BOREAL ENVIRONMENT RESEARCH 28: 195–206
ISSN 1797-2469 (online)

Helsinki 30 August 2023

Resolving species-specific abundances of three cold-water dinoflagellates using a simple staining technique

Tobias Lipsewers

Finnish Aquaponics Society, Gartenstraße 73, 33397 Rietberg, Germany
*(*corresponding author's e-mail: tobias.lipsewers@gmail.com)*

Received 1 Feb. 2023, final version received 4 Jun. 2023, accepted 15 Jun. 2023

Lipsewers T. 2023: Resolving species-specific abundances of three cold-water dinoflagellates using a simple staining technique. *Boreal Env. Res.* 28: 195–206.

The dinoflagellates *Gymnodinium corollarium* (Gc), *Biecheleria baltica* (Bb), and *Apocalathium malmogiense* (Am) are very difficult to separate using traditional light microscopy. Their dominance patterns may have ecosystem-wide consequences in terms of, e.g., the Baltic Sea carbon cycle. This study describes a protocol to separate them, determines their relative abundances, and suggests the adjustment of monitoring programs. A relatively simple staining technique was found to be suitable to identify Gc, Bb, and Am. Opposite trends were observed for Gc (increasing) and Bb (decreasing) along with the spring bloom, indicating different niches. Two clusters of sub-basins were identified: 1) almost absolute dominance of the usually grouped biomass by Gc; and 2) Bb also became dominant. Gc occasionally dominated even the overall microscopy-derived biomass, Bb clearly contributed, and Am contributed minor proportions. The proposed strategy can be implemented in monitoring protocols. This will facilitate the tracking of changes within Baltic Sea phytoplankton assemblages.

Introduction

Phototrophic motile dinoflagellates are a group of phytoplankton and key aquatic primary producers. For instance, in the Baltic Sea, the phytoplankton spring bloom is the most important event in terms of primary production (Lignell *et al.* 1993). The biomass-relevant spring bloom dinoflagellates in the Baltic Sea form resting stages (Kremp *et al.* 2018). These resting stages are not directly bioavailable as a source of energy for other organisms and thus, may function as a sink for inorganic nutrients and carbon (e.g., Spilling *et al.* 2018). Therefore, depending on the dominant species, dinoflagellates might provide beneficial ecosystem services. On the other hand, low proportions of dinoflagellates (< 0.5, relative to diatoms) are considered an indication of better ecosystem health (Wasmund *et al.* 2017). Thus, understanding the phytoplankton community composition is important. This is highlighted by the fact that higher proportions of dinoflagellates (compared with diatoms) have been detected in several parts of the Baltic Sea (Wasmund and Uhlig 2003, Klais *et al.* 2011), due to changes in climatic conditions such as a reduction in sea ice in the last decades (Klais *et al.* 2013).

Species-specific differences exist within the phylum of dinoflagellates so the present study was motivated by the need to differentiate between three species, belonging to different systematic orders and (based on previous knowledge) may constitute a major part of

Editor in charge of this article: Johanna Mattila

the phytoplankton biomass produced during the spring bloom in the Baltic Sea (e.g., Sundström *et al.* 2010). *Gymnodinium corollarium* (Gc), *Biecheleria baltica* (Bb), and *Apocalathium malmogiense* (Am) are very difficult to separate using traditional light microscopy and are usually grouped under a single name (referred to henceforth as dino-group) without knowing the species-specific abundances and biomasses. In fact, the three species are considered inseparable when analysed according to traditional monitoring protocols (light microscopy, mixed samples preserved with acidic Lugol's solution, using 125-fold magnification). Important findings are lost when these species are not separated, since they differ ecologically (laboratory study by Sundström *et al.* 2009, life cycle modelling study by Warns *et al.* 2013) and have different effects on aspects such as, the biogeochemistry of the Baltic Sea (Spilling *et al.* 2018). For example, Bb prefers lower temperatures and can be abundant under the sea ice even before the onset of the spring bloom (Sundström *et al.* 2010). The sedimentation of its resting stages may contribute as much as 45% to the total export of particulate organic carbon (POC) after the spring bloom in the Gulf of Finland (GOF, Heiskanen 1993). A study on the GOF sediment revealed that drastically increasing abundance of Bb resting stages has coincided with increasing eutrophication since the 1930's (Kremp *et al.* 2018). Gc occurs in high abundance during the spring bloom in several sub-basins (Sundström *et al.* 2010) such as the Baltic Proper (Sundström *et al.* 2009). The resting stages of Gc (an athecate species) were found to be comparably fragile (Sundström *et al.* 2010) and thus, it can be assumed that they degrade more quickly on the sea floor compared with thecate species (e.g., Bb and Am). The resting stages of Am are far less abundant compared with Bb in the GOF and therefore, contribute only a minor fraction of the POC-pool in the spring (Kremp *et al.* 2018). Due to their different strategies for resting stage formation (encystment) and germination (hatching of vegetative cells), nutrient uptake efficiencies, and longevity of resting stages, different dominance patterns of these species may have ecosystem-wide consequences. Therefore, species-level identification is important for improving the understanding of interactions between biotic and abiotic factors as well as between different organisms. The dominant species defines the quantity and quality of the food web's base, and the fate of nutrients and carbon.

Fortunately, separation of the three species is possible using several approaches (e.g., Kremp *et al.* 2005). Different identification methods are available with various advantages and disadvantages to each method. For instance, the analysis of calcofluor white MR2-stained samples by inverted epifluorescence microscopy (used here) allows the separation of dinoflagellate species based on compositional and structural differences in their cell walls (Fritz and Triemer 1985). Compared with molecular tools, this method has multiple advantages: it is easy to learn for a trained phytoplankton microscopist; it can be used on live samples (relevant for establishing new cell lines) and with alkaline fixatives; it allows the separation of different life cycle stages (relevant for, e.g., Bb and Am); it results in qualitative and quantitative results; and it is less costly (given the availability of a suitable microscope).

The findings of Sundström *et al.* (2009 and 2010) and Kremp *et al.* (2018) described above, initiated this study, which aims to: 1) show that the described method is suitable for separating Gc, Bb, and Am; 2) highlight the additional knowledge gained by using species-level identification for samples collected in different phases of the spring bloom in and sub-basins of the Baltic Sea; and 3) propose a strategy to implement the separation of the three species in phytoplankton monitoring programs.

Material and methods

Origin of samples

The water samples (n = 62) were collected during three research cruises on board R/V Aranda (2014: n = 15; 2015: n = 38; 2016: n = 9) with the aid of a CTD-rosette sampler equipped with Niskin bottles. Surface waters (z = 3 m) of the Baltic Sea (Fig. 1a, HELCOM 2017) were sampled at 51 different stations located in seven different sub-basins during different bloom-phases. Some of the stations were

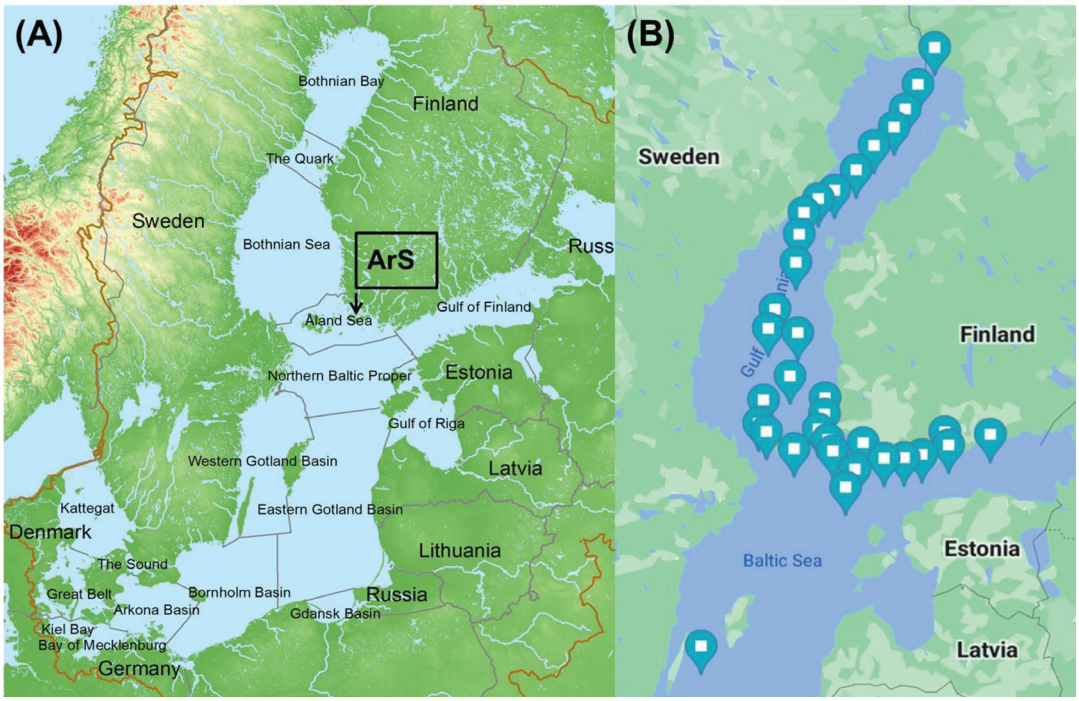

Fig. 1. (**a**). The Baltic Sea with all of its sub-basins (modified after HELCOM 2017). The location of the Archipelago Sea (ArS) is indicated by the black arrow. (**b**) The sampled part of the Baltic Sea showing the distribution of the visited stations (prepared with google maps using the coordinates of the sampling stations in WGS-84 format, Google Maps 2023). Following the link (Google Maps 2023), all stations can be seen separately. The samples were collected during three research cruises (2014, 2015, and 2016). Some of the stations were sampled more than once (one station was visited twice in 2016; others were revisited in different years).

visited repeatedly (one twice in 2016; others in different years), resulting in 62 individual samples. Combining the samples of all three cruises, largely the Northern half of the Baltic Sea was covered (Fig. 1b, Google Maps 2023). The southernmost station was located between the Swedish islands Gotland and Öland. Further details can be obtained from Lipsewers *et al.* (2020).

Identification of the three cold-water dinoflagellates by inverted epifluorescence microscopy of calcofluor white MR2-stained samples

The staining method and the data analyses are described in detail. Details concerning the definition of bloom-phases and Microscopy Derived Carbon (MDC) are described in Spilling *et al.* (2019) and Lipsewers *et al.* (2020), respectively.

Monoalgal cultures of the three species were used for dye testing and for validating the identification of species in field samples (strains: WHTV-S1 (*Biecheleria baltica*, Bb), GCTV-C1 (*Gymnodinium corollarium*, Gc), and SHTV-JR (*Apocalathium malmogiense,* Am); FINMARI culture collection). This approach to identifying and quantifying the three dinoflagellate species Gc, Bb, and Am, forming the dino-group, is based on inverted epifluorescence microscopy of samples stained with calcofluor white MR2 (Fluorescence Brightener 28, Sigma-Aldrich). This dye absorbs ultraviolet (UV)-radiation (340–400 nm range) and re-emits visible blue light (Fritz and Triemer 1985). Thus, the microscope equipment and settings must be selected accordingly. The amount of cellulose in the dinoflagellate cell wall determines the staining intensity. The original method (Fritz and Triemer 1985) was slightly modified and optimised for Baltic Sea samples by Anke Kremp

(unpublished) and used in this study. Environmental samples preserved with neutral Lugol's solution were used, as the fluorescent dye is pH-dependent and does not work with acidic fixatives. Firstly, the cells were settled according to Utermöhl (1958), and sedimentation volumes of 25 to 100 ml were used, depending on the known abundance of the dino-group in the total plankton counts. Five drops (Pasteur pipette, evenly distributed) of a calcofluor white MR2 working solution (1 mg ml^{-1}) were added to the concentrated sample (Hydro-Bios counting chamber, 2.973 ml) after sedimentation (24–72 hours). Samples were incubated for at least five minutes in the dark at room temperature before analysis with the aid of an inverted epifluorescence microscope (Leica DMI 3000 B) and a camera (Leica DFC 490) at 40-fold magnification. Micrographs were taken at different magnifications. It was aimed to analyse at least 100 cells of the dino-group per sample. For samples with very low biomass/dino-group abundance, the maximum sedimentation volume of 100 ml and one half of the counting chamber area were examined to determine the relative abundances of Gc, Bb, and Am. The relative proportions (henceforth referred to as relative contribution/abundance) of the three species were determined (e.g., 30 cells of Bb amongst 100 cells of the group = 30% Bb). By knowing the species-specific counts and the dino-group biomass in the overall nano- and microplankton community, which was previously determined for each sample (details on the method in Lipsewers and Spilling 2018), the percentage relative contributions can be used to calculate an estimate of the species-specific biomass.

Data analysis

To calculate average values and standard deviations of the selected variables in the different bloom-phases and sub-basins, the sampling stations, originating from different cruises, were grouped accordingly. A species was considered dominant if it contributed > 50% of the dino-group abundance. Calculation of the Gc/Bb-index (based on relative contributions of Gc and Bb to the dino-group) was inspired by Wasmund *et al.* (2017). This index ranges from zero to one, where zero indicates an absolute dominance of Bb and one an absolute dominance of Gc. The equation is the following:

$$\text{Gc/Bb – index} = \text{Gc\%} / (\text{Gc\%} + \text{Bb\%}) \quad (1)$$

The index was calculated for each sample to identify significant differences between the bloom-phases and sub-basins. For this, a Student's *t*-test (unpaired) was performed with all possible combinations of bloom-phases and sub-basins using the untransformed Gc/Bb-indices (SigmaPlot 10). A *t*-test determines if mean values of two data columns are significantly different by testing the hypothesis that the means of these two groups are equal. An unpaired *t*-test can be performed on different sized columns, since no relationship is assumed between the groups. For two comparisons a paired *t*-test was also performed, since the number of samples (column size) was equal. The degrees of freedom are henceforth abbreviated as df and the compared column sizes (number of samples, *n*) are given in subscript of a given df-value. *p*-values of < 0.05 represent significant differences. A maximum of four decimals is shown for *p*- and *T*-values.

Results

Suitability of the staining method and separation of the three dinoflagellates of interest

The first aim of this study was to show that the described method is well suited to separate the three species, which are usually grouped (*Gymnodinium corollarium* (Gc), *Biecheleria baltica* (Bb), and *Apocalathium malmogiense* (Am)), based on their distinct cell wall characteristics. When the fluorescent dye is excited with UV-light, the obvious differences in cell wall morphologies between Gc, Bb, and Am are visualised and they can be distinguished in samples preserved with neutral Lugol's solution (Fig. 2, panel B). The comparison to their appearances when the dye is not excited (as seen usually) is astonishing (Fig. 2, panel A).

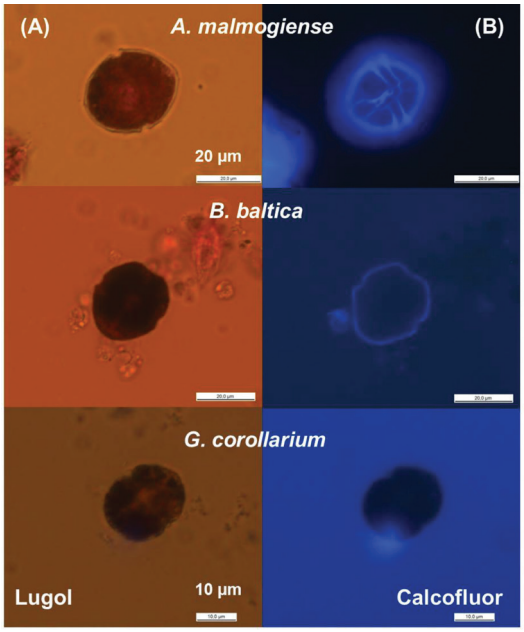

Fig 2. Comparing the appearances of the three dinoflagellates in samples preserved with neutral Lugol's solution (panel A) to the calcofluor-stain of the identical cells (panel B). Note the different scale bars for *Gymnodinium corollarium* (10 µm) and the other species (20 µm).

Gc is athecate and not stained at all; Bb has thin thecal platelets and its cell wall appears as a uniformly blue margin around the cell; and Am has thick distinct thecal plates and features the most intense colour. Due to these obvious differences, this method is especially useful to separate the studied species.

The relative species-specific abundances of Gc, Bb, and Am in different bloom-phases and sub-basins

The second aim was to highlight the additional knowledge gained by using species-level identification in terms of the relative abundances of Gc, Bb, and Am in different phases of the spring bloom in and sub-basins of the Baltic Sea. The statistical analyses focused on the most remarkable findings: the development of the dino-group composition along with the bloom and the different clusters of sub-basins based on this community (details below). Based on the results (Fig. 3), it was decided to exclude Am from further analyses and consider the Gc/Bb-index (Eq. 1), exclusively. All Student's *t*-tests were considered as successful by the software, meaning that the data fit the assumptions met by the *t*-test so there are no error biases.

Bloom-phases

Based on the large and overlapping standard deviations, there was no difference between the average contributions of the dino-group to the total Microscopy Derived Carbon (MDC) comparing the bloom-phases (minimum: 9.4 ± 13.7 SD%, peak phase; maximum: 19.8 ± 13.8 SD%, growth phase; average ± standard deviation (SD); Fig. 3a).

Gc and Bb featured opposite developments from growth to post-bloom phase after starting out with similar relative contributions to the dino-group (~50%; Fig. 3b). The contribution of Gc increased (growth phase: 52.01 ± 24.08 SD%; post-bloom phase: 93.97 ± 5.17 SD%) whereas that of Bb decreased with decreasing phototrophic biomass (growth phase: 47.50 ± 24.12 SD%; post-bloom phase: 5.66 ± 4.68 SD%). Based on the standard deviations (no overlap), the contributions of Bb and Gc clearly differed during the decline phase and post-bloom conditions (Fig. 3b). The Gc/Bb-indices differed significantly during post-bloom conditions in comparison to all other bloom-phases (Table 1). Based on the average values, Am contributed less than one percent to the dino-group throughout the bloom (maximum: 0.52 ± 1.40 SD%, decline phase) and did not show a clear trend.

Since the column sizes (number of samples) of two of the comparisons were equal, a paired *t*-test was also performed and confirmed statistically significant differences (paired *t*-test; Baltic Proper (BP) *vs*. Bothnian Sea (BS): $p = 0.0001$, $df_{10,10} = 9$, $T = 6.8741$; The Quark (Kv) *vs*. Aland Sea (AS): $p = 0.0047$, $df_{4,4} = 3$, $T = 7.5931$).

Sub-basins

At three of the seven sub-basins, the dino-group contributed more than 10% (average values) to the total MDC (BP: 25.35 ± 17.42 SD%; Gulf

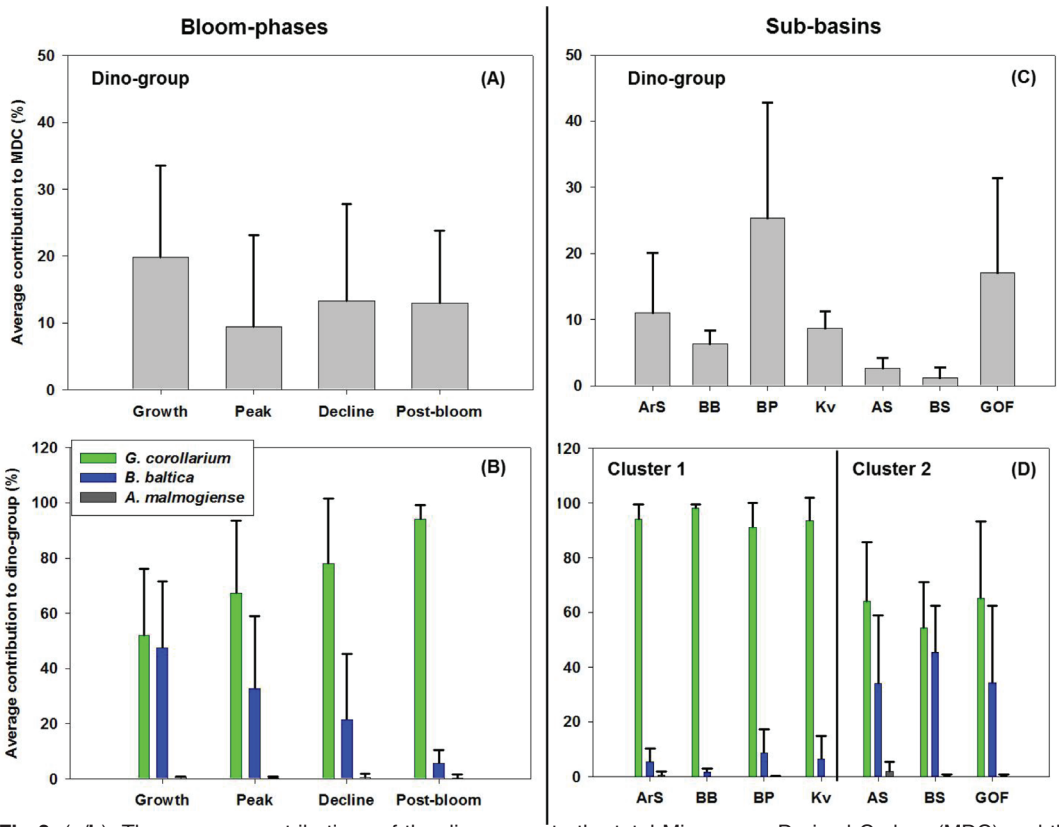

Fig 3. (**a**/**b**). The average contributions of the dino-group to the total Microscopy Derived Carbon (MDC) and the average contributions of *Gymnodinium corollarium* (Gc), *Biecheleria baltica* (Bb), and *Apocalathium malmogiense* (Am) to the dino-group in the different growth phases of the Baltic Sea spring bloom, respectively. The number of samples differed between the bloom-phases (Growth: 3, Peak: 17, Decline: 29, Post-bloom: 13). (**c**/**d**) The average contributions of the dino-group to the total MDC and the average contributions of Gc, Bb, and Am to the dino-group in the different sub-basins (ArS = Archipelago Sea, AS = Aland Sea, BB = Bothnian Bay, BP = Baltic Proper, BS = Bothnian Sea, GOF = Gulf of Finland, Kv = The Quark), respectively. The number of samples differed between most of the different sub-basins (ArS: 11, AS: 4, BB: 5, BP: 10, BS: 10, GOF: 18, Kv: 4). (**b**/**d**) The colour code is valid for both plots. (**a**, **b**, **c**, **d**) Average values represent the percentage relative contributions and the error bars represent the standard deviations. Data from three different cruises were used. The label of the y-axis of plot (**a**) is also valid for (**c**) and the one of (**b**) is also valid for (**d**).

of Finland (GOF): 17.07 ± 14.27 SD%; Archipelago Sea (ArS): 11.09 ± 9.01 SD%; Fig. 3c). The standard deviations did not overlap in the following comparisons: AS *vs*. BP and Kv, Bothnian Bay (BB) *vs*. BS, BP *vs*. BS, and BS *vs*. Kv. Based on average values, Gc clearly dominated the dino-group in four of the sub-basins with low standard deviations (ArS: 93.99 ± 5.37 SD%; BB: 98.24 ± 1.26 SD%; BP: 91.02 ± 9.09 SD%; Kv: 93.51 ± 8.48 SD%; Fig. 3d). In fact, this species dominated every sample taken from ArS, BB, BP, and Kv (n = 30). Viewing the data revealed that Bb was able to become dominant in the other sub-basins (BS, GOF, and AS), also indicated by higher average contribution values to the dino-group (BS: 45.45 ± 17.13 SD%; GOF: 34.47 ± 28.08 SD%; AS: 34.11 ± 24.74 SD%; Fig. 3d). Again, Am contributed the least to the dino-group compared with Gc and Bb (maximum: 1.82 ± 3.64 SD%, AS).

The composition of the dino-group was found to be comparable in separated sub-basins (ArS and Kv) but also differed between connected sub-basins (BS and Kv; Fig. 3d). Based on the relative contributions of Gc, Bb, and Am to the dino-group, the sub-basins can be grouped

into two clusters as indicated in Fig. 3d: 1) ArS, BB, BP, and Kv; 2) AS, BS, and GOF. In cluster one, the standard deviations for Gc and Bb were lower compared with cluster two and did not overlap whereas there was overlap in cluster two. In summary, Gc dominated the sub-basins in cluster one and Bb also dominated in cluster two. The statistical tests supported identification of the two clusters based on the dino-group's composition (Fig. 3 and Table 1). Except for the comparison of Kv and GOF, all sub-basins differed significantly between the two clusters in terms of the Gc/Bb-indices (Table 1). No significant differences were found between sub-basins of one cluster.

Maximum contributions of the dino-group, Gc, Bb and Am to the overall biomass (MDC)

In 9.7% of the samples, the dino-group contributed >30% of the total MDC (maximum: 61.9%, GOF, decline phase). The maximum separate contributions were 51% for Gc (BP, peak phase) and 18% for Bb (GOF, decline phase) and both species were detected in all samples. Am was detected in 24.2% of the samples and contributed a maximum of 7.3% to the dino-group (AS, decline phase), which was equivalent to 0.35% of the total MDC. Am never became dominant.

Strategy proposal for implementation of this method in monitoring programs

The third aim of this study was to propose an implementation strategy for separation of the three dinoflagellate species in phytoplankton monitoring programs. Thus, an approach to achieve a higher resolution of the community structure is suggested in the following:

1) As these species are only present during the vernal bloom, it would be sufficient to separate them in samples collected during the spring sampling campaign organised by HELCOM (Baltic Marine Environment Protection Commission).

Table 1. The numerical outcomes of the unpaired Student's *t*-tests on untransformed data (all successful according to SigmaPlot 10). Merely, the significant differences ($p < 0.05$) in the *Gymnodinium corollarium* (Gc)/*Biecheleria baltica* (Bb) – indices (Eq. 1) between different bloom-phases and different sub-basins (see Fig. 3 for abbreviations) are presented. The number of samples differed between both bloom-phases and most of the sub-basins (*n*). df = degrees of freedom. The compared column sizes (number of samples, *n*) are given in subscript.

The differences in the Gc/Bb-index between the different bloom-phases

Comparison	*T*-value	*p*-value	Degrees of freedom
Growth / post-bloom	–6.4676	0.0000	$df_{3,13} = 14$
Peak / post-bloom	–3.6243	0.0011	$df_{17,13} = 28$
Decline / post-bloom	–2.3719	0.0226	$df_{29,13} = 40$

The differences in the Gc/Bb-index between the different sub-basins

Comparison	*T*-value	*p*-value	Degrees of freedom
ArS / AS	16.4141	0.0000	$df_{11,4} = 13$
ArS / BS	7.4692	0.0000	$df_{11,10} = 19$
ArS / GOF	3.3728	0.0023	$df_{11,18} = 27$
BB / AS	30.3778	0.0000	$df_{5,4} = 7$
BB / BS	5.6319	0.0001	$df_{5,10} = 13$
BB / GOF	2.5663	0.0180	$df_{5,18} = 21$
BP / AS	8.9506	0.0000	$df_{10,4} = 12$
BP / BS	6.0761	0.0000	$df_{10,10} = 18$
BP / GOF	2.8084	0.0093	$df_{10,18} = 26$
Kv / AS	9.4959	0.0001	$df_{4,4} = 6$
Kv / BS	4.2994	0.0010	$df_{4,10} = 12$

2) To reduce the additional workload, only samples within a certain threshold (e.g., > 5%) of dino-group contribution to total MDC could be analysed.
3) To identify Gc, Bb, and Am in spring samples, an additional sample should be collected and preserved with Lugol's solution of a neutral instead of acidic pH to allow the use of calcofluor white MR2. The extra sample should be taken from the same integrated sample used to prepare the usual monitoring sample.

To realise this, HELCOM could consider including identification of Gc, Bb, and Am in the phytoplankton expert group's routine work or set up a new expert group in collaboration with external research groups to carry out additional analyses of monitoring samples. The required funding could be sourced from, e.g., the EU-government to foster the development of tools and measures contributing to the conservation of the Baltic Sea ecosystem. Ideally, the same person analysing the usual phytoplankton monitoring sample would apply this proposed staining technique. Alternatively, the additional analyses could be the topic of Master's theses. In this case, the microscopist should be provided with micrographs of the dino-group cells found in the corresponding integrated sample. It requires some training on cultured cells and environmental samples (approximately one week depending on previous experience) to become familiar with and be able to distinguish the three species from each other. Subsequently, several samples can be analysed daily.

Discussion

General remarks

The results are a snapshot of the bloom dynamics, as only one surface water sample was collected at each station visit during the day. The results (average ± standard deviation) for different sub-basins and bloom-phases were obtained from samples originating partially from different research cruises. Furthermore, the number of stations sampled varied between different bloom-phases and most of the sub-basins. However, this approach did not result in biased findings as shown by the characteristic species succession of different taxonomic units along with the bloom (Lipsewers *et al.* 2020). Additionally, the findings are in accordance with other studies (see below).

Suitability of calcofluor white MR2 in separation of three cold-water dinoflagellates (1st aim)

As previously mentioned, species-level identification is important for understanding the ecology of the Baltic Sea in more detail. For instance, *Gymnodinium corollarium* (Gc), *Biecheleria baltica* (Bb), and *Apocalathium malmogiense* (Am) are ecologically different in terms of nutrient uptake efficiencies and encystment strategies (Sundström *et al.* 2009, Warns *et al.* 2013). Thus, depending on the dominant species, their effects on biogeochemical cycles for example, might vary substantially. Usually (e.g., in monitoring samples), the species-specific abundances of the three species are not determined.

Comparison of Gc, Bb, and Am when stained and unstained (Fig. 1) clearly underscores the advantages of calcofluor white MR2 and validates its applicability in identifying some of the major players of the phytoplankton spring bloom community of the Baltic Sea. In cases of doubt, characteristic patterns and features of the thecal plates (especially of Am) can be studied further (e.g., using micrographs and a well-suited image editor) and/or experts may be consulted. In the present dataset, Am was virtually absent and distinguishing between Gc and Bb was straightforward. Cell sizes vary within all three species and cells of Gc, Bb, and Am can be very similar in size (length of the cells in Fig. 1; Am: 31.4 µm, Bb: 26.4 µm, and Gc: 21.1 µm). Thus, this feature is unsuitable for separation. Additionally, the appearance of non-stained cells differs between samples, magnifications, and microscope settings, making identification using the current monitoring protocol for Baltic Sea phytoplankton impossible.

Depending on the budget and research question, molecular tools could be a good alternative to microscopy. Unlike some other nucleic acid-

based methods, quantitative polymerase chain reaction (qPCR) leads to qualitative (identification) and quantitative (abundances) results. Furthermore, this method features a lower detection limit and allows a high sample-throughput, but it is also more costly. In addition to PCR-based methods, metabarcoding could be used to develop a method for the separation of Gc, Bb, and Am. Nevertheless, the staining method is best suited to be implemented in existing monitoring programs.

The relative contributions of the three species in different bloom-phases and sub-basins (2nd aim)

General findings

This study revealed that the combination of Gc and Bb may dominate the nano- and microplankton biomass (> 50% of the total Microscopy Derived Carbon (MDC)), highlighting their role as important primary producers in the Baltic Sea. Previously, it was thought that Am would also bloom but it contributed only a minor fraction to the dino-group biomass (< 10%), highlighting the importance of separating these species. The results clearly show the important information gained by species-level identification, especially the different trends of the dino-group and single species during the spring bloom. Therefore, the following will focus on the species-specific findings, exclusively.

Bloom-phases

Generally, overlapping standard deviations of the relative abundances (e.g., comparing two species in one bloom-phase or one species in different bloom-phases) indicate no clear differences in the dominance patterns. Considering the standard deviations (no overlaps), the contribution of Gc clearly increased as the bloom progressed (comparing the growth phase and post-bloom conditions) whereas the one of Bb decreased. The later the stage of the bloom, the more pronounced were the differences observed between the relative abundances of Gc and Bb based on the species-specific standard deviations in the decline and post-bloom phase. According to the statistical analyses, the Gc/Bb-indices differed significantly ($p < 0.05$), comparing the post-bloom phase to all other phases. These findings indicate that they occupy different ecological niches during the Baltic Sea spring bloom and thus, follow species-specific succession. For instance, Bb can be abundant under the sea ice before the onset of the spring bloom (Sundström *et al.* 2010), explaining its higher contribution in the initial phase of the bloom, when the temperatures are still low. Furthermore, Kremp *et al.* (2018) found increasing abundances of Bb resting stages coinciding with increasing eutrophication, supporting the fact that this species was most abundant in the beginning of the bloom, when the concentration of dissolved inorganic nutrients is still high. Low abundances of Am were detected throughout the bloom and no obvious trend was observed, indicating that it does not occupy a distinct ecological niche during the spring bloom. These findings agree with the records of resting stages from sediments in the GOF (Kremp *et al.* 2018).

Sub-basins

Some of the sub-basins of the Baltic Sea differ significantly in terms of temperature, salinity and growth-limiting nutrients. For instance, the GOF is a highly eutrophicated sub-basin, generally supporting high algal biomass (Pitkänen *et al.* 2001), and features comparably low salinities. In cluster two of the sub-basins, the variation in the relative abundances of Gc and Bb was higher with no obvious differences (larger and overlapping standard variations), meaning that both species were able to dominate the dino-group. The GOF was part of this cluster and Bb featured its maximum contribution to the overall MDC (18%) at one station situated within it. A study on a 100-year-old sediment core from the GOF, connected drastically increasing abundances of Bb resting stages to increasing eutrophication (Kremp *et al.* 2018), supporting the presented findings of the pelagic environment. The fact that Bb was also dominant in 40% of the stations in the BS, belong-

ing to the Northern Baltic Sea, might also be related to its tolerance for low temperatures. In contrast, Gc has shown almost absolute dominance in the sub-basins of cluster one, which included the BP. In this cluster, the standard deviations for the biomass-relevant Gc and Bb were lower compared with cluster two and did not overlap, indicating differences in their relative abundances within these sub-basins. The statistical analyses confirmed separation of the two clusters based on the proportions of Gc and Bb, except for the comparison of two sub-basins (unpaired *t*-test; Kv *vs*. GOF: $p = 0.0660$, $df_{4,18} = 20$, $T = 1.9451$). Furthermore, Gc was able to dominate the entire nano- and microplankton biomass in the BP alone (maximum contribution to MDC = 51%). This species was originally described in samples collected in this sub-basin (Sundström *et al.* 2009). The BP is characterised by higher salinities compared with GOF (for instance). Generally, Gc can become abundant in several sub-basins of the Baltic Sea during the spring bloom (Sundström *et al.* 2010). Am was not found at stations located in the Bothnian Bay (BB) and The Quark (Kv), which could have been due to the microscopy detection limit. Also, its overall abundance in the entire dataset was negligible (maximum contribution to dino-group = 7.3%), indicating that its contribution was previously overestimated. This is supported by the findings in the sediment records of Kremp *et al.* (2018). Findings of the composition of the dino-group based on the sub-basins were somewhat controversial: relative abundances of Gc, Bb, and Am were comparable in sub-basins that are not in direct proximity to each other (Archipelago Sea (ArS) and Kv) but also different in directly connected ones (BS and Kv). ArS and Kv are not part of the open Baltic Sea, meaning that their plankton communities could be influenced by their proximity to land. However, in a recent study (Lipsewers *et al.* 2020), the distance to the shore did not significantly affect community composition during the Baltic Sea spring bloom. Thus, geography alone does not determine the composition of the dino-group. The data for the different sub-basins included samples from different bloom-phases and *vice versa*, indicating that different factors interact to shape the phytoplankton community composition. In fact, abiotic factors (e.g., temperature, depth of the upper mixed layer, and dissolved inorganic nutrients) are the major drivers of the nano- and microplankton community composition during the Baltic Sea spring bloom (e.g., Lipsewers *et al.* 2020). These factors are modified by factors such as climate change so the plankton community composition is expected to change further, with implications for biogeochemical cycles, primary production, and food web dynamics amongst others.

Relevance of this study for the Baltic Sea ecosystem and monitoring programs (3rd aim)

Marine ecosystems are facing increasing environmental pressures arising from climate change and other anthropogenic activities. Simultaneously, our dependency on marine resources is increasing and it is necessary to monitor and understand the effects of environmental change on marine food webs (Mulvihill 1990). For this purpose, different monitoring programs exist. Several plankton groups are suggested to thrive due to climatic changes, and it will be challenging for monitoring programs to register these alterations to the community composition. Phytoplankton forms the base of aquatic food webs, and negative impact on this base will also affect higher trophic levels (e.g., Andersson *et al.* 2015). The plankton community composition affects the biogeochemistry of the ocean and modelling studies which consider the different functional groups may contribute considerably to understanding marine material fluxes (Litchman *et al.* 2015, Vichi *et al.* 2015). Time series data can be used to evaluate the ecosystem structure and functioning, i.e. community composition, community assembly, and food web assessment (Wasmund *et al.* 2011, Lehtinen *et al.* 2016, Klais *et al.* 2017). Monitoring programs may also facilitate decisions to adjust management practices to maintain or improve environmental conditions (Borja *et al.* 2016). Data harmonisation and publicly available datasets are required to allow and promote scientific use and increase the value of monitoring data (Klais *et al.* 2015, Zingone *et al.* 2015).

This study has shown that epifluorescence microscopy of calcofluor white MR2-stained samples is suitable for unravelling the species-specific contributions of three cold-water adapted dinoflagellates in the Baltic Sea. Overall, species-level identification in a dataset spanning different bloom-phases and sub-basins has resulted in relevant and novel findings. Thus, the identification of these species would be very valuable for studying long-term phytoplankton trends and improving our understanding of the ecosystem-wide effects of changing communities in the Baltic Sea. This technique could be applied to samples currently used for phytoplankton identification and quantification (with minimal changes, i.e. pH of the fixative), is rather inexpensive, and relatively easy to learn. Monitoring programs are important and meaningful, however, additional tasks would exceed the scope of what is possible. Nevertheless, this strategy could solve an unsustainable increase in workload while simultaneously obtaining higher resolution of the phytoplankton community. Ideally, this method (modified after Fritz and Triemer 1985) will be implemented in national monitoring programs (coordinated by the Baltic Marine Environment Protection Commission (HELCOM)). Even with the aforementioned compromise (see proposed strategy), the additional knowledge will increase the scientific potential of long-term datasets and allow for assessment of possible far-reaching consequences of changes to plankton community composition may have on the Baltic Sea ecosystem.

Acknowledgements: The following people and institutions are acknowledged: Dr. Pia Steinrücken (Norwegian Research Centre, Bergen, Norway) for the highly constructive feedback on finalising the manuscript for submission; Danielle Bansfield (Marine and freshwater solutions, Finnish Environment Institute, Helsinki (SYKE); Aalto University, Espoo, Finland) for the thorough assessment of the linguistic quality of this article which surely resulted in an improvement; Dr. Anke Kremp (Leibniz-Institute for Baltic Sea Research, Warnemünde, Germany) for the discussion of the method and microscopy results prior to manuscript preparation; Johanna Oja (SYKE) for carrying out part of the "Lugol-microscopy", which was essential for the planning of the subsequent epifluorescence microscopy; Dr. Riina Klais-Peets (Information Technology Centre of the Ministry of Environment, Tallinn, Harjumaa, Estonia) for the idea to work with a Gc/Bb-ratio during a collaboration in 2016; SYKE for the opportunity to work in the C-FluxII-project and to participate in the cruises; and the crew of R/V Aranda for great technical support and fabulous catering. During a large part of the practical work, funding was available from the Academy of Finland (decision numbers 259164 and 263862) for the C-fluxII-project and personal grants were awarded to Tobias Lipsewers by the Onni Talas Foundation and the University of Helsinki. This study utilised SYKE's marine research infrastructure as a part of the national FINMARI consortium.

References

Andersson A., Meier H.M., Ripszam M., Rowe O., Wikner J., Haglund P., Eilola K., Legrand C., Figueroa D., Paczkowska J., Lindehoff E., Tysklind M. & Elmgren R. 2015. Projected future climate change and Baltic Sea ecosystem management. *AMBIO: A Journal of the Human Environment*. 44 :345–356.

Borja Á., Chust G., Rodríguez J.G., Bald J., Mª Belzunce-Segarra J., Franco J., Garmendia J.M., Larreta J., Menchaca I., Muxika I., Solaun O., Revilla M., Uriarte A., Valencia V. & Zorita I. 2016. 'The past is the future of the present': Learning from long-time series of marine monitoring. *Science of The Total Environment.* 566: 698–711.

Fritz L. & Triemer R.E. 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. *Journal of phycology.* 21(4): 662–664.

Google Maps 2023: https://shorturl.at/cEX09 (accessed: 31 May 2023).

Heiskanen A.S. 1993. Mass encystment and sinking of dinoflagellates during a spring bloom. *Marine Biology*. 116(1): 161–167.

HELCOM 2017. Our Baltic Sea, available at: http://stateofthebalticsea.helcom.fi/in-brief/our-baltic-sea/ (accessed: 31 May 2023).

Klais R., Cloern J.E. & Harrison P.J. 2015. Resolving variability of phytoplankton species composition and blooms in coastal ecosystems. *Estuarine, Coastal and Shelf Science*. 162: 4–6.

Klais R., Tamminen T., Kremp A., Spilling K. & Olli K. 2011. Decadal-scale changes of dinoflagellates and diatoms in the anomalous Baltic Sea spring bloom. *PloS one.* 6: e21567, doi: 10.1371/journal.pone.0021567.

Klais R., Norros V., Lehtinen S., Tamminen T. & Olli K. 2017. Community assembly and drivers of phytoplankton functional structure. *Functional Ecology*. 31: 760–767.

Klais R., Tamminen T., Kremp A., Spilling K., An B.W., Hajdu S. & Olli K. 2013. Spring phytoplankton communities shaped by interannual weather variability and dispersal limitation: Mechanisms of climate change effects on key coastal primary producers. *Limnology & Oceanography*. 58: 753–762.

Kremp A., Elbrächter M., Schweikert M., Wolny J.L. &

Gottschling M. 2005. *Woloszynskia halophila* (Biecheler) comb. nov.: A bloom-forming cold-water dinoflagellate co-occurring with *Scrippsiella hangoei* (dinophyceae) in the Baltic Sea. *Journal of Phycology*. 41(3): 629–642.

Kremp A., Hinners J., Klais R., Leppänen A.-P. & Kallio A. 2018. Patterns of vertical cyst distribution and survival in 100-year-old sediment archives of three spring dinoflagellate species from the Northern Baltic Sea. *European Journal of Phycology*. 53: 135–145.

Lehtinen S., Suikkanen S., Hällfors H., Kauppila P., Lehtiniemi M., Tuimala J., Uusitalo L. & Kuosa H. 2016. Approach for Supporting Food Web Assessments with Multi-Decadal Phytoplankton Community Analyses—Case Baltic Sea. *Frontiers in Marine Science*. 3: 220, available at https://doi.org/10.3389/fmars.2016.00220.

Lignell R., Heiskanen A., Kuosa H., Gundersen K., Kuuppo-Leinikki P., Pajuniemi R. & Uitto A. 1993. Fate of a phytoplankton spring bloom: sedimentation and carbon flow in the planktonic food web in the northern Baltic. *Marine Ecology Progress Series*. 94: 239–252.

Lipsewers T., Klais R., Camarena-Gómez M.T. & Spilling K. 2020. Effects of different plankton communities and spring bloom phases on seston C: N: P: Si: chl a ratios in the Baltic Sea. *Marine Ecology Progress Series*. 644: 15–31, available at https://doi.org/10.3354/meps13361.

Lipsewers T. & Spilling K. 2018. Microzooplankton, the missing link in Finnish plankton monitoring programs. *Boreal Environment Research*. 23: 127–137.

Litchman E., Edwards K.F. & Klausmeier C.A. 2015. Microbial resource utilization traits and trade-offs: implications for community structure, functioning, and biogeochemical impacts at present and in the future. *Frontiers in microbiology*. 6: 254, doi:10.3389/fmicb.2015.00254.

Mulvihill S. (editor) Marine Board of the National Research Council. 1990. Managing troubled waters: the role of marine environmental monitoring. National Academies Press. *From the division on Engineering and Physical Sciences, Commission on Engineering and Technical Systems*. Washington, DC: The National Academies Press, available at https://doi.org/10.17226/1439.

Pitkänen H., Lehtoranta J. & Räike A. 2001. Internal nutrient fluxes counteract decreases in external load: the case of the estuarial eastern Gulf of Finland, Baltic Sea. *AMBIO: A Journal of the Human Environment*. 30: 195–202.

Spilling K., Fuentes-Lema A., Quemaliños D., Klais R. & Sobrino C. 2019. Primary production, carbon release, and respiration during spring bloom in the Baltic Sea. *Limnology and Oceanography*. 64(4): 1779–1789.

Spilling K., Olli K., Lehtoranta J., Kremp A., Tedesco L., Tamelander T., Klais R., Peltonen H. & Tamminen, T. 2018. Shifting diatom—dinoflagellate dominance during spring bloom in the Baltic Sea and its potential effects on biogeochemical cycling. *Frontiers in Marine Science*. 5: 327, available at https://doi.org/10.3389/fmars.2018.00327.

Sundström A.N., Kremp A., Tammilehto A., Tuimala J. & Larsson U. 2010. Detection of the bloom-forming cold-water dinoflagellate *Biecheleria baltica* in the Baltic Sea using LSU rRNA probes. *Aquatic microbial ecology*. 61(2): 129–140.

Sundström A.M., Kremp A., Daugbjerg N., Moestrup Ø., Ellegaard M., Hansen R. & Hajdu S. 2009. *Gymnodinium corollarium* sp. nov. (dinophyceae) - A new cold-water dinoflagellate responsible for cyst sedimentation events in the Baltic Sea. *Journal of Phycology*. 45: 938–952.

Utermöhl H. 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik: Mit 1 Tabelle und 15 Abbildungen im Text und auf 1 Tafel. *Mitteilungen der Internationalen Vereinigung für theoretische und angewandte Limnologie*. 9: 1–38.

Vichi M., Lovato T., Lazzari P., Cossarini G., Gutierrez Mlot E., Mattia G., Masina S., McKiver W. J., Pinardi N., Solidoro C., Tedesco L. & Zavatarelli M. 2015. The Biogeochemical Flux Model (BFM): Equation Description and User Manual. BFM version 5.1. Release 1.1, *BFM Report Series 1*, Bologna, Italy, available at http://www.bfm-community.eu.

Warns A., Hense I. & Kremp A. 2013. Modelling the life cycle of dinoflagellates: a case study with *Biecheleria baltica*. *Journal of Plankton Research*. 35: 379–392.

Wasmund N. & Uhlig S. 2003. Phytoplankton trends in the Baltic Sea. *ICES Journal of Marine Sciences*. 60: 177–186.

Wasmund N., Tuimala J., Suikkanen S., Vandepitte L. & Kraberg A. 2011. Long-term trends in phytoplankton composition in the western and central Baltic Sea. *Journal of Marine Systems*. 87 :145–159.

Wasmund N., Kownacka J., Göbel J., Jaanus A., Johansen M., Jurgensone I., Lehtinen S. & Powilleit M. 2017. The diatom/dinoflagellate index as an indicator of ecosystem changes in the Baltic Sea. Principle and handling instruction. *Frontiers in Marine Science.* 4: 22, available at https://doi.org/10.3389/fmars.2017.00022.

Zingone A., Harrison P. J., Kraberg A., Lehtinen S., McQuatters-Gollop A., O'Brien T., Sun J. & Jakobsen H.H. 2015. Increasing the quality, comparability and accessibility of phytoplankton species composition time-series data. *Estuarine, Coastal and Shelf Science*. 162: 151–160.