshina *et al.*, 2003), in investigating the spatial and temporal distribution of specific taxa as well as long-term changes (Hällfors, 2013) and factors affecting the phytoplankton community (Forsblom *et al.*, 2019). Since the use of ferrybox sampling has successfully reported the dynamics of the fragile mixotrophic ciliate *Mesodinium rubrum* (Lips and Lips, 2017), the use of this sampling technique for the whole heterotrophic protist community would be a valuable option in supporting the sampling performed onboard research vessels during dedicated monitoring cruises.

Other aspects to consider and future perspectives

Routine monitoring should produce data to assess the state of the ecosystem and to track changes in different regional scales (e.g. European Commission 2008). In addition to sampling frequency and timing, it is important to also address the choice of sampling and analytical methods in order to produce reliable data. This is underlined by the fact that one of the reasons why the abundance and therefore also the importance of heterotrophic protists in planktonic food webs was long underestimated, was that they were targeted with unsuitable methods for sample collection and preservation. Aloricate ciliates, in particular, are fragile and easily destroyed during sampling and can pass through or be destroyed by nets (Pierce and Turner, 1992). In addition, ciliates also easily shrink and "explode" with commonly used fixatives (Choi and Stoecker, 1989). Another challenge related to the analysis of preserved samples and community composition is that all preservatives have some deleterious effects, distorting or masking the features used for species identification. When investigating the protist community, it comes down to a choice between a detailed study of the species composition in a few samples using comparatively laborious or expensive specialized methods (such as the light microscopical analysis of live samples, or electron microscopy), or alternatively, studying the occurrence of fewer taxa using the less advanced methods applied within monitoring, but thereby achieving a higher spatial and temporal resolution. In the current study, the methods used facilitated identification to species level of only part of the community, the remaining specimens were identified to genus or higher level. Hence, the number of observed taxa should be taken as exactly that; it gives an idea of, but should not be interpreted as a definite expression of community diversity, since the higher than species level taxa in reality each comprise an unknown (and not necessarily constant) number of species.

Ideally, the sampling depths and sampling methods used in heterotrophic protist monitoring would be similar to the ones used in phytoplankton monitoring in order to reduce workload and save resources utilized for sampling. The samples for the present study were collected using a Niskin bottle at 10 m depth. Various sampling techniques have been employed for heterotrophic protist or ciliate sampling for research purposes for example in the Baltic Sea (e.g. tube samplers of various design and volume, Setälä, 2004; automatic flow-through sampling systems, Lips and Lips, 2017; plastic hoses, Johansson *et al.*, 2004), indicating that any sampler appropriate for quantitative phytoplankton sampling seems suited for the purpose of protistan heterotrophic microplankton monitoring providing their fragile nature is taken into consideration.

As has been repeatedly stated, the balancing act between limited resources and acquiring high-quality data, is an ever-present issue in marine monitoring. In addition to time- or resource-saving developments in sampling procedures such as ferrybox, there are some promising developments in the suite of analysis methods which potentially could supplement traditional sample analysis. Microscopy, an inherent part of all plankton monitoring, is very labour-intensive, and only researchers with adequate training are able to perform reliable community composition analyses. Thus, the counting procedure is a bottleneck in present plankton monitoring programmes. Semi-automatic techniques have been developed, such as particle counting (e.g. flow-cytometry; Sieracki *et al.*, 1998) and imaging-based techniques (Benfield *et al.*, 2007; Uusitalo *et al.*, 2016) and with their further improvement they are expected to complement the conventional traditional microscopical sample analysis currently routinely used in plankton monitoring. Such methods have already been used to study the coupled dynamics of ciliates and phytoplankton (Haraguchi *et al.*, 2018). When these procedures are connected with deep learning tools such as convolutional neural networks the classification accuracy of plankton organisms and handling of huge image databases may immensely increase the potential temporal resolution of plankton sampling and analysis (e.g. Kerr *et al.*, 2020). These methods analyse samples and produce data at a fraction of the time compared to microscopical analyses and could increase the temporal frequency of data. However, using conventional monitoring methods, i.e. light microscopy provides better taxonomical precision for nanoplankton and microplankton compared to for example, flow-cytometry (Haraguchi *et al.*, 2017), and imaging-based techniques require an investment

of time to build training sets of images of focal biota to be used by the software in taxonomic identification before they can be routinely taken into use. In due time, we expect the novel methods to contribute valuably to conventional microscopy-based plankton monitoring, and thus enable more extensive temporal monitoring data collection.

One of the most intensive regional sea monitoring programmes is conducted in the Baltic Sea, however, there is still room for improvement. Within the phytoplankton monitoring programme in the Baltic Sea (HELCOM, 2017), the mixotrophic ciliate *M. rubrum* as well as heterotrophic nano- and microsized flagellates and heterotrophic dinoflagellates are counted and included in the abundance and biomass data of phytoplankton, even though the heterotrophic fraction is subsequently excluded from those phytoplankton data analyses in which the focus is on primary producers. However, even though e.g. heterotrophic dinoflagellates are counted from the phytoplankton samples, the counted numbers for especially large-sized heterotrophs are usually very low due to the counting method. Counting is stopped after a certain number of phytoplankton cells is reached, which are usually more numerous than the large-sized heterotrophs. Thus, the data on heterotrophic protists is compromised in the counting stage. To get representative biodiversity, abundance and biomass data on all heterotrophic protists, it would be valuable to include them in the heterotrophic protist monitoring with adequate methods focusing specifically on the heterotrophs.

Conclusions

We tested different sampling frequencies on a unique dataset collected on a weekly basis from a marine pelagic system. Infrequent sampling of four times a year and also once a month, despite giving a reliable estimate on total annual protist biomass, failed to give accurate information on species or biomass succession. However, a simulated twice a month sampling compared well with the original weekly sampling, but did not match it: while the mean total annual biomass was not greatly affected by sampling only every other week, the mean annual number of observed taxa decreased by 7%. For the purposes of heterotrophic succession based parametrizations, such as for assessing biomass ratios in the food web or carbon flow to higher trophic levels, sampling only four times per year may give a proper estimate. However, a more frequent sampling scheme is required to assess the changes in seasonal succession of community composition, in biodiversity and in the role of individual taxa in the food web. This is also true if the goal is to reveal trends in comparison to abiotic or biotic changes. An important question concerning microscopical analysis is its usability in predicting the true number of taxa in each sample, e.g. for comparison of species number based indices. Rarefaction can be used to evaluate if microscopical enumeration produces an accurate taxon number, but we did not include rarefaction in our analysis. Thus we cannot answer this specific question. However, we found that the temporarily intense sampling program is superior to a low-frequency sampling in revealing the species ensemble of a location. This is of importance if the aim is to observe year-to-year changes in the species composition. Based on the experience of our test case, we recommend that if weekly sampling is not possible due to limited resources, heterotrophic protists should be sampled at least twice a month, or with a hybrid sampling programme with infrequent sampling during low biomass periods and more frequent sampling during the productive season, in seasonal marine areas like the Baltic Sea. However, we should bear in mind that present global change promotes the lengthening of the growing season and unprecedented annual successions, which may compromise the saved resources if a hybrid sampling programme has been chosen. Similarly, modelling studies of spring bloom formation have shown that grazing in autumn has the potential of affecting next year's spring bloom dynamics (e.g. Behrenfeld and Boss, 2014), therefore, acquiring year-round heterotrophic protist abundance data would provide an opportunity to test this hypothesis. A frequently sampled but sparse sampling network is preferable to a large number of stations sampled infrequently. We recommend using similar sampling depths and sampling methods as are used in current phytoplankton monitoring, since the same methods can be used for heterotrophic protist monitoring, and a joint sampling would save the often limited monitoring resources.

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Data availability statement

The research data are maintained at the Plymouth Marine Laboratory and are available online at the Western Channel Observatory website.

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