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Impacts of counting protocols for light microscopy on estimates of biodiversity and algal density of phytoplankton

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Abstract

Knowledge on the biodiversity and abundance of phytoplankton is key for many ecological and societal (e.g., blue growth) questions. Gathering temporal variation and spatial patterns on key indicators requires reliable and standardized protocols on sampling, species identification and counting. Numerous methods are used but consequences for comparing the biodiversity and abundance of phytoplankton of these different techniques are not well known. We evaluated the consequences of different counting protocols using light microscopy (i.e., subsampling transects or wedges within counting chambers) for these indices using samples collected weekly to bi-weekly ($n = 398$, 2009–2018) from the Wadden Sea (southern North Sea). Phytoplankton cells were counted (by one person under similar conditions) in a fixed number of viewing fields (58, 70, and 29) at three respective magnifications (10 × 100, 10 × 40, and 10 × 10). Patterns in the spatial distribution of phytoplankton cells varied among species and clustering of cells occurred in more than one-fifth of the samples. This will induce error in the conversion from counts (per viewing field) to abundance (cells mL^{-1}). Our present effort resulted in a high accuracy (95%) in overall cell abundances. This was not the case for species richness, for example, capturing 90% of all species present in the sample would require an almost threefold increase in effort for the 10 × 40 and 10 × 10 magnifications. We recommend that counting methods be tailored to the main research objectives and that counting protocols should quantify uncertainty as well as potential bias to provide an estimation of the error in phytoplankton abundance and species composition.

Phytoplankton community compositions are highly dynamic in both time and space (Thyssen et al. 2008; Beltrán-Abaunza et al. 2017). Drivers of change in phytoplankton species composition include temperature, light, and nutrients as well as grazing pressure and sinking/resuspension dynamics. Because variation in the concentrations and species composition of phytoplankton directly impacts primary consumers (Lévy et al. 2015), establishing monitoring programs that allow one to track and understand phytoplankton dynamics is considered to be important in understanding ecosystem change (Hajnal and Padisák 2008). Long-term monitoring allows one to distinguish seasonal succession from long-term trends and to reveal how human-induced changes including eutrophication and climatic change (e.g., increased temperatures, hydrological change) impact the base of marine food webs. Monitoring also allows for the detection of invasive species and the presence of toxic species (HELCOM 2021). Essential for long-term monitoring programs is the use of reliable and standardized sampling, species identification and counting protocols (e.g., Olrik et al. 1998).

Whilst numerous methods exist for assessing phytoplankton community species composition (e.g., flow cytometry, HPLC, electronic microscopy), this study will focus on methods using light microscopy. The most common method to identify and quantify phytoplankton species composition in water samples is counting cells according to the Utermöhl

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Additional Supporting Information may be found in the online version of this article.

inverted light microscope method (Utermöhl 1958). This method involves the gravitational sedimentation of preserved phytoplankton in a counting chamber and is considered suitable for counting algal cells in low (< 10^2–10^4 cells mL^{-1}) concentrations (Sournia 1978).

Taxonomic composition is generally determined from subsamples of water samples (e.g., 250 mL from 10 liters) that are preserved and stored. To determine phytoplankton species and abundance, a second subsample (e.g., 3.5 mL) is taken from the preserved material and put into a counting chamber. Once all preserved algae of this subsample have settled to the bottom, the counting chamber is viewed using an inverted microscope, and the counting starts. Counting phytoplankton cells of different sizes is generally done at different (fixed) magnifications (Karlson et al. 2010), either using the same counting chamber for all magnifications (Andersen and Throndsen 2003; Brierley et al. 2007) or using separate chambers for different magnifications (this study).

Protocols on sampling, subsampling, settlement procedures, and the use of different counting chambers have been thoroughly reviewed (e.g., Edgar and Laird 1993; Hamilton et al. 2001). With respect to the actual counting, there are various common strategies which differ in the number of counting fields per counting chamber, in the distribution of these counting fields and in the use of single or multiple counting chambers. Different strategies are often based upon different assumptions on the distribution of the preserved cells in the counting chamber. In this study, we explored the assumptions underlying the different counting strategies and how the violation of these assumptions might result in biased outcomes of phytoplankton abundances and species composition.

To limit the time and effort of analyzing a sample, counting of small- and medium-sized species is generally done within a part (selected number of fields of view) of a counting chamber, for example, by counting small cells in a limited number of random fields of view, and counting medium-sized cells in transects within the counting chamber. For large cells, it is generally advised to count the complete volume of a counting chamber (Brierley et al. 2007). A prerequisite for reliably counting only a fraction of the volume is a random distribution of cells on the bottom of the counting chamber (Lund et al. 1958; Edgar and Laird 1993). In other words, the distribution of the cells of all algal species within the counting chamber must follow the hypothesis of complete spatial randomness (CSR). While a random distribution of cells is considered a prerequisite for a reliable estimate, not many studies have reported on the actual distribution within a counting chamber nor on the consequence for abundance estimates if the distribution is non-random (but *see* Edgar and Laird 1993; Sandgren and Robinson 1984). Despite a careful sedimentation procedure, however, a random distribution is not always achieved (Edgar and Laird 1993; Hamilton et al. 2001; HELCOM 2021). There have been, for example, reports of large cells accumulating at the edges of a counting chamber and small cells in the center (Olrik et al. 1998), while others report more cells at the edges unrelated to cell size (Sandgren and Robinson 1984). Haberyan and Haddock (2013) hypothesized that the distribution of diatom cells could reflect valve morphology and that taxa with long spines and thick walls would be prone to non-random distribution.

While Brierley et al. (2007) advised to use "alternative protocols or methods" for phytoplankton samples with a non-random distribution, others claim that this unequal distribution is taken into account by counting diagonals, grids along a diagonal, or by counting the entire bottom of a counting chamber (Olrik et al. 1998; Andersen and Throndsen 2003). Sandgren and Robinson (1984) stated that "random distribution of cells can never be consistently achieved" and that the counting of multiple transects does not solve the problem since, in circular areas, central regions are more heavily sampled compared to peripheral regions. Those studies concluded that counting cells in random views was the most reliable strategy.

Systematic phytoplankton counts of water samples taken at high tide from the Marsdiep (the westernmost tidal inlet of the Wadden Sea, adjacent to the southern North Sea) started in 1965 (Cadée and Hegeman 1991). Historically, the density of phytoplankton species was derived from algal counts in viewing fields that were lined along transects in the counting chamber, the so-called "transect strategy." In 2008, part of the samples were also analyzed using the so-called "proportional strategy" where the viewing fields are positioned in a wedge of the counting chamber (*see* Supporting Information S1). Using these two methods on the same samples resulted in different plankton concentrations (cells mL^{-1}). One potential reason for the difference was thought to be a non-random distribution of algal cells in the counting chambers. However, the two methods also differ in the respect that the counts conducted with the method developed at the Royal Netherlands Institute for Sea Research (NIOZ) (*see* Materials and procedures and Supporting Information S1) were performed without an a priori fixed magnification and used a different threshold to terminate the counts, which could also have contributed to the observed differences in cell abundances.

To explore the impact of counting strategy, we developed a new counting protocol in 2009 which would enable us to determine underlying assumptions in the "transect strategy" with respect to random distribution and threshold values, as well as the consequences for plankton abundances and richness if these assumptions were violated. In this study, analyses were performed on almost 400 water samples from one location in the Dutch Wadden Sea sampled between 2009 and 2018. The aim was to investigate the distribution of cells in a counting chamber in detail and to report on the consequences of the distribution on the estimates of total cell counts. In addition, a method is presented to ascertain the reliability

of the counts, both when the aim is to estimate cell concentrations and species richness. This paper describes the outcome of these tests in particular and the consequences of these findings for plankton counts in general.

Materials and procedures

Counting protocol

For this study, 398 water samples were collected at one location (NIOZ Jetty, 53°00′06.5″N; 4°47′20.5″E) between January 2009 and December 2018 at local high tide, with an average effort of 40 samples per year. After the water samples were collected using a bucket (10 liters), a subsample of 600 mL was taken, homogenized and 100 mL of this subsample was fixed in 300 μL alkaline Lugol solution (3‰ final concentration). Samples were stored in the dark at 4°C until further analysis. After careful homogenization and acclimatization of the sample in the storage bottle, a fixed volume of 3.25 mL was pipetted into each of the four sedimentation chambers to be used for counting at the different magnifications (*see* Supporting Information S1). All counting and preparation have been processed by the same person under similar conditions (e.g., microscope, laboratory) in order to limit the human effect.

To compare the consequences for outcomes on phytoplankton abundances of different counting methods, counts were done in different numbers and distribution of viewing fields per chamber (using an inverted light microscope equipped with a ×10 magnifying eyepiece lens, and with ×100, ×40, and ×10 magnifying scanning objective lenses):

- Counting chamber 1: 58 fields at a 10 × 100 magnification
- Counting chamber 2: 70 fields at 10 × 40 magnification
- Counting chamber 3: 29 fields at 10 × 10 magnification
- Counting chamber 4: counted completely at 10 × 10 magnification, but results will not be included in the present analysis as these counts were not recorded per field of view.

The sedimentation of subsamples took place on an even and horizontal surface, in absence of vibrations, under constant (room) temperature and under dim light conditions. A fixed list of species was used, and for each species, it was indicated up to which magnification the species was still recognizable, and thus counted. The counting strategy in short consisted of first counting species in chamber 1 (at ×100), counts in the second chamber are continued for species larger than 6 μm and when the species count was less than 15 individuals. Counts in chamber 3 continued for species larger than 10 μm and when species count in chambers 1 and 2 was (still) smaller than 15 individuals. For each species within each viewing field, a distinction was made between "observations" (if a species was present or not) and "counts" (the number of cells within one viewing field). In the present analysis, only the actual counts (numbers of cells per species per viewing field) were used.

Overall spatial distribution of plankton cells

To test whether the distribution of cells in a counting chamber satisfied the hypothesis of complete spatial randomness (CSR), a modified "quadrat method" (Bijkerk and Beers 2010; Smith 2014) was used. Under the CSR hypothesis, it was expected that the counts in the fields follow a homogeneous spatial Poisson distribution. For each counting chamber, a number of fields (58, 70, and 29 fields at 10 × 100, 10 × 40, and 10 × 10 magnification, respectively) were randomly selected from the data records, and the sum of the cell counts of all species within each of the selected fields was determined. Hereafter, the mean number of counts, the variance and the variance/mean (index of dispersion) of those chosen fields were determined. The process of selection of the fields and calculation was then repeated using a bootstrap resampling method ($n = 1000$).

Under the Poisson distribution, the variance of the population equals the mean (so the variance/mean ratio = 1). A ratio < 0.8 indicates a dispersed distribution (regular or under-dispersed), a ratio > 1.2 points to a clustered distribution (contagious or over-dispersed). Samples with a ratio between 0.8 and 1.2 were considered to have a random distribution of cells, so not causing an error in the estimates of plankton abundances if these are based upon phytoplankton counts for only a part of the counting chamber (Edgar and Laird 1993; Bijkerk and Beers 2010) (Fig. 1).

Radial gradient within the counting chamber

To explore whether or not species were evenly distributed from the center to the edge of the counting chambers, the counts (number of cells per viewing field) in center and edge fields were compared at different magnifications (*see* Supporting Information S1 for the specific selection of edge and center fields). If there is a gradient in counts from the center to the edge, the strength of this gradient might vary with phytoplankton cell size. Therefore, this test was performed for large and small species, where large species were defined as those that were counted at the lowest (10 × 10) magnification, and small species (< 6 μm) as those that were only counted at 10 × 100 magnification (*see* Supporting Information S1).

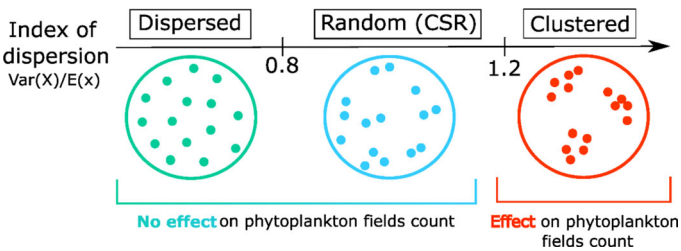

Fig. 1. Value of index of dispersion (I = variation/mean) of algal cells in a counting chamber when the cells are dispersed ($I < 0.8$), completely spatially random ($0.8 \leq I = \leq 1.2$), or clustered ($I > 1.2$). If cells are clustered, then counting for a selection of viewing fields instead of the full chamber may result in error for the estimation of cell abundances.

In practice, for each species or species group of interest, the abundance of cell per field was compared for edge and center fields using a χ^2 test ($p < 0.05$).

Within each of the groups of small and large species (*see* Supporting Information S2), the 10 most dominant species were selected for this analysis (with the minimum number of 5 cells as a prerequisite). The selection of common species (or taxonomic or functional group of species) included *Phaeocystis* species, an abundant and nuisance plankton group that occurs in two life forms (colony and flagellate cells), both of which were considered for this study.

To investigate whether other characteristics than size (silicate skeleton, flagella, chain formation) influenced the distribution of cells during settlement in the counting chambers, some additional less common species were also considered. For example, the group of *Chaetoceros* species was included because of their long spines and chain formation, potentially influencing their settling behavior (Bienfang 1981; Laurenceau-Cornec et al. 2015). In addition, freshwater species (green algae and cyanobacteria separately) were included to check for a possible difference in settling "behavior" compared to marine phytoplankton species.

Since all species, except for the smallest ones (< 6 μm), are counted at multiple magnifications, all analyses for this test have generally been performed at the magnification where the species (group) was most abundant. Only for freshwater species, that were equally abundant in the 10 × 100 and 10 × 40 magnifications, the distribution was investigated for both magnifications separately (*see* Supporting Information S1).

In addition, potential gradients were explored in more detail using the distance between all fields and the center of the counting chamber. Therefore, at each magnification, an x–y-coordinate was calculated for the fields of view, with x indicating the distance (in mm) from the center horizontally and y the distance from the center vertically. Then multiple sorts of regression were applied to identify potential trends: linear, order 2 (quadratic), order 3 (cubic) polynomial and generalized additive model (GAM). The GAMs were fitted using thin-plate splines and the Poisson distribution (adapted to counts) using the "mgcv" package (Wood 2011). For all models, the significance (p value) and R^2 were computed and, in addition, deviance (%) for the GAMs. The Akaike Information Criterion (AIC) was computed to identify which model (within each phytoplankton group) fitted the data best taking the different number of model parameters into account.

Consequences of counting strategies

Two counting methods were compared regarding the estimate of total cell numbers in a counting chamber. The first method was called the "transect" method, here the counts in the cells in the fields of view along a transect were summed (Fig. 2). This method was compared to the "proportional" method, where cells were counted in a pie-part (Fig. 2), so that

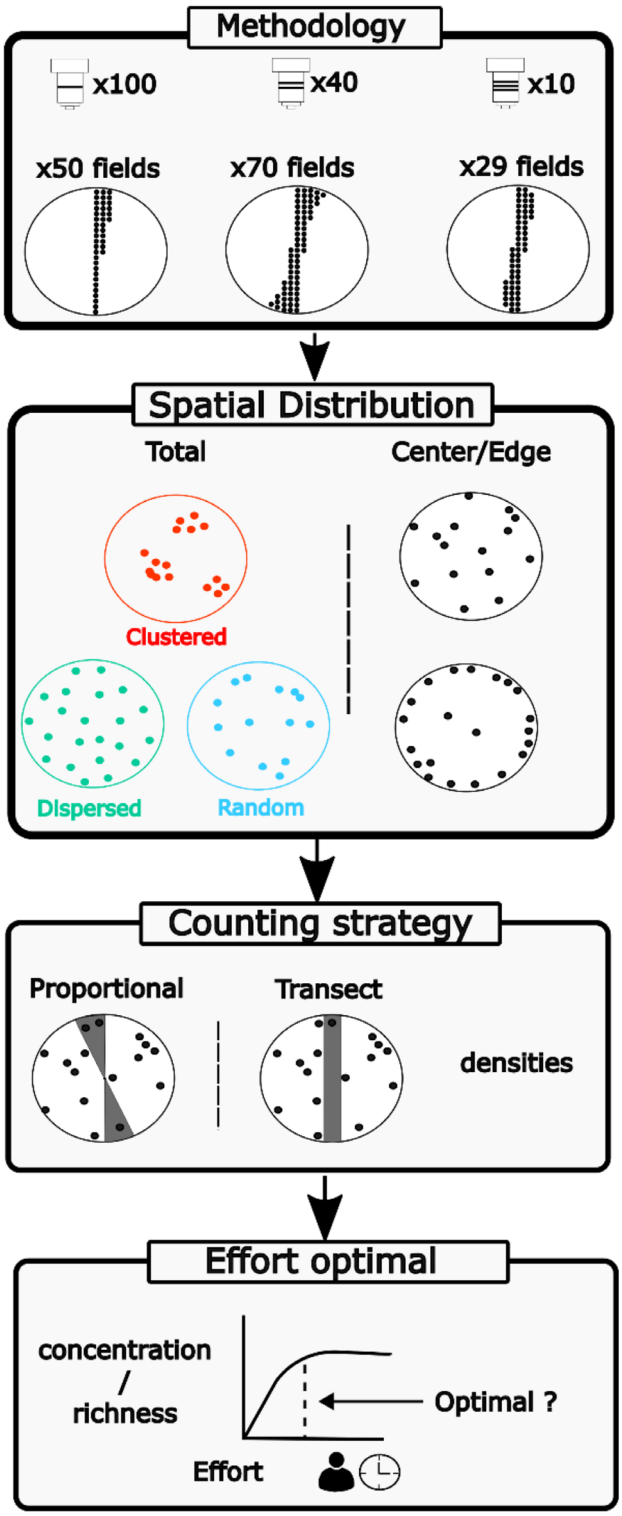

Fig. 2. Graphic summary of the procedure used to evaluate potential bias and variance resulting from different spatial distributions of cells within a counting chamber viewed using two counting strategies (proportional vs. transect) at different magnifications (10 × 100, 10 × 40, and 10 × 10) and from calculating the relationship between effort (number of fields viewed) and the abundance or species richness of each sample.

the number of fields counted at the center or edge of a chamber is proportional to the area this region makes up in a counting chamber (*see* Supporting Information S1).

To compare both methods for the same number of fields of view, fields of view were randomly chosen from the pie-part, in such a way that the number of fields per region (center or edge) was proportional to the total fraction of the region in the total chamber. To compare the estimate of total cell numbers between the two counting methods, the differences between the estimates were tested using a paired *t*-test. The normality of the differences was inspected visually on a histogram of the differences (data not shown).

Optimal number of fields

Phytoplankton abundances

To explore the effect of the number of fields counted on the estimate of phytoplankton abundance (number of cells mL^{-1}), the error in relation to the number of fields of view counted was calculated. This was done by estimating the count based on one field of view and comparing this value with the actual count (sum of algal cells in all fields of view at

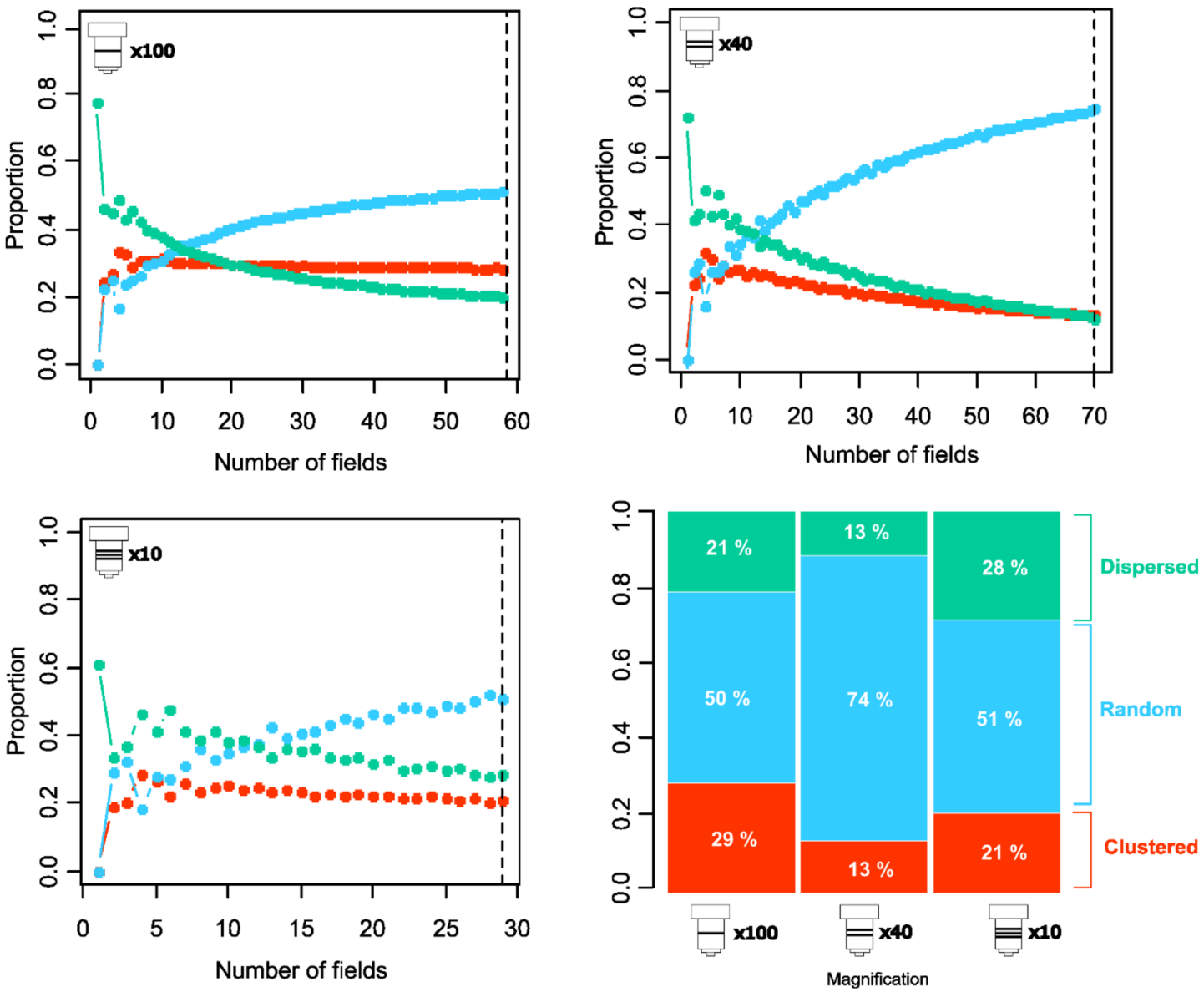

Fig. 3. Proportion of samples (%) at which algal cells displayed a clustered (red), random (blue) and dispersed (green) spatial distribution using different magnifying scanning objective lenses: ×100 (top-left), ×40 (top-right), and ×10 (bottom-left) depending on the number of fields counted. The dashed line display the applied number of fields used for this study (50 for ×100, 70 for ×40, 20 for ×10). The barplot (bottom-right) displays the same proportion for the number of fields used for the analysis of our study. $N = 396$ for ×100, and $N = 397$ for ×40 and ×10 magnifying scanning objective lenses.

a specific magnification). Then a count was estimated based on two fields of view et cetera until the maximum number of fields of view (f_{max}) was reached. The selected fields of view (from 1 to $f_{max} - 1$) were randomly drawn.

The error (%) was calculated according to Eq. 1:

$$\text{Error} = |\text{count}_{\text{true}} - \text{count}_{\text{estimated}}|/\text{count}_{\text{true}} \times 100 \quad (1)$$

This error is assessed by repeating the drawing procedure through a bootstrap with replacement, allowing the calculation of the mean error of the bootstrap and the mean squared error estimating the total number of observations in all fields of view, and comparing this with the actual observation done in all fields of view using a bootstrap routine ($n = 1000$). This was also done for the 10 × 40 and 10 × 10 magnifications for 1–70 and 1–29 fields of view, respectively. Based upon these curves, the critical value of effort (e.g., the number of fields to count) was set at a 5% error in the estimates of phytoplankton abundances.

Phytoplankton richness

To estimate the completeness of species diversity (no. species mL^{-1}) in relation to the number of fields of view counted, the following procedure was applied for each magnification. Using presence-absence (per field of view, per sample and per species). The number of expected species per sample was calculated using the function "assempar" from the R library "SSP" (Guerra-Castro et al. 2020; R Core Team 2020). The function used four Jackknife-like functions to estimate the expected species richness. For this, the original data set (observations rather than presence-absence) was used. If observed/expected richness was < 90% it was calculated what the extra effort should have been to come to 90% completeness for each sample. Then a species accumulation curve (number of species present depending on fields of view) was constructed per sample and per magnification using the function "specaccum" from the R library "vegan" (Oksanen et al. 2020) and then fitting a "Clench" function using non-linear regression (Moreno and Halffter 2000; Mourthé 2013) using Eq. 2:

$$S_f = af/(1 + bf) \quad (2)$$

where f is the effort (number of fields of view counted), S_f is the species richness (number of species present) at effort f, a is the rate of increase at the start of counting (species/effort) and

Table 1. Results of the comparison of the number of cells in viewing fields at the edge and at the center of a counting chamber, where *N* indicates the number of samples in which the species was present, and *n* the total number of cells in the respective viewing fields in the edge and center of the counting chambers of those *N* samples. Significant *p* values (< 0.05) of the outcome of the χ^2 test are displayed in bold, the significance threshold is not corrected for multiple comparisons. For species (or species groups) with significant differences in numbers between edge and central viewing fields, the highest value is underlined. Phytoplankton samples ($n = 398$) were taken at high tide from the NIOZ jetty in the Marsdiep, the westernmost tidal inlet of the Wadden Sea, between January 2009 and December 2018.

Small species (< 6 μm)	*N*	n_{edge}	n_{center}	χ^2	*p* Value
Small flagellates (unidentified, colored)	358	751	534	36.65	**<0.0001**
Small flagellates (unidentified, colorless)	326	474	493	0.37	0.54
Chlorococcales (solitary)	194	262	288	1.32	0.27
Hemiselmis group	189	232	212	0.90	0.34
Chroococcaceae	89	106	162	11.70	**<0.001**
Prasinophyceae Pseudocourfieldia group	93	145	81	18.12	**<0.0001**
Cyanophyta (unidentified)	73	59	67	0.51	0.48
Thalassiosiraceae (unidentified)	64	72	47	5.25	**0.022**
Prymnesiales	16	12	13	0.04	0.84

Large species (> 6 μm)	*N*	n_{edge}	n_{center}	χ^2	*p* Value
Mediopyxis helysia	118	214	296	13.18	**<0.001**
Rhizosolenia imbricata group	105	218	207	0.28	0.59
Gymnodiniaceae (10–30 μm)	166	176	203	1.92	0.17
Guinardia delicatula	112	166	153	0.53	0.47
Odontella aurita	85	144	157	0.56	0.45
Thalassiosiraceae (10–30 μm)	111	105	107	0.02	0.89
Bacillariales (10–30 μm)	107	80	85	0.15	0.70
Pseudonitzschia fraudulenta group	60	73	91	1.98	0.16
Ditylum brightwellii	40	77	69	0.44	0.51
Chaetoceros spp. (chains)	16	34	15	7.37	**<0.01**

b relates to the shape of the curve with species being added (1/effort). Parameters a and b are derived from the regression.

The asymptote of the species accumulation curve is a/b (species). The effort (number of fields of view) needed to count a pre-set fraction (f_q) of the expected number of species (q) can then be calculated according to Eq. 3 (Soberón and Llorente 1993):

$$f_q = q/[b(1-q)] \quad (3)$$

For our analyses, this fraction was set on 0.9, implying that we calculated how many fields of view are needed to include 90% of all species present in the total sample.

All analyses were performed in R (R Core Team 2020).

Assessment

Overall spatial distribution of plankton cells

For all three magnifications, the spatial distribution of plankton cells was clustered for more than one-fifth of the samples (Fig. 3). The highest proportion for the number of fields selected for this study of samples displaying a clustered distribution (29%, 114 of the 396 samples) was found for the 10 × 100 magnification, the percentage of clustered samples counted at other magnifications was 14% (55 of the 397 samples) at 10 × 40 and 21% (85 of the 397 samples) at 10 × 10 magnification.

Gradients within the counting chamber

Four out of nine small and 2 out of 10 large species (or species groups) displayed significant differences in the number of cells in the center and at the edge of a counting chamber (Table 1). There was no generic pattern of cells accumulation in the center nor edge, however, as Thalassiosiraceae and *Chaetoceros* were observed more in the edge field of view while numbers of *Mediopyxis helysia* and Chroococcaceae were relatively high in the central fields of view.

Based upon the AIC, a linear model gave the best fit for the relationship between counts (number of algal cells per viewing field) of all plankton cells in viewing fields and distance from the center (Fig. 4A). The fit of this model (showing a slight increase in counts from the center to the edge of the counting chamber) was, however, not significant and only explained less than 1% of the variation in the data (Supporting Information S3). For the group of small unidentified colored flagellates, however, the GAM showed a significant (non-linear) fit with counts per viewing field more or less decreasing until 9 mm from the center followed by an increase (Fig. 4B; Supporting Information S3). The deviance of this fit was 13% which implies that 87% of the variance in the data was not captured by this model. As for plankton cells in general, also the linear model with an increase from center to edge explained counts of the large diatom

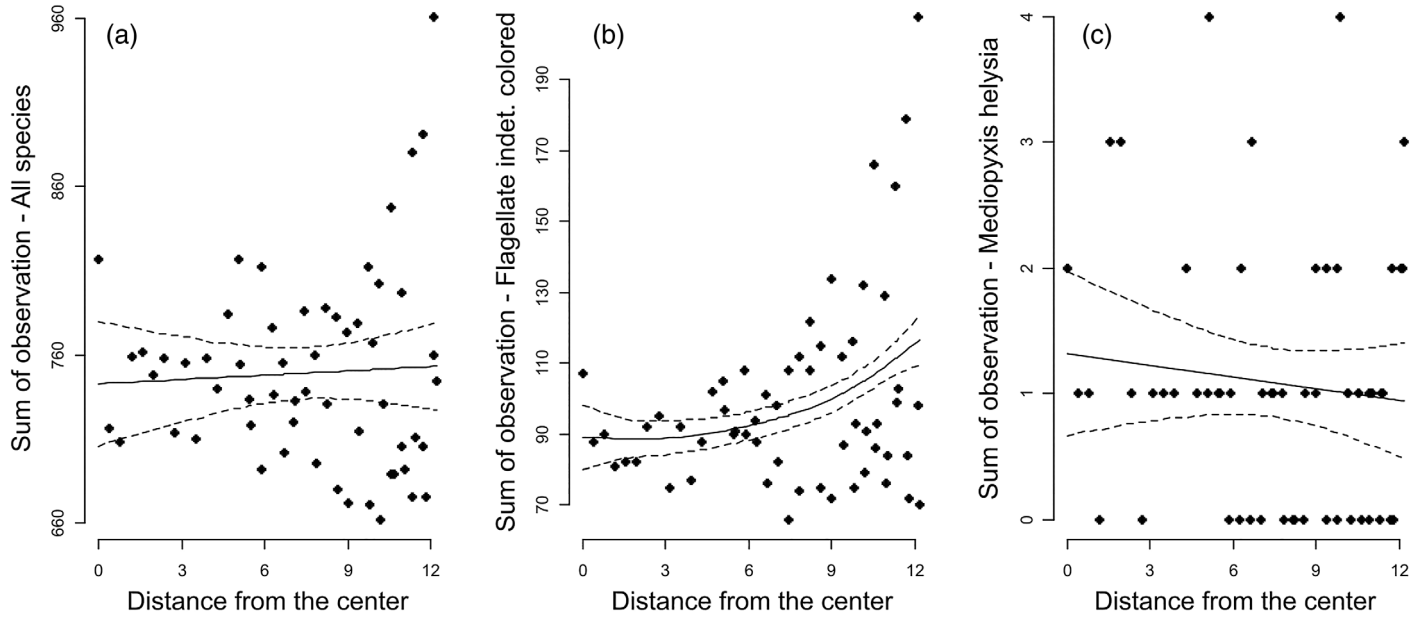

Fig. 4. Best fits (based upon AIC values) of the relationships between the counts (number of algal cells per viewing field) and the distance of that viewing field from the center of the counting chamber (in mm) for (**A**) all species combined (n = 43,438 cells) as the sum of counts at all magnifications, (**B**) small unidentified colored flagellates (n = 5748 cells) counted at the 10 × 100 magnification, and (**C**) the large diatom species *Mediopyxis helysia* (n = 63 cells) counted at 10 × 40 magnification. Black solid lines indicate outcomes of the best models, black dashed lines their 95% confidence intervals. This analysis is based upon phytoplankton samples taken from the Marsdiep tidal inlet (2009–2018). *See* Supporting Information S3 for details on the statistical analyses.

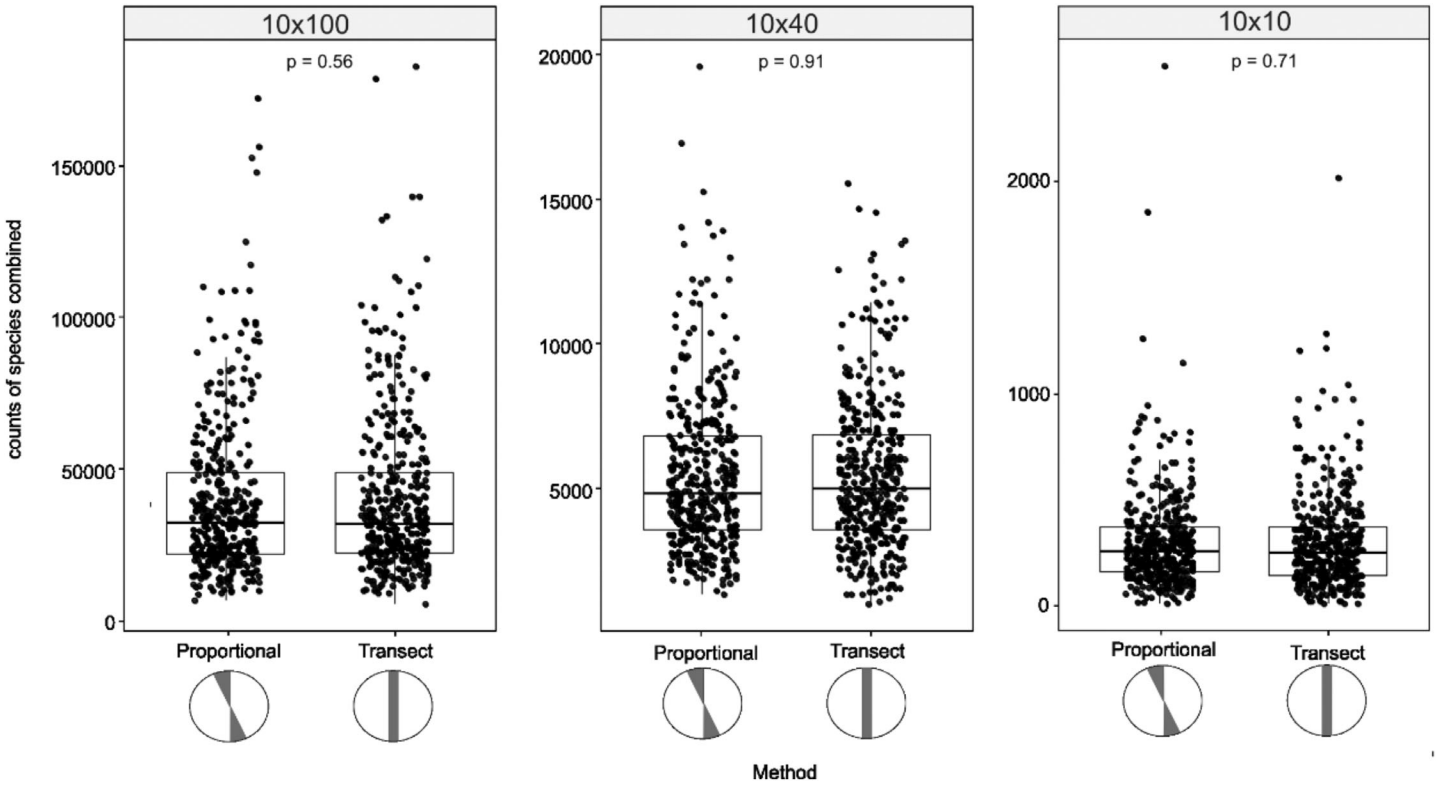

Fig. 5. Comparison of the summed numbers of algal cells for all species at each of three magnifications (10 × 100, 10 × 40, and 10 × 10) using proportional vs. transect counting strategies. At each magnification, there was no significant difference in the total number of species (paired *t*-test, $p > 0.05$). Phytoplankton samples were collected from the Marsdiep tidal inlet, western Wadden Sea (2009–2018).

M. helysia best (Fig. 4C; Supporting Information S3). The fit of this model was, however, not significant and explained just over 1% of the variation in de data set (Supporting Information S3).

Consequences of counting strategies

The use of different counting strategies (the proportional vs. the transect method) did not result in different phytoplankton cell abundances for all species combined (Fig. 5). These findings are in line with the absence of edge-center differences and absence of gradients when all species were combined (*see* previous results).

No significant difference in cell concentrations was observed between the proportional and transect counting strategies for those single species for which we observed significant differences between counts in the center and edge of the counting chambers (*see* Fig. 6; Table 1).

Optimal number of fields

The critical effort (e.g., the number of fields to count) necessary to obtain < 5% error on estimates of phytoplankton abundance was 48, 55, and 27 fields at ×100, ×40, and ×10 magnification, respectively (Fig. 7). That effort was less than the effort used in our study (58 at ×100, 70 at ×40, and 29 at ×10 magnification) indicating high confidence (> 95%) in our estimates of phytoplankton abundances.

The median effort necessary to detect 90% of the species present in a sample was 88, 191 and 79 fields at ×100, ×40, and ×10 magnifications, respectively (Fig. 8). This effort was greater than that used in our study (58 at ×100, 70 at ×40, and 29 at ×10 magnification) indicating that our estimates of phytoplankton richness were below an accuracy of 90%.

Discussion

The determination of phytoplankton abundances from subsampling of viewing fields during microscopic counting generally assumes that algal cells are not clustered and do not show spatial patterns in the counting chambers. Algal cells were clustered in more than one-fifth of the samples at all magnifications (10 × 10, 10 × 40, and 10 × 100). This observation for coastal waters confirms the frequent occurrence of aggregated phytoplankton samples as reported by others (Edgar and Laird 1993; Salonen et al. 2021).

Clustering of algal cells in counting chambers may be influenced by ecological processes that occurred at the time of sample collection (e.g., flocculation of *Phaeocystis* spp. during bloom [Passow and Wassmann 1994], aggregation of cells due

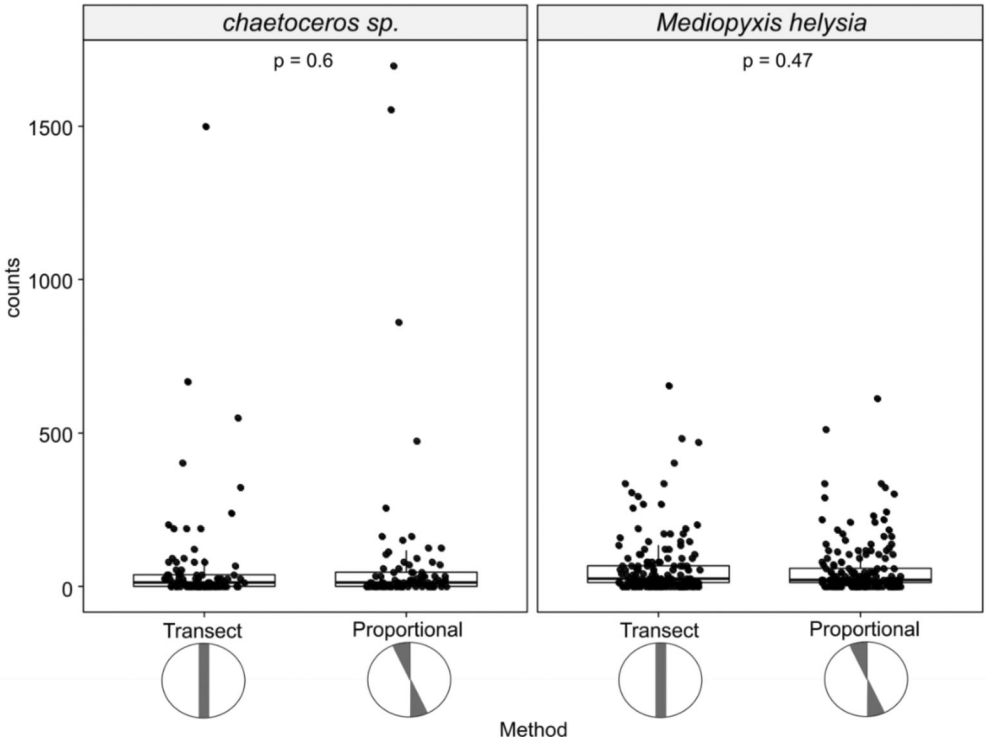

Fig. 6. Comparison of the total numbers of algal cells of the group of *Chaeotoceros* species (left panel) and the large diatom *Mediopyxis helysia* (right panel) using proportional vs. transect counting strategies. For each magnification, there was no significant difference in the total number of species (paired *t*-test, $p > 0.05$). Phytoplankton samples were collected from the Marsdiep tidal inlet, western Wadden Sea (2009–2018).

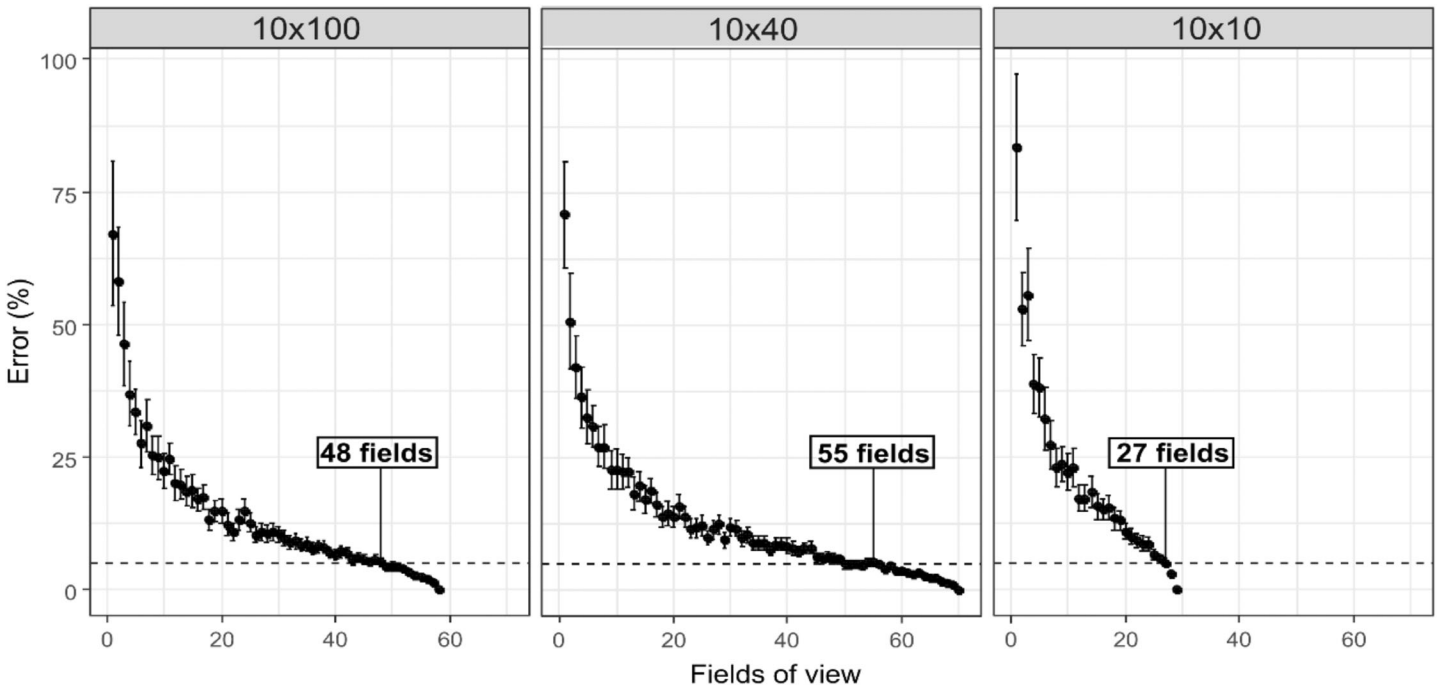

Fig. 7. Relationships between the mean error (%) of calculated abundance (cells mL^{-1}) vs. the number of fields of view per counting chamber for different magnifications (10 × 100, 10 × 40, and 10 × 10). Points represent the mean errors and bars the confidence intervals of the bootstrapped abundance estimates. The horizontal dashed line indicates a 5% error, the number of fields at which this error occurred is also displayed. This analysis is based upon phytoplankton samples taken from the Marsdiep tidal inlet ($n = 398$, 2009–2018).

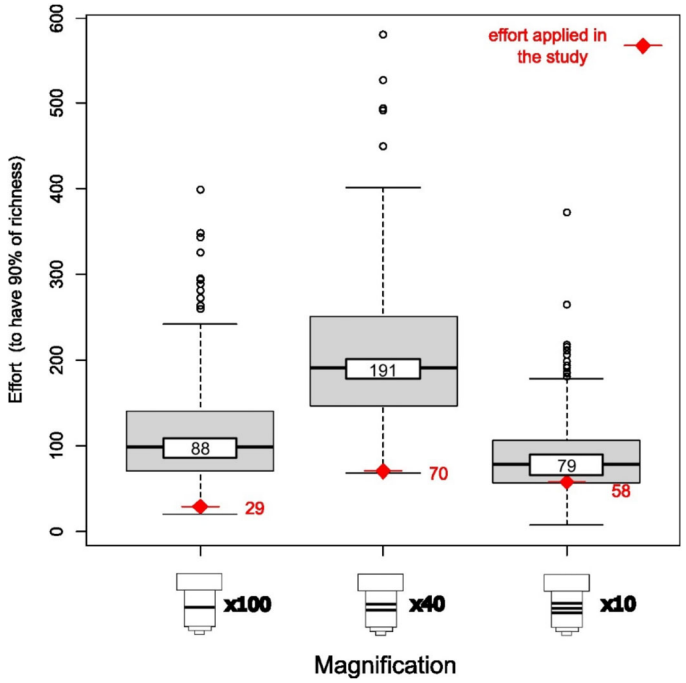

Fig. 8. Effort (e.g., number of viewing fields per counting chamber) that is needed to determine 90% of species richness in the sample for different magnifying scanning objective lenses (×100, ×40, and ×10). The black-boxed value indicates the median of the distribution of the needed effort, values in red indicate the effort applied in this study. This analysis is based upon phytoplankton samples taken from the Marsdiep tidal inlet ($n = 398$, 2009–2018).

to exopolymer production [Logan et al. 1995]), but may also occur (or be strengthened) due to the human effect during sampling, preservation, subsampling, and settling (Salonen et al. 2021). This difference in spatial distribution will lead to increase of error in the estimation of the number of cells if the counting strategy cannot correct for this clustering.

Identifying which parameter (e.g., flocculation, preservation, settling) exactly influences spatial distribution would need extensive research as well as isolation for each process. Nonetheless, spatial patterns in counting chambers such as lateral and radial distributions of the algal cells are generally considered to have been produced by variations in temperature or vibrations during settling (Sandgren and Robinson 1984; Berthold and Resagk 2012). So far, abundances of plankton cells were generally found to be higher at the margins than at the center of counting chambers (Sandgren and Robinson 1984), and large and small cells accumulated at the edges and center of counting chambers (Olrik et al. 1998).

For the most dominant species ($n = 19$) in our samples, we found that 30% ($n = 6$) of the species showed significantly different abundances at the margins compared to the center of the counting chamber, with 11% (1 large [> 6 μm] and 1 small [< 6 μm] species) having higher abundances at the center than at the edge, and 19% (5 large and 5 small species) having the highest abundances at the margins. Our findings, therefore, confirm previous findings on the occurrence of spatial patterns in counting chambers and also revealed that such patterns (if present) are not consistent between samples and between species. Although for all plankton species combined and for *M. helysia*, linear models showed the best fit, these fits were not significant and the explanatory values of the fits were very low (around 1%). Non-linear significant fits, such as found for the small unidentified colored flagellates, only explained less than 15% of the variation and are, therefore, also not applicable to be used as a correction method for a (potential) bias. This implies that any counting method that is based upon subsampling within the counting chamber is expected to result in an error, and that this error should be made explicit when presenting algal densities.

The "proportional" counting strategy better balances the sampling effort in central and peripheral regions (cf. Sandgren and Robinson 1984). In case of the presence of a gradual gradient, this counting strategy would yield different (better) estimates of phytoplankton abundance than the "transect" counting strategy. For our samples, however, we did not find significant differences in the estimates of phytoplankton concentration (total or for individual species) between the two strategies. This result is not surprising because in the case of our samples, there were no linear gradients in cell numbers from the center to the edge (numbers per viewing field) and, thus, the "proportional" counting strategy is not expected to improve the estimates compared to counting using the "transect" strategy.

Alternatively, it has been suggested to count algal cells in randomly chosen fields of view in the counting chambers instead of transects, grids or proportions (Sandgren and Robinson 1984). This approach does, however, not address clustered distribution (ca. 21% of our samples) nor takes the occurrence of gradients from the center to the margins (ca. 21% of our samples with dominant species) into account. In addition, finding the location of a specific field of view in a counting chamber takes extra time (in comparison of counting adjacent fields when following transect or proportional counting strategies) and effort and also poses the risk of counting another field of view than originally intended. Putting this extra time and energy into counting more viewing fields per counting chamber (using a transect method to enable the detection of more accurate trends in counts per field of view from the edge to the center of the counting chambers) and/or in analyzing more samples per station might then be a better option to decrease variability and get more precise estimates of the spatial, seasonal, and long-term dynamics of phytoplankton.

With respect to the number of viewing fields per counting chamber, it appears that our present efforts are sufficient to allow for a reliable estimate (5% error) of phytoplankton abundances (Salonen et al. 2021). This outcome only applies to the effort needed for an estimate of the abundances of all species combined and not for individual species. On the other hand,

the results of the current study showed that the applied effort was insufficient to correctly depict the species richness in a sample.

This result is in agreement with Rodríguez-Ramos et al. (2014), who stated that the effort applied in most studies is too low to cover a good proportion of species thus affecting the estimate of phytoplankton diversity. As the volume of sampling used in the current study should be sufficient for a correct richness representation (Cermeño et al. 2014), the insufficient effort (number of viewing fields per counting chamber) is here pinpointed as the main driver.

Our present efforts seem also to be insufficient when considering the contribution of rare species to phytoplankton community structure (Ignatiades and Gotsis-Skretas 2014) and Shannon–Weiner or Pielou indices (Chiarucci et al. 2011). Depending on the diversity index used and the number of individuals counted, it is recommended to use a high (> 90%) value of species richness (Soetaert and Heip 1990; Chao et al. 2009) showing the importance of those rare species. Increasing counting efforts will take more time and resources per sample, however, and therefore potentially limit the number of samples that can be analyzed per year and/or per area.

Recommendations

Based on our and previous findings, the numbers of algal cells within viewing fields of the same counting chamber can be highly variable affecting the estimates of phytoplankton abundance and species richness of phytoplankton communities. First, our findings underscore the need for intra-laboratory standardization of protocols (Rott et al. 2007) that help prevent clustering (from sampling to settling) and composing spatial patterns during settling and counting (e.g., resulting from vibrations and temperature gradients). Since visual inspection cannot detect non-random patterns (Salonen et al. 2021), counting protocols should include regularly checking for clusters and patterns in viewing fields by means of statistical cluster analyses of the count data. Although a posteriori correction is not possible if such deviations from a random distribution are identified, the outcomes of this analysis will at least supply information on error and assist in reducing this source of variation by testing the consequences of changes in protocols and procedures.

Because spatial patterns observed in the counting chambers are not consistent between samples or between species (Olrik et al. 1998; Sandgren and Robinson 1984; our study), no counting strategy will be fully able to capture the actual numbers of all species within a counting chamber if these numbers are estimated from counts in a selection of viewing fields. However, for a unique species of importance, a specific counting strategy can be applied to correct the result if the spatial distribution is known. Thus, selecting the least time-consuming and most robust method of selecting viewing fields within a counting chamber (e.g., transects) has utility because it would allow for more samples to be analyzed in time and space. This larger sample size might ultimately aid in getting better estimates of the spatiotemporal variation in concentrations and species composition of phytoplankton communities. The balancing between the accuracy of single phytoplankton counts and that of increasing sampling efforts might vary between regions and seasons, and (most importantly) for the scientific questions addressed.

As was already stated by Rodríguez-Ramos et al. (2014) and others, most of the protocols used to count samples are not suitable to yield robust estimates of phytoplankton diversity and, hence, track changes in that aspect of phytoplankton communities over time. We would require a three-fold increase in the number of viewing fields per counting chamber (for the ×40 and ×100 magnifying scanning objectives) to have 90% accuracy in the estimate of the species diversity in our samples. Given our current effort in long-term sampling and analysis of phytoplankton, such an increase in counting effort would result in a reduction from 40 to less than 20 samples per year. Considering the strong seasonal and year-to-year dynamics of phytoplankton communities in our waters (Philippart et al. 2010; Jacobs et al. 2020), such a reduction of sampling resolution would result in less power to determine long-term changes in the abundances of the most dominant species. We recommend optimizing the effort used in sampling and analysis efforts of phytoplankton abundances to address the main research questions and accepting (and realizing) the limits of this choice for addressing other challenges.

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