Eesti Maaülikool
Estonian University of Life Sciences

PIGMENT-BASED CHEMOTAXONOMY – EFFICIENT TOOL TO QUANTIFY PHYTOPLANKTON GROUPS IN LAKES AND COASTAL SEA AREAS

PIGMENTIDEL PÕHINEV KEMOTAKSONOOMIA – TÕHUS VAHEND FÜTOPLANKTONI KOOSSEISU SELGITAMISEKS JÄRVEDES JA RANNIKUMERES

MARJU TAMM

A Thesis
for applying for the degree of Doctor of Philosophy
in Applied Biology

Väitekiri
filosoofiadoktori kraadi taotlemiseks rakendusbioloogia erialal

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"Just because you can read, write and do a little math, doesn't mean that you're entitled to conquer the universe."

— Kurt Vonnegut, Hocus Pocus

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, referred to in the text by the relevant Roman numerals. Papers are reproduced by the kind permission from the publishers.

I **Tamm, Marju;** Freiberg, Rene; Tõnno, Ilmar, Nõges, Peeter; Nõges, Tiina (2015). Pigment-based Chemotaxonomy - A Quick Alternative to Determine Algal Assemblages in Large Shallow Eutrophic Lake? PLOS ONE 10, e0122526.

II **Tamm, Marju;** Laas, Peeter; Freiberg, Rene; Nõges, Peeter; Nõges, Tiina (2018). Parallel assessment of marine autotrophic picoplankton using flow cytometry and chemotaxonomy. Science of the Total Environment 625, 185–193.

III **Tamm, Marju;** Ligi, Martin; Panksep, Kristel; Teeveer, Karolin; Freiberg, Rene; Laas, Peeter; Paavel, Birgot; Kutser, Tiit; Reinart, Anu; Tõnno, Ilmar; Nõges, Tiina. (2019). Boosting the monitoring of phytoplankton in optically complex coastal waters by combining pigment-based chemotaxonomy and in situ radiometry. Ecological Indicators 97, 329–340.

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ABBREVIATIONS

PPP	phototrophic picoplankton
CDOM	chromophoric dissolved organic matter
Chl *a*	chlorophyll *a*
HPLC	high performance liquid chromatography
PC	phycocyanin
PE	phycoerythrin
Peuk	coccoid picoeukaryote group detected by flow-cytometry
WFD	European Union's Water Framework Directive

1. INTRODUCTION

Autotrophic phytoplankton is accountable for around half of the global net primary production (Boyce et al., 2010). As a key element of aquatic food webs and biogeochemical cycles, phytoplankton biomass plays an important part in environmental assessment. The monitoring networks aiming to evaluate water quality and status of aquatic ecosystems are compelled to measure phytoplankton taxonomic groups. For example, the European Union's Water Framework Directive (WFD; EC, 2000) uses phytoplankton as one of the four biological quality elements employed for assessment of the ecological status of surface waters.

Monitoring, however, is constantly struggling between necessary sampling frequency (able to reflect natural processes) and the financial budget limits. Rather often the latter dictates the monitoring effort to be made and therefore the data obtained may lack sufficient spatial and temporal coverage and the dynamics of phytoplankton groups is very poorly reflected (Schaeffer et al., 2013; Skeffington et al., 2015; Bergkemper & Weisse, 2018). Aside traditional phytoplankton counting with a microscope, the modern technologies have a great potential to extend the reach of monitoring networks without increasing the overall cost.

Utermöhl (1958) counting technique that employs inverted microscope paired with highly skillful specialist provides very detailed information about phytoplankton composition. However, the sample analysis time is very high and the acquired species resolution is needlessly detailed for the aims of regular monitoring. Here the phytoplankton marker pigments provide a powerful alternative to the traditional approach.

This thesis analyses the potential of employing pigment-based chemotaxonomy in phytoplankton monitoring in specific types of environments. Phytoplankton groups are described via pigments and microscopy in large shallow and eutrophic Lake Võrtsjärv and in a eutrophic coastal sea area (the West-Estonian Archipelago Sea), both rich in chromophoric dissolved organic matter (CDOM). Algal pigments are further used to examine the community composition of phototrophic picoplankton in the West-Estonian Archipelago Sea. Since this thesis aims to improve the monitoring of phytoplankton, also a region-specific remote sensing algorithm was developed to calculate cyanobacteria biomass from reflectance spectra in brackish and eutrophic coastal areas.

2. REVIEW OF THE LITERATURE

2.1. Challenges in phytoplankton monitoring

All members of the aquatic food web rely on the autotrophic phytoplankton as food supply. The amounts and dynamics of phytoplankton groups reflect the rate of eutrophication, biogeochemical cycling and formation of potentially harmful algal blooms (Falkowski, 1994; Heisler et al., 2008; Cloern & Jassby 2010). Consequently, phytoplankton is one of the key elements in aquatic ecosystem and should be followed in ecological monitoring of aquatic environments. Small size of phytoplankton species (10^{-3}–10^{-7} m) together with short lives (10^{4}–10^{7} s) and high growth rates (3-3.6 doublings day^{-1}) makes targeting these organisms very challenging for meaningful observations (Furnas, 1990; Padisák et al., 2010). The fact that some phytoplankton communities may contain hundreds of different species (e.g. Armonies et al., 2018) further complicates the quantification of these life forms.

Rapid changes and the heterogeneous nature of phytoplankton communities require assessment systems that are able to sensitively track the changes on the spatio-temporal scale (Smayda, 1998; Gallegos & Neale, 2015). Effective monitoring is essential for evaluating the well-being of aquatic systems and for establishing successful management strategies that are able to minimise the negative impacts of the stressors on their ecological status.

Traditionally, phytoplankton community composition is revealed via inverted microscopy (Utermöhl, 1958). This approach provides detailed information about the phytoplankton species, but several drawbacks need to be faced: (1) microscopy is very time consuming; (2) it requires highly qualified specialists; (3) the results are subjective; (4) the smallest fraction of phytoplankton (i.e. picoplankton 0.2 to 2 μm) is left out of the analysis (Schlüter et al., 2000; Lassen et al., 2010). Considering these aspects, the high cost of time and expertise sets significant limits for the sampling frequency.

Alternatively, the molecular approach provides a good phytoplankton community profile and allows a much more comprehensive and time-effective view on community composition compared to conventional

methods, but due to high taxonomic diversity of phytoplankton samples, the taxonomic resolution of the method is still rather coarse as the reference barcode databases are far from being complete. Therefore, microscopic analysis still needs to be carried out side by side with metabarcoding until the reference DNA sequence databases would advance to sufficient level (Abad et al., 2016).

Pigment-based chemotaxonomy has attracted much attention as a valuable alternative and extension to microscopy since the analysis is fast, objective and sensitive. Lower cost compared to the above-mentioned methods is a strong advantage in large-scale studies and monitoring although the taxonomic resolution is much lower (Havskum et al., 2004; Sarmento & Descy, 2008; Swan et al., 2016; Schlüter et al., 2018).

2.2. Phytoplankton pigments as markers

Around 750 carotenoids and 20 chlorophylls have been isolated from natural sources (Britton et al., 2004). It is apparent that these numbers will increase in near future since many of the pigments still remain to be identified. Compared to the high biodiversity of phytoplankton, the diverse ontogenetic and phylogenetic molecular adaptations at pigment level to light conditions have been studied in very few species so far (Serive et al., 2017).

Pigments serve for two basic purposes in algal cells: (1) photoprotection – promoting the dissipation of excess excitation energy as heat in the photosynthetic antennae; (2) light harvesting – using most of the wavelengths of visible light available for photosynthesis (Jeffrey et al., 2011). Complex distribution of both photoprotective and light harvesting pigments among phytoplankton groups has led to a considerable research interest in both ubiquitous and unambiguous pigments. Pigment-based chemotaxonomy aims to employ the unique marker pigments to identify algal taxa and assess their abundance. Because of methodological issues, most commonly the fat-soluble pigments i.e. carotenoids and chlorophylls are used as markers in chemotaxonomic analysis. However, the water-soluble phycobiliproteins are also very beneficial in targeting some specific groups, e.g. cyanobacteria, red algae, cryptophytes and glaucocystophytes (Sidler, 1994).

Chemotaxonomy commonly consists of two basic steps – first the marker pigment concentrations are determined from the water sample with High Performance Liquid Chromatography (HPLC) and second, the obtained data is used to calculate the composition and chlorophyll *a* (Chl *a*) biomass of the algal classes by Chemtax (CHEMical TAXonomy) software [Fig. 1, (Mackey et al., 1996; Wright et al., 1996)]. There is a number of alternative ways to interpret pigment data, but Chemtax stands out with the ability to include several marker pigments for one algal class thus improving the estimation of algal groups that share the dominant pigments – e.g. chrysophytes and diatoms that share fucoxanthin.

While Jeffrey et al. (2011) has provided an exhaustive list of chemotaxonomic markers for oceanographers (74 pigments across 25 algal classes), the pigment analysis is not without complications. The major problem relies upon the calculation procedure. To convert the pigment concentrations into algal groups, Chemtax requires appropriate marker pigment:Chl *a* ratios. The ratios between accessory pigments and Chl *a* are loaded into the program in the form of a matrix together with pigment concentrations to determine the contribution of each phytoplankton group. However, these ratios are dependent on the species composition, growth rate, light intensity and nutrients, making them very variable in time and space (Goericke & Montoya, 1998; Schlüter et al., 2000; Lauridsen et al., 2011). Finding the precise diagnostic pigment ratios that are suitable for the specific environment is one of the most crucial issues of chemotaxonomy. Our knowledge about constructing proper initial pigment ratio matrices for calculation in different types of water bodies is far from perfect and needs validation with microscopy counts (Schlüter et al., 2000; Schlüter et al., 2006; Laurindsen et al., 2011). Although Chemtax has been extensively applied and is a well acknowledged method in marine environments, very little research has focused on using Chemtax in estuaries and freshwater systems. Hence, more work is still needed to verify the literature-based marker pigment:Chl *a* ratios for accurate analysis in the diverse range of brackish and freshwater environments. The correct marker pigment:Chl *a* ratios would decrease the analysis time of a water sample and the sample processing would include more automated steps. Most importantly, phototrophic picoplankton (PPP) that is omitted by routine microscopy would be included in Chemtax analysis.

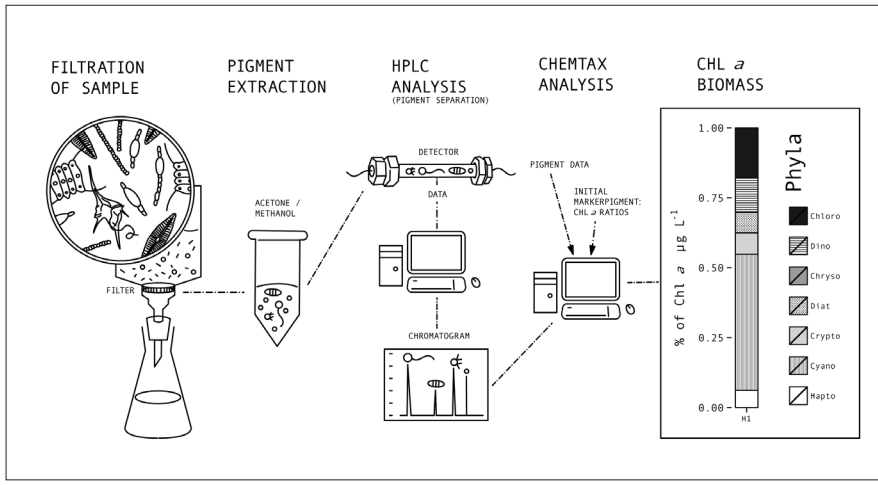

Fig. 1. Work flow in pigment-based chemotaxonomy

2.3. Phototrophic picoplankton in pigment analysis

Up to now, far too little attention has been paid to incorporating PPP in regular monitoring. There is a growing body of literature that recognises PPP (often referred to as autotrophic picoplankton in literature which is not entirely correct) as a considerable contributor to primary production, hence playing important role in the carbon flow. Most of the world's oceans are dominated by the PPP and it has been observed that the contribution of PPP to the total primary production in oligotrophic lakes and oceans may even reach up to 50-90% (Caron et al., 1985; Goericke & Montoya, 1998; Richardson & Jackson, 2007; Iversen & Seuthe, 2011). Mostly due to methodical motives, phytoplankton monitoring has been generally focused only on the larger species that are visually distinguishable with inverted microscopy and/or retained on the GF/F filters (0.7 µm pore size) that are commonly used to give estimates of the total phytoplankton Chl *a* and biomass.

PPP consists of two large components – picocyanobacteria, consisting in marine systems mainly of genera *Synechococcus* and *Prochlorococcus*, and picoeukaryotes, represented by a broad range of algal classes (Vaulot et al., 2008).

During the development of marine microbiology, focus on picoeukaryotes has been smaller and they have been treated as a bulk assemblage for decades (Massana, 2011). Although molecular methods

have exposed the wide diversity of this group, the taxonomic overviews of marine eukaryotic picoplankton are still rare. Comparisons of different studies tend to be further complicated since the size range for the pico-size eukaryotes is often considered to be 3 μm or less, opposed to the size limit proposed for picoplankton in general (<2 μm) (Vaulot et al., 2008; Gaulke et al., 2010; Massana, 2011). Several studies have provided evidence that the contribution of the smallest eukaryotes to carbon biomass is significant. Nevertheless, knowledge about their role in ecosystems and biogeochemical cycles is especially poor in productive coastal waters that hold considerable amounts of large phytoplankton (Worden et al., 2004; Bec et al., 2005; Vargas et al., 2007; Collado-Fabbri et al., 2011).

Chemtax method can be applied to study PPP by fractionating the water samples using membrane filters of different pore sizes. It is generally acknowledged that sequential filtration may cause over- or underestimating the real values of PPP. Either some large cells could pass through small-pore- size filters allowing them to stop on 0.2 μm filters or some smaller cells could be trapped in filters with larger pore sizes if the pores become clogged. The results of sequential filtering therefore need to be interpreted with some caution. Nevertheless, the method is still used and valued in studies of PPP that involve catching the algal material on filters – e.g. for pigment analysis and DNA amplification (Ansotegui et al., 2003; Lovejoy et al., 2006; Gaulke et al., 2010; Collado-Fabbri et al., 2011). A notable example of this approach can be seen in a paper published by Lepere et al. (2009) about photosynthetic picoeukaryote community structure in the South East Pacific Ocean where Chemtax analysis revealed Prymnesiophytes as a major component of eukaryotic picoplankton. Even though nearly all described species of this group are larger than 3 μm, it was recently established using fluorescent in situ hybridization that there are indeed pico-size cells in this group. Hence, Chemtax can provide very valuable insight to picoeukaryote communities.

2.4. Chl *a* measurements with remote sensing methods

While the taxonomic groups can be easily revealed with pigment-based chemotaxonomy, harmful algal blooms can be tracked most effectively with remote sensing methods that are able to provide detailed information about the total Chl *a* over very large areas. The concentration of Chl

a serves commonly as a proxy for phytoplankton biomass and the ecological status of water-bodies.

The Baltic Sea is one of the most polluted seas globally. Human induced growth of primary production has increased the magnitude and frequency of the naturally occurring cyanobacterial blooms in the Baltic Sea (Bianchi et al., 2000; Kahru & Elmgren, 2014). Spatially and temporally heterogeneous nature of the blooms complicates the research and comprehension of the occurrence and magnitude of these events (Kutser, 2004). However, this information is critical and modern monitoring networks should aim at tracking finer-scale changes in phytoplankton community composition.

Despite remote sensing is one of the finest and most cost-effective tools for capturing the blooms, the quantification of blooms is very complicated in shallow coastal areas. The presence of CDOM further complicates the correct quantification of Chl *a*. In open ocean waters (case-1 waters), the main optically active components are typically associated to the life cycle of phytoplankton, hence well correlated with Chl *a*. In turbid and productive coastal and lacustrine waters (case-2 waters), however, the constituents, such as suspended matter and CDOM, can be of various origin and therefore mostly not correlate with each other. This makes the optical properties of the water in these areas region-specific. Consequently, the coastal waters of the Baltic Sea require regional parameters and additional validation for trustful calculations of Chl *a* concentrations (Kutser, 2004; Cui et al., 2010; Harvey et al., 2015; Matthews & Odermatt, 2015).

3. AIMS AND HYPOTHESES OF THE STUDY

The main objective of this thesis is to encourage the use of pigment-based chemotaxonomy in lakes and coastal areas. A broader aim is to urge the employment of the benefits that modern methods provide for phytoplankton quantification additionally to traditional methods.

In the present thesis we have set the following aims:

1. To analyze the effectiveness of the pigment-based method to detect changes in phytoplankton seasonally and during short bloom periods in a large shallow and eutrophic lake (I).

2. To assess the sufficiency of suggested specific marker pigments and published marker pigment:Chl *a* ratios for tracking seasonal changes in eutrophic freshwater environment (I).

Hypothesis: *Chemtax method is applicable and the published marker pigment:Chl a ratios provide a realistic basis for proper estimation of the seasonal dynamics of phytoplankton groups in large shallow eutrophic lakes.*

3. To assess the structure and abundance of late summer PPP community in a brackish and eutrophic coastal sea area via algal pigments (II).

4. To determine the taxonomic composition of picoeukaryotes and their contribution to the total PPP in a brackish and eutrophic coastal sea area (II).

Hypothesis: *Picoeukaryotes, not distinguishable by traditional microscopic methods, display broad taxonomic diversity and constitute a substantial part of PPP in productive coastal sea areas.*

5. To assess phytoplankton community composition by pigment-based chemotaxonomy and validate the results with microscopic counting method in a brackish and eutrophic coastal sea area (III).

6. To improve the retrieval of information about phytoplankton communities by combining remote sensing with laboratory based approaches and develop a region-specific algorithm to calculate

cyanobacteria biomass from reflectance spectra in a brackish and eutrophic coastal sea area (III).

Hypothesis: *Different pigment-based methods and combining them with standard phytoplankton monitoring would boost the power of detection of harmful algal blooms in optically complex coastal areas.*

4. MATERIALS AND METHODS

4.1. Study sites and sampling

Studies were conducted in the West-Estonian Archipelago Sea (Väinameri in Estonian, part of the Baltic Sea) and Lake Võrtsjärv (Fig. 2). The Väinameri is a shallow (average depth <10 m) area between the mainland Estonia and its western islands located in the north-eastern Baltic Sea. Sampling points in the Väinameri covered the area with variable depth, optical properties and Chl *a* concentration (III, Table 3). The summer phytoplankton community in the area features different, mostly cyanobacterial, dominants – e.g. *Pseudanabaena limnetica*, *Aphanizomenon flos-aquae*, *Nodularia spumigena* (Kotta et al., 2008). In the Väinameri studies (II, III), the surface water samples were collected from 14 stations in July 2013 simultaneously with optical measurements. All samples were kept refrigerated in the dark until onshore processing where subsamples were taken for spectrophotometric measurements, flow cytometry, molecular analysis (not described in this thesis), microscopy and HPLC.

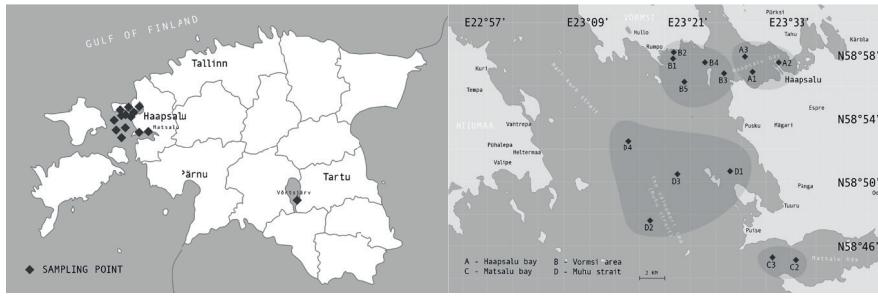

Fig. 2. Study sites and sampling points in Estonia (left). Closer view of the study site and sampling stations in the Väinameri (right; II, III).

Lake Võrtsjärv is a large (270 km^2) shallow and eutrophic lake. Phytoplankton community is dominated by cyanobacteria – around two-thirds of the total phytoplankton biomass during summer, followed by diatoms. Most of the biomass is built up by two filamentous cyanobacteria species *Limnothrix planktonica* and *L. redekei* (Nõges & Nõges, 2012). Depth-integrated samples from the lake were collected monthly in 2009–2012 and fortnightly during the vegetation period of 2013 near the lake's deepest point (Fig. 2). Subsamples for water

chemistry, spectrophotometric measurements, HPLC and microscopy were subtracted from the water sample in laboratory.

In both study sites Secchi depth and water temperature were also recorded.

4.2. HPLC and spectrophotometric measurements

For HPLC measurements of pigment content, water samples (50–200 mL in Võrtsjärv and 500 mL in the Väinameri) were vacuum filtered through Whatman glass fibre filters GF/F (47 mm, 0.7 μm pore size). Filters were placed in plastic vials, frozen immediately and stored at -70 C° until further analysis. For the picoplanktonic fraction in the Väinameri, seawater was vacuum filtered sequentially through the following 47-mm diameter filters with decreasing pore size: 8 μm, 2 μm (both Whatman Nuclepore) and 0.2 μm (Whatman nylon membrane filters). Pigments were extracted with mixture of 100% acetone/internal standard (2 mL) and sonicated (Branson 1210) for 5 minutes. Extracts were kept in dark at −20 °C for 24 h and filtered through 0.45 μm syringe filters (Millex LCR, Millipore) before HPLC analysis.

All samples were further processed with reversed-phase HPLC (Shimadzu Prominence, Japan) with a photodiode-array (PDA) and fluorescence (excitation at 440 nm, emission at 660 nm) detectors for separation of phytoplankton pigments. The latter provided correct measurements of Chl *a* even at low concentrations. 0.5 M ammonium acetate was added in a volume ratio of 2:3 to each sample before the injection. To avoid chemical decomposition of the pigments, the autosampler was cooled to +5 °C (Reuss & Conley, 2005). Pigment separations were completed in a reversed-phase mode by using two Waters Spherisorb ODS2 3 μm columns (150 mm × 4.6 mm inner diameter) in-line with a pre-column (10 mm × 5 mm inner diameter). A binary gradient elution method was used (isocratic holds 0–2 and 30–43 min) with a fixed flow rate (0.8 mL/min) throughout the elution. Absorbance was detected from 350 to 700 nm. Data analysis was done with the 'LC solution ver. 1.22' (Shimadzu) software. For peak identification and quantification the external standards (DHI, Denmark) were used. The HPLC-Chl *a* data was compared with both spectrophotometric Chl *a* and microscopy biomass data for all samples.

Spectrophotometric measurements were always calculated according to Jeffrey & Humphrey (1975). In both study sites the same pigment extract was used for HPLC and spectrophotometric measurements. In the Väinameri the additional spectrophotometric measurements were performed with ethanol (96%) extracted pigments.

4.3. Chemotaxonomic analysis

HPLC pigment data was further processed with matrix factorization program Chemtax (version 1.95) which calculates the relative abundance of phytoplankton classes in the total Chl *a* of a water sample (Mackey et al., 1996). Chemtax employs a steepest-descent algorithm to find the best fit to the data based on an initial estimate of pigment ratio matrix for different algal classes. Exact calculation procedures are described in Mackey et al. (1996, 1997). Initial pigment ratio matrices were constructed using published marker pigment:Chl *a* ratios (Mackey et al., 1996; Marinho & Rodrigues, 2003; Schlüter et al., 2006; Greisberger & Teubner, 2007), which were applied according to previous knowledge about phytoplankton classes present in Võrtsjärv and the Väinameri area. Several initial pigment ratio matrices were constructed and tested to find the best marker pigments, Table 1) and marker pigment:Chl *a* ratios for each phytoplankton group (I, Table 2; II, Table 1) according to microscopy. Calculation settings for Chemtax were fixed according to the suggestions of Mackey et al. (1997).

Table 1. Phytoplankton classes and their associated marker pigments in the study sites

Algal class	**Marker pigment**	**Site**
Cyanobacteria	Zeaxanthin	Lake Võrtsjärv; Väinameri
Chrysophytes	Fucoxanthin, diadinoxanthin	Lake Võrtsjärv; Väinameri
Dinoflagellates	Peridinin	Lake Võrtsjärv; Väinameri
Cryptophytes	Alloxanthin	Lake Võrtsjärv; Väinameri
Chlorophytes	Chlorophyll-*b*; lutein	Lake Võrtsjärv; Väinameri
Diatoms	Fucoxanthin	Lake Võrtsjärv; Väinameri
Haptophytes	Diadinoxanthin	Väinameri

4.4. Microscopy counts

Phytoplankton samples were fixed with acid Lugol's solution and stored in cool and dark. Samples were settled according to Utermöhl (1958) and counted under inverted microscope using 200-600x magnifications. The volume and settling time varied between 3–25 ml and 12–24 h, respectively. All taxa were identified to the lowest possible level (species or genus). Volume for each counting unit was calculated by applying the formulae of the closest geometric shape and converted into wet biomass assuming a specific gravity of 1 g/cm^3 (Kovala & Larrance, 1966; Hillebrand et al., 1999; Olenina et al., 2006). For comparision with Chemtax data, microscopic counts of phytoplankton were summed up by main classes: diatoms, cryptomonads, cyanobacteria, chlorophytes, dinoflagellates chrysophytes and haptophytes (only in the Väinameri).

4.5. Flow cytometry

In addition to pigment-based chemotaxonomy, the PPP biomass was assessed with flow cytometry in the Väinameri area (II). Samples were fixed with paraformaldehyde and glutaraldehyde and processed with Accury C6 flow cytometer (Becton Dickinson, USA). For details see Tamm et al. (2018). Based on the results, three significant PPP groups were distinguished according to the size and pigmentation of the algal cells: (1) phycoerythrin (PE) containing *Synechococcus* (PE-type *Synechococcus*); (2) PE-lacking *Synechococcus* that likely are phycocyanin-rich strains of *Synechococcus* (PC-type); (3) coccoid eukaryotic (Peuk) cells that lack orange pigments. Carbon biomass of PPP was calculated by conversion from cell abundances (Campbell et al., 1994; Buck et al., 1996).

4.6. Optical in situ measurements

The optical measurements were a part of the study conducted in the Väinameri (III) to upgrade the retrieval information about phytoplankton community. The reflectance measurements above water were conducted with handheld instrument WISP-3. The details about the instrument and measurements are described in Tamm et al. (2019). The Chl *a* concentrations from reflectance were calculated using two approaches that have shown good results in Estonian coastal areas (Ligi et al., 2017): (1) 693.5 nm and 679 nm (Dierberg & Carriker, 1994); (2) 706.5 nm and

677.5 nm (Kallio et al., 2001). To obtain a region-specific algorithm to calculate cyanobacteria biomass from reflectance spectra, the suitability of the 709 nm to 620 nm ratio (Simis et al., 2005) was tested.

4.7. Statistical analysis

Data analysis was carried out using the statistical package R (version 2.15.3, R Core Team, 2014). Since the data retrieved with microscopy and Chemtax about phytoplankton groups almost entirely lacked normal distribution, logarithmic transformation was applied before the Pearson correlation coefficients (r_p) were calculated and the regression analysis performed. If log-transformation did not result in normal distribution, the Spearman rank order correlation coefficient (r_s) was calculated instead. To analyse the spatial variation of picoplankton (II), Chemtax and microscopy data (III), the aggregated data from four studied regions was used. To visualise the dynamics of phytoplankton groups in Lake Võrtsjärv described by microscopy and Chemtax (I), Loess curve was fitted and overlaid on data.

5. RESULTS AND DISCUSSION

5.1. Phytoplankton cell counts and Chemtax

The pigment-based approach proved to be a suitable tool to quantify the seasonal cycle of phytoplankton in a large shallow and eutrophic lake (Fig. 3). Microscopy and Chemtax demonstrated significant correlations for nearly all phytoplankton groups (Fig. 4). Strongest correlations occurred for the dominant groups in the lake – cyanobacteria (r_s=0.87, $p<0.001$), diatoms (r_s=0.77, $p<0.001$) and chlorophytes (r_s=0.74, $p<0.001$). During the vegetation periods, nearly two-thirds of the total Chl *a* in Lake Võrtsjärv was contained in cyanobacteria, almost always dominated by *L. planktonica* and on rarer occasions by *L. redekei* (Fig. 3). Statistically significant but weaker correlations appeared for cryptophytes (r_s=0.52, $p<0.001$) and chrysophytes (r_s=0.27, $p<0.05$) which had relatively small share in the total phytoplankton biomass. Surprisingly the only statistically non-significant correlation appeared for dinoflagellates (I). Our study confirmed that the published marker pigment:Chl *a* ratios are applicable to quantify algal groups, however, calibration for specific type of environment is needed (I).

Similarly in the Väinameri, chemotaxonomic analysis of the major phytoplankton groups, such as cyanobacteria (r_p=0.73, $p<0.01$) and dinoflagellates (r_p=0.64, $p<0.05$), displayed strong correlations with microscopy counts (III). *A. flos-aquae* was the most abundant species (around 85% of all cyanobacteria) in all areas except the Matsalu Bay. In this area the relative share of the species was smaller (around 50%) and co-dominated by *Dolichospermum flos-aquae* (~25%). Among the less abundant algal groups the correlations between microscopy data and Chemtax were statistically significant only for cryptophytes (r_p=0.71, $p<0.01$).

Several reports have likewise demonstrated weak correlations between microscopy counts and Chemtax analysis for the minor phytoplankton groups (Eker-Develi et al., 2008; Goela et al., 2014; Schlüter et al., 2016). One of the most important reasons for the inconsistency between these methods could be attributed to the sample volume. For the microscopy, sample volumes remain commonly around 50 mL (Paxinos & Mitchell, 2000). In the Väinameri, the quantity was between 10–25 mL and in

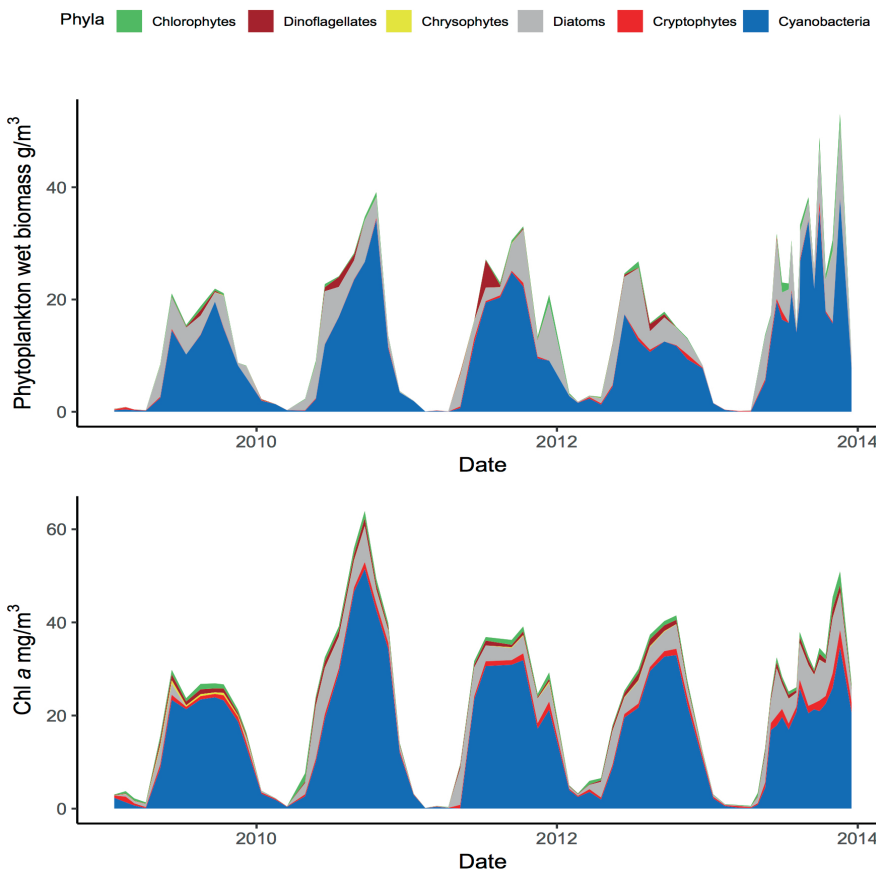

Fig. 3. Dynamics of phytoplankton communities in Lake Võrtsjärv (2009–2013) according to microscopy (upper) and Chemtax (lower) (I).

eutrophic Võrtsjärv around 3 mL (summer samples diluted 5x). For HPLC samples the water volumes filtered were considerably bigger – e.g. in the Väinameri generally 500 mL and in Lake Võrtsjärv around 150 mL. Large sample volume increases the odds of the rare species included in the analysis (Irigoien et al., 2004; Rodríguez-Ramos et al., 2014; Jakobsen et al., 2015). Rodríguez-Ramos et al. (2014) has demonstrated that conventional small volume samples miss around 20-40% of the species, stating that the inadequate sample volume severely underrates the species richness of marine phytoplankton communities. Similarly Schlüter et al. (2016) showed that, compared to microscopy, pigment analysis and Chemtax determined higher Shannon's diversity index and steadily revealed a larger number of phytoplankton groups in a study conducted in more than 40 lakes.

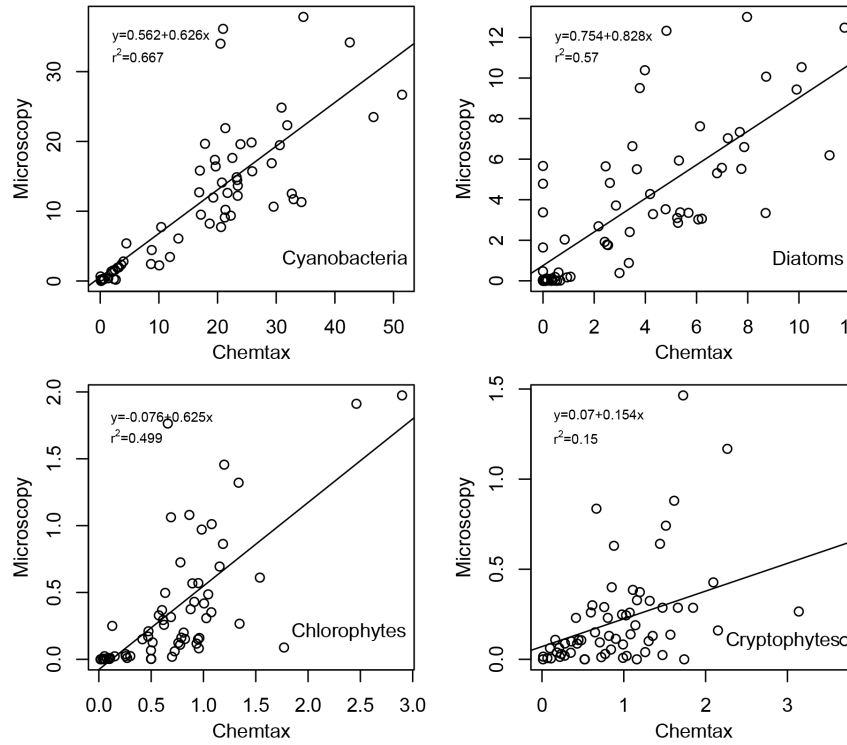

Fig. 4. Linear regression of Chemtax and microscopy results for cyanobacteria, diatoms, chlorophytes and cryptophytes in Lake Võrtsjärv during the years 2009–2013 (I).

In our studies the most obvious underestimation due to microscopy counting error of a minor group occurred in case of dinoflagellates in Lake Võrtsjärv (I, Fig. 5). Two-sample t-test revealed a statistically significant difference between the mean relative contribution of dinoflagellates analysed by Chemtax and microscopy ($t=3.064$, $df=92.7$, $p<0.01$). As HPLC traced the peridinin, an unambiguous marker for dinoflagellates, the group was very likely present in the phytoplankton community of Võrtsjärv. However, the rare large individuals of, e.g. *Peridinium* sp. and *Ceratium hirundinella* that built up the biomass, increased the variability of the microscopy counting results. While higher abundances result in higher microscopy counting precision, the less abundant species can produce very large variation in counting results (Jakobsen et al., 2015). The number of counting units (cells, trichomes, colonies) has a direct effect on the counting accuracy and it has been proposed that at least 400 units of each phytoplankton species should be counted to obtain +/- 10% precision with 95% confidence limits (Cassell, 1965). Therefore,

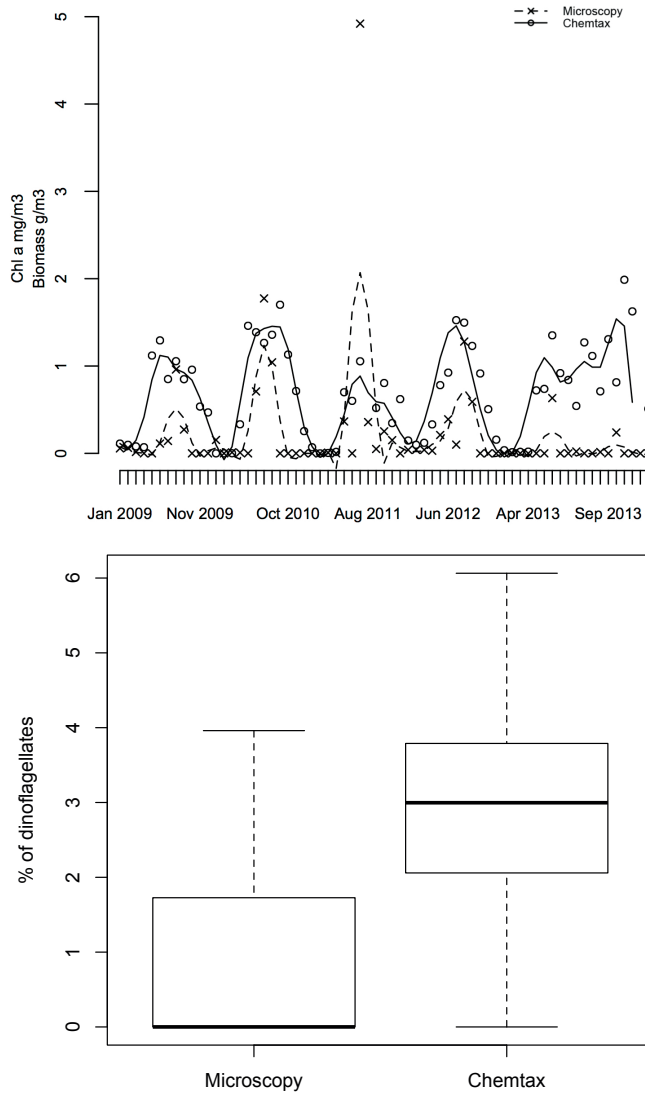

Fig. 5. Dynamics of dinoflagellates in Lake Võrtsjärv (2009–2013) described by microscopy (phytoplankton wet biomass g/m^3) and Chemtax (Chl *a* mg/m^3), loess curve is fitted and overlaid on data (upper, I). Percentage of dinoflagellates in Lake Võrtsjärv according to microscopy and Chemtax (lower).

it is not so surprising that an inter-calibration exercise carried out in Denmark by Jakobsen et al. (2015) that assessed the performance of six phytoplankton taxonomists, showed very discouraging results. Analysis of subsamples from the same sample displayed a 2-fold difference for species abundance and cell volume and the mean total carbon biomass among the analysts showed very high variability (CV 43%).

5.2. Picoplankton in the Väinameri

Pigment analysis revealed that in the Väinameri the contribution of PPP was around 20% of the total Chl *a* (II). Both Chemtax and flow-cytometry suggested that in addition to picocyanobacteria, picoeukaryotes may be equally important and display remarkable taxonomic diversity within the group. Some of the phytoplankton groups detected with Chemtax (e.g. dinoflagellates) might be related to filtering artefacts. Nevertheless, pigment-based chemotaxonomy is a very efficient and cost-effective tool providing a robust estimation about PPP groups. The knowledge about the taxonomic composition of picoeukaryotes is scarce. For instance, the eukaryotic component of freshwater picoplankton has received full attention only from the beginning of the last decade (Callieri, 2008).

Around 50% of the total PPP biomass in the Väinameri was built up by eukaryotes (II). The variety of picoeukaryotes was large, consisting of cryptophytes, chlorophytes, diatoms and small amounts of haptophytes and chrysophytes (Fig. 6, III). Our study revealed a somewhat bigger role of picoeukaryotes (average amount of Chl *a* was 0.087 mg/m^3) than in some previous studies done by Kuosa (1991) and Morán (2007), most probably due to the lower water temperatures in our sampling periods. Picoeukaryotes are known to dominate during colder periods and their significance decreases during summer months in the Baltic Sea. This is partly related to the cyanobacterial blooms that are triggered by the higher water temperatures during summer months (Kuosa, 1991). Very few studies have targeted the taxonomic composition of picoeukaryotes in the Baltic Sea. Hu et al. (2016) used metabarcoding to describe the diversity of pico- to mesoplankton along salinity gradient of the Baltic Sea, but the outcomes of this study are somewhat hard to compare with our study. Although metabarcoding provides very precise information about the taxa, comparing the relative abundances among different taxonomic groups is misleading and no quantitative information is provided (Hu et al., 2016; Sawaya et al., 2019). Therefore, the Chemtax method has an advantage over the metabarcoding since the analysis yields Chl *a* concentrations of each algal class.

The other half of the picoplanktonic Chl *a* in the Väinameri belonged to cyanobacteria (II). Flow cytometry specified that most of the carbon biomass of this group was built up by genus *Synechococcus* – one of the most widespread genera of picocyanobacteria in the open water areas of the Baltic Sea (Kuosa, 1991; Albertano et al., 1997; Laas et al., 2015; Hu

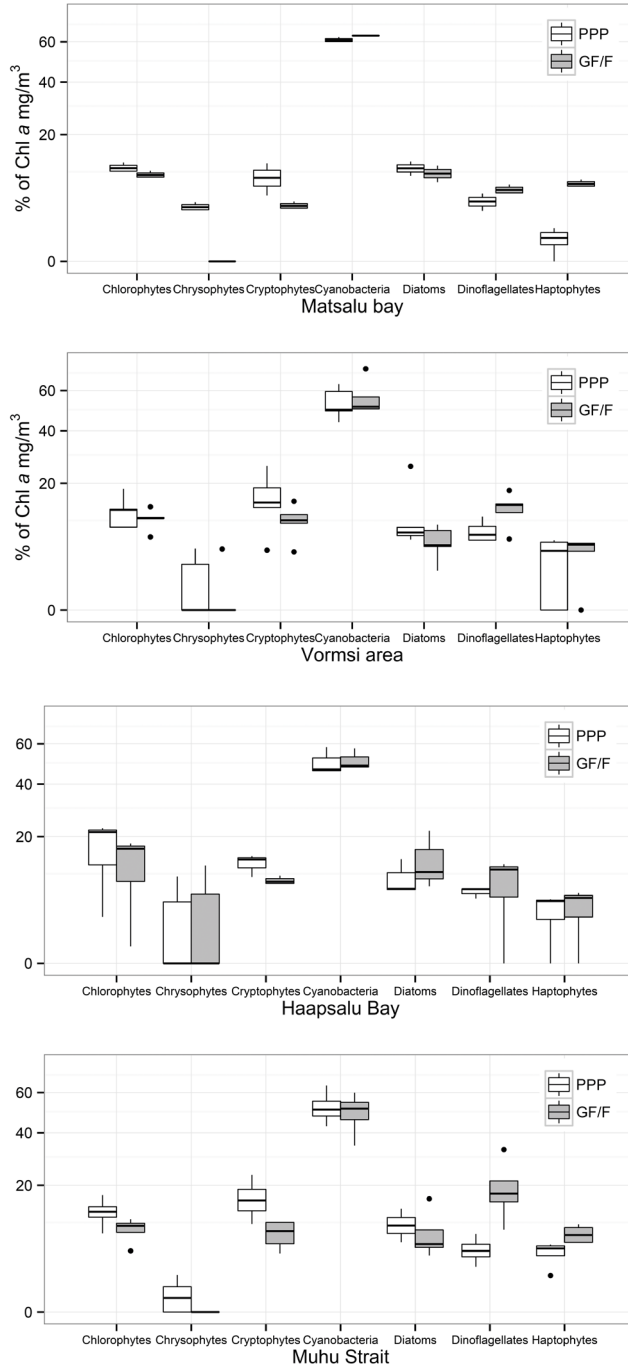

Fig. 6. Proportion of phytoplankton groups on GF/F filters (grey boxplots) and in phototrophic picoplankton size fraction (PPP, white boxplots) in different areas of the Väinameri, calculated by Chemtax (III). Note that on y-axis scale square root transformation is applied (% of Chl *a* values remain original).

et al., 2016). Along with other picocyanobacteria species, two different *Synechococcus* groups were detected – PE-type *Synechococcus* and PC-type *Synechococcus* (II). These two groups are known to coexist as equal players in the coastal areas of the Baltic Sea (Albertano et al., 1997; Stomp et al., 2007; Haverkamp et al., 2009). Our results demonstrated an obvious dominance of PE-type *Synechococcus* in the late summer picocyanobacterial community of the Väinameri (II). The climate change will likely increase the abundance of *Synechococcus* since the maximum growth rates are achieved at much higher temperatures than for other cyanobacteria (Moore et al., 1995; Mackey et al., 2013).

Flow cytometry revealed that *Synechococcus* was more numerous in the open water areas and less important in the bays (II, Fig. 7). This spatial distribution is likely caused by the considerably lower salinity of the bays that have freshwater inflows from rivers.

Our findings suggest that phototrophic picoplankton constitutes an important part of phytoplankton community in the coastal area of the Baltic Sea. The fact that picoplankton has been left out of the current monitoring practices according to EU Water Framework Directive (EC, 2000) and Marine Strategy Framework Directive (EC, 2008), is considered a great mistake by several authors (e.g. Caruso et al., 2015; Bergkemper & Weisse, 2018). In future monitoring networks picoplankton assessment should be included as they are significant contributors to total phytoplankton biomass and sensitive indicators of anthropogenic stressors. There is much space for further progress in determining the role of PPP in the food webs in the context of eutrophication of the Baltic Sea and changing climate. Also the picoeukaryotes require more attention, both at class and species level.

5.3. Optical measurements

Our study in the Väinameri (III) aimed at further improving the remote sensing algorithms in the optically complex coastal area. Hence optical measurements were combined with laboratory measurements. The Chl *a* values calculated from reflectance spectra overestimated the laboratory values (III, Fig. 2), but the correlations were comparable with a previous study done in Estonian coastal waters by Ligi et al. (2017). The r_p remained between 0.73 and 0.8 in both studies. The seemingly bigger overestimation in our study is partly related to the Chl *a* lab measurements (III). It is well known that spectrophotometry

and HPLC hold different degrees of accuracy, the latter being the most accurate. Ligi et al. (2017) performed the Chl *a* measurements only with the spectrophotometer (ethanol extraction), which can generate notably bigger measurement errors due to the Chl *a* degradation products (pheopigments) and accessory pigments with overlapping spectra (Pickney et al., 1994; Kumari, 2005). The methodological issues of spectrophotometry, such as extraction solvent and filter type, may further amplify these differences (Aminot & Rey, 2002; Pápista et al., 2002). Our measurements in ethanol-extracted Chl *a* resulted in about 30% higher Chl *a* concentrations than in acetone-extracted Chl *a* (III). This clearly shows that the methodological issues need more extensive research since the validation of remote sensing algorithms is mostly done only spectrophotometrically. Moreover, the limited comparability of the radiation-based and photometric measurements should be also always kept in mind.

During our study (III), the phytoplankton community of the Väinameri had not reached its late summer peak commonly built up by variety of cyanobacteria. According to microscopy, most of the measuring stations were dominated by filamentous species *A. flos-aquae* and *D. flos-aquae*. Regardless of the low biomass, correlation between cyanobacteria wet biomass and the 709 nm/620 nm reflectance ratio was strong (r_p = 0.75, $p < 0.01$) and the signal was not affected too much by the noise. Our results demonstrate that the longer wave lengths can provide adequate information even at lower cyanobacterial biomass. Hence, the cyanobacterial phycocyanin can be detected and tracked even before the formation of heavy blooms (III). Monitoring of CDOM-rich coastal areas and bloom dynamics research may significantly benefit from the use of the algorithm developed in our study (III, Fig. 5).

5.4. Recent approaches to phytoplankton monitoring

All our results lead to a general fundamental issue regarding modern phytoplankton monitoring. The Utermöhl (1958) method has served scientists and the monitoring networks faithfully for more than a half of century. However, our knowledge about phytoplankton has grown and needs to further improve and adapt to the challenges brought on by climate change since numerous studies have predicted increasing cyanobacteria abundances both in marine and freshwater environments due to elevated temperatures (Elliott et al., 2006; Jeppesen et al., 2009; Paerl & Paul, 2012; Wiedner et al., 2007; Ullah et al., 2018).

Up to now there is no ideal universal method for phytoplankton quantification for any purpose and waterbody. A recent study of Bergkemper & Weisse (2018) points out that WFD (EC, 2000) that relies on inverted micrsocopy for phytoplankton assessment, fails to adequately capture coccal and picocyanobacteria and reflect the biomass fluctuations of phytoplankton species that are sensitive to extreme weather events. Several reports have stated clearly that the conventional monitoring based only on inverted microscopy needs to be combined with newer instruments – e.g. remote sensing, flowCAMs, molecular tools, pigment-based chemotaxonomy (Schaeffer et al., 2013; Bennke et al., 2018; Bergkemper & Weisse 2018, Dörnhöfer et al., 2018). The trade-off that remains between finer-scale monitoring on spatio-temporal range and the financial capacity could substantially benefit from employment of the novel methods.

Our studies confirm that pigment-based approach is a very suitable addition to microscopy (I, II, III). While there are some inaccuracies in quantifying the minor pigments and problems with Chemtax treating them, the combination of Chemtax and microscopy significantly enhances phytoplankton monitoring (Havskum et al., 2004; Irigoien et al., 2004; Schlüter et al., 2014).

Similarly to our studies Schlüter et al. (2014) demonstrated in the Fehmarn Belt area (Baltic Sea) that the large monitoring programmes can benefit a great deal from merging different approaches of phytoplankton quantification. The development of region-specific calculation matrices for Chemtax would therefore lead to more effective monitoring with higher sampling frequency of regions most sensitive to eutrophication such as lakes and coastal areas. In addition to determining phytoplankton groups for ecological assessment, HPLC pigment data can be employed also for other purposes – to capture growth/grazing rate of phytoplankton, to improve the remote sensing algorithms for distinguishing phytoplankton functional groups and to analyse historic changes in phytoplankton communities from sediments (Fig. 7).

While our studies did not specifically focus on employing molecular tools together with Chemtax analysis, there is a great potential in combining them. E.g. Kirchman et al. (2017) applied tag pyrosequences of the 16S rRNA gene successfully together with Chemtax analysis to look into the relationships between bacterial and phytoplankton communities along the salinity gradient in an estuarine system. Molecular tools would also

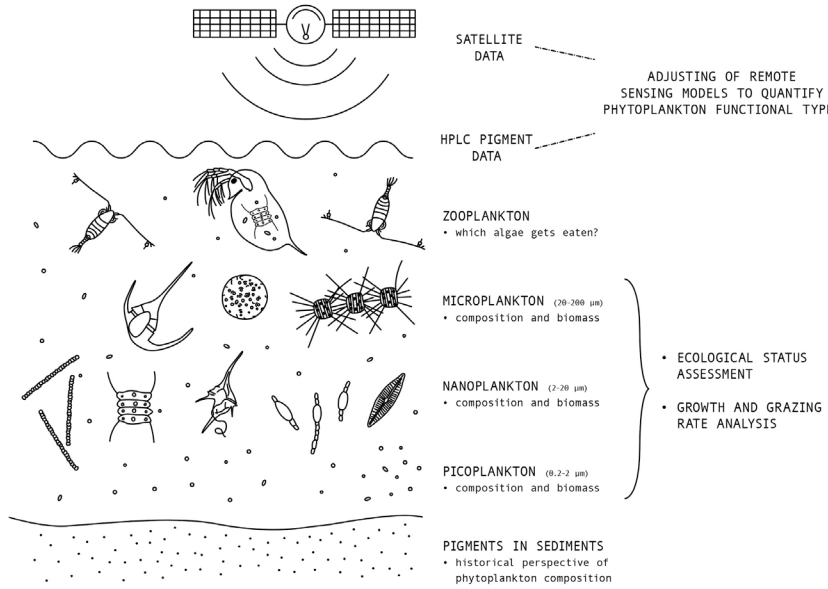

Fig. 7. Potential application of phytoplankton pigments measured with HPLC.

be beneficial to improve the understanding about changes in marker pigment:Chl *a* ratios on the cellular level. This could aid obtaining correct marker pigment:Chl *a* ratios to quantify harmful algal blooms.

It is essential to keep in mind that HPLC pigment data and its interpretation with Chemtax should always be accompanied by microscopy (e.g. quick screening) to guarantee most accurate results (Llewellyn et al., 2011; Schlüter et al., 2016). Chemtax analysis can also be improved significantly by following the suggestions laid out by Higgins et al. (2011), illustrated in Figure 8. Our studies (I, III) confirm that the complementary data about phytoplankton groups and environmental conditions are equally important since they provide basis for constructing proper initial pigment ratio matrices. While these ratios can be found from literature, it is important to ensure that they indeed take into account the properties of the considered ecosystem – e.g. nutrient levels, irradiance, mixing (Descy et al., 2009; Higgins et al., 2011). Since pigment composition and concentrations also change with depth, the analysis should consider using specific marker pigment:Chl *a* ratios. For the Baltic Sea, mathematical formulas have been recently developed to describe the depth profiles of pigments (Stoñ-Egiert et al., 2019).

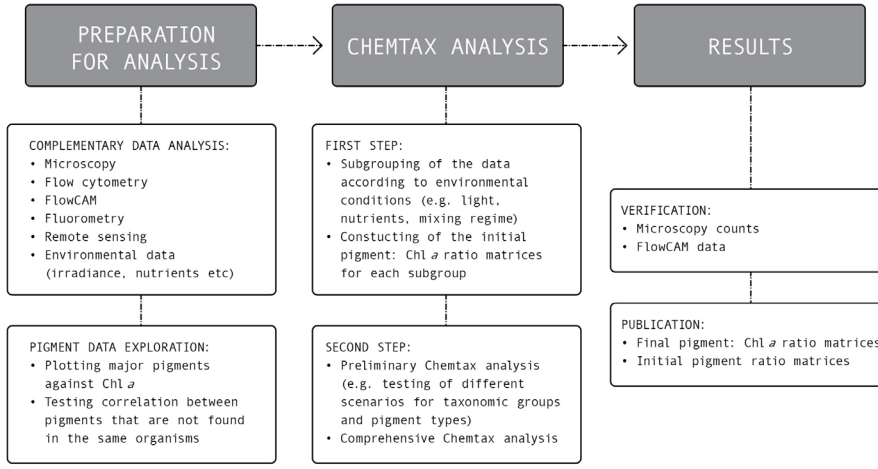

Fig. 8. Guidlines to improve the accuracy of Chemtax analysis, compiled according to Higgins et al. (2011).

Before actual Chemtax analysis, samples also need to be grouped according to similar conditions, e.g. samples from different depths should be analysed separately. This issue has received little attention, but since Chemtax is an optimization method basing on an input matrix of different pigment:Chl *a* ratios, analyzing the same samples in different groupings could give different results (Higgins et al., 2011; Simmons et al., 2016). Preliminary Chemtax analysis is useful to test different scenarios that include various taxonomic groups and pigment types and indicate outliers in the data (Higgins et al., 2011; I). To aid future research, the final pigment:Chl *a* ratio matrices should be published together with ratio boundaries and initial "seed" values (Higgins et al., 2011).

Our studies confirmed that integrated monitoring approach is the only way to improve the understanding of phytoplankton communities and the problems related to them. In addition to pigment-based chemotaxonomy the improvement of remote sensing methods for case-2 waters would boost the phytoplankton monitoring efficiency in nutrient rich coastal areas. We observed a promisingly efficient remote sensing detection of even low cyanobacterial biomass in CDOM-rich waters. This encourages further joint employment of remote sensing and pigment-based chemotaxonomy especially in tracking cyanobacterial blooms. Ideally the future monitoring networks would include remote sensing paired with *in situ* measurements to assess the water quality as proposed also by Schaeffer et al. (2013).

6. CONCLUSIONS

The main objective of this thesis was to promote the use of pigment-based chemotaxonomy for phytoplankton monitoring in lakes and coastal sea areas. Our work elucidates the potential of these applications and provides recommendations how to improve Chemtax techniques for such estimations.

In Lake Võrtsjärv Chemtax provided highly comparable results with microscopy. Our study demonstrated that the Chemtax method is applicable and the published marker pigment:Chl *a* ratios provide a realistic basis for proper estimations of phytoplankton groups' seasonal dynamics in large shallow eutrophic lakes. Good agreement between microscopy and Chemtax was obtained for most phytoplankton groups – cyanobacteria, chlorophytes, diatoms and cryptophytes. The pigment-based method also helps to describe adequately the less abundant groups that are poorly quantified by microscopy counting due to small sample volume (I).

Our analysis showed that Chemtax can provide valuable insight into picoplankton community in a brackish and eutrophic coastal sea area. We saw that in late summer, phototrophic picoplankton can constitute an important part of phytoplankton population – about 20% of the total Chl *a* in the area belonged to PPP. The parallel use of Chemtax and flow cytometry revealed the share of eukaryotic and prokaryotic part of PPP in Väinameri area. We determined that pico-size eukaryotes formed about half of the late summer phototrophic picoplankton community consisting of a large variety of algal groups – cryptophytes, chlorophytes, diatoms and small amount of haptophytes and chrysophytes. Flow cytometry specified that picocyanobacteria were clearly dominated by phycoerythrin containing *Synechococcus*. Although molecular methods provide most detailed information about the picoeukaryote species composition, pigment-based chemotaxonomy is an efficient and more cost-effective alternative (II).

Chemtax calculations about phytoplankton groups showed good agreement with microscopy also in the brackish and eutrophic coastal area of the Baltic Sea (Väinameri). The integrated monitoring by combining remote sensing with laboratory based approaches improved

the knowledge on phytoplankton communities in optically complex coastal area of the Baltic Sea. Hence, we strongly recommend to pair these methods in economically efficient way to monitor this type of areas. The promisingly efficient remote sensing detection of cyanobacteria even at low concentrations boosts the detection of harmful algal blooms in CDOM-rich waters and encourages the further joint employment of remote sensing and pigment-based chemotaxonomy (III).

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SUMMARY IN ESTONIAN

Pigmentidel põhinev kemotaksonoomia – tõhus vahend fütoplanktoni koosseisu selgitamiseks järvedes ja rannikumeres

Autotroofne fütoplankton toodab ligi poole globaalsest primaarproduktsioonist (Boyce et al., 2010). Kuna fütoplankton on veekogude toiduahelate ja biogeokeemiliste ringete võtmekomponent, mängib ta ka veekeskkondade seires väga olulist rolli. Igasugune seiretegevus, mille eesmärgiks on hinnata vee kvaliteeti ja veeökosüsteemide seisundit, ei saa üle ega ümber fütoplanktoni taksonoomiliste rühmade määramisest ja mõõtmisest. Samal põhjusel kasutab ka näiteks Euroopa Liidu veepoliitika raamdirektiiv (EC, 2000) fütoplanktonit ühena neljast bioloogilisest veekvaliteedi näitajast, mis võimaldavad hinnata veekogu ökoloogilist seisundit.

Seiretegevuses peab pidevalt otsima tasakaalu piisava mõõtmissageduse ja piiratud eelarve vahel. Just finantsplaan dikteerib alatihti võimaliku mõõtmissammu, kuid saadud andmestik ei pruugi olla piisav fütoplanktoni rühmade ajalis-ruumiliste muutuste kirjeldamisks (Schaeffer et al., 2013; Skeffington et al., 2015; Bergkemper & Weisse, 2018). Kaasaegsete tehnoloogiate kasutusele võtmine traditsioonilise mikroskoobiga fütoplanktoni loendamise kõrval laiendab oluliselt seirevõrgustike ulatust, seejuures märkimisväärselt kulutusi suurendamata.

Traditsiooniline Utermöhl'i (1958) loendusmetoodika võimaldab saada üksikasjalikke andmeid fütoplanktoni koosluse kohta, kuid nõuab vilunud spetsialisti rohket tööd pöördmikroskoobiga. Sellisele analüüsimisele kulub palju aega ja tööjõudu ning tavaseire tarvis on selline detailne liigiline eristamine ka tarbetu. Siinkohal on fütoplanktoni markerpigmentide kasutamine (Chemtax mudeliga) suurepäraseks alternatiiviks traditsioonilisele lähenemisele.

Käesolev doktoritöö analüüsib pigmentidel põhineva kemotaksonoomia rakendamisvõimalusi erinevates veekogudes. Suures, madalas ja eutroofses Võrtsjärves ning eutroofses rannikumeres (Väinameri) kirjeldatakse fütoplanktoni gruppe nii pigmentide alusel kui ka mikroskoopiale toetudes. Mõlema uurimisala veed on rikkad värvunud lahustunud orgaanilise aine (CDOM) poolest, mis muudab nad optiliselt küllaltki

keerukateks. Fütoplanktoni pigmente rakendatakse ka fototroofse pikoplanktoni koosluse uurimiseks Väinameres. Doktoritöö laiemaks eesmärgiks on täiustada fütoplanktoni seiret ning selleks töötati välja riimveelisele ja eutroofsele rannikuveele sobilikud kaugseire algoritmid arvutamaks sinivetikate biomassi peegeldusteguri spektrist.

Selgitasime välja, et Chemtax võimaldab edukalt kirjeldada fütoplanktoni koosluse struktuuri ning sesoonset dünaamikat suures, madalas ja eutroofses järves. Ühtlasi nägime, et teiste uurijate poolt avaldatud markerpigmentide:Chl *a* suhete kasutamine arvutuste alusena võimaldas saavutada igati realistlikke hinnanguid ka meie uuritavates veekogudes. Pigmentide rakendamine aitas paremini kirjeldada ka vähemarvukaid vetikarühmi ja nende hulka, millele mikroskoopia annab proovi väikese mahu tõttu tihti üsna ebatäpseid hinnanguid (I).

Saime teada, et fütoplanktoni pigmentide kasutamine uuringutes aitab tunduvalt paremini mõista pikoplanktoni koosluses toimuvat ning et fototroofne pikoplankton võib moodustada väga olulise osa kogu fütoplanktoni populatsioonist riimveelises eutroofses rannikuvees. Tegime kindlaks, et piko-suuruses päristuumsed vetikad moodustasid ligi poole kogu hilissuvisest fototroofse pikoplanktoni kooslusest ja nende mitmekesisus oli muljetavaldav – esines neelvetikaid, rohevetikaid, ränivetikaid ja väikeses koguses ka haptofüüte ning koldvetikaid (II).

Nägime, et integreeritud lähenemine fütoplanktoni seirele, mis ühendab kaugseire meetodid laboris tehtud mõõtmistega, parandab oluliselt meie teadmisi fütoplanktoni koosluste kohta Läänemere optiliselt keerukates rannikuvetes. Ohtlike veeõitsengute avastamine ja seire CDOM-rikastes vetes paraneb, kui kasutada sinivetikate tuvastamiseks käesolevas töös välja pakutud kaugseire algoritmi, mis näitas häid tulemusi ka juba madalate rakutiheduste juures. Meie tulemused julgustavad suurendama fütoplanktoni pigmentidel põhineva kemotaksonoomia kasutamist koos kaugseire meetoditega (III).

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RESEARCH ARTICLE

Pigment-Based Chemotaxonomy - A Quick Alternative to Determine Algal Assemblages in Large Shallow Eutrophic Lake?

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Abstract

Pigment-based chemotaxonomy and CHEMTAX software have proven to be a valuable phytoplankton monitoring tool in marine environments, but are yet underdeveloped to determine algal assemblages in freshwater ecosystems. The main objectives of this study were (1) to compare the results of direct microscopy and CHEMTAX in describing phytoplankton community composition dynamics in a large, shallow and eutrophic lake; (2) to analyze the efficiency of the pigment-based method to detect changes in phytoplankton seasonal dynamics and during rapid bloom periods; (3) to assess the suitability of specific marker pigments and available marker pigment:chlorophyll *a* ratios to follow seasonal changes in eutrophic freshwater environment. A 5-year (2009-2013) parallel phytoplankton assessment by direct microscopy and by CHEMTAX was conducted using published marker pigment:chlorophyll a ratios. Despite displaying some differences from microscopy results, the pigment-based method successfully described the overall pattern of phytoplankton community dynamics during seasonal cycle in a eutrophic lake. Good agreement between the methods was achieved for most phytoplankton groups - cyanobacteria, chlorophytes, diatoms and cryptophytes. The agreement was poor in case of chrysophytes and dinoflagellates. Our study shows clearly that published marker pigment:chlorophyll a ratios can be used to describe algal class abundances, but they need to be calibrated for specific freshwater environment. Broader use of this method would enable to expand monitoring networks and increase measurement frequencies of freshwater ecosystems to meet the goals of the Water Framework Directive.

Introduction

Quantification of phytoplankton biomass by taxonomic groups serves as an excellent tool for evaluating water quality and status of aquatic ecosystems [1, 2]. Being a key element of aquatic food webs, phytoplankton has a considerable role in ecological monitoring. European Union has implemented the Water Framework Directive (WFD, [3]), which requires member states

data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

to assess water quality in all water-bodies spanning from ground water to coastal marine waters. Phytoplankton is one of the four biological water quality elements used in the WFD to determine the ecological status of a water-body. While boosting the sensitivity of assessment systems, the application of biological indicators brings about new challenges, such as the growing need for time-consuming taxonomic analyses in the expanding monitoring networks and high variability of biological indicators implying increased measurement frequency.

Phytoplankton community composition and abundance are traditionally determined by microscopy and therefore require much time and highly skilful professionals [4]. Despite strong efforts, results may vary notably among specialists and fail to reflect the physiological status of the taxa. Gained very detailed species level information has in general needlessly high resolution for ecological evaluation during monitoring (e.g. [5, 6]). Another major shortcoming is the high lower size limit (5 μm) of standard microscopy that does not allow determining the amount of autotrophic picoplankton (<3 μm) present [7, 8]. The contribution of autotrophic picoplankton to total primary production may reach 50–90% in oligotrophic lakes and oceans [9] and 30–70% in meso/eutrophic lakes [10].

An advantageous alternative to microscopy is the use of phytoplankton marker pigments for quantification of phytoplankton groups. Estimation of phytoplankton composition is usually achieved combining high performance liquid chromatography (HPLC) and a matrix factorization program called CHEMTAX [11]. Unlike morphological, optical, genetic or biochemical methods, the pigment-based method is suitable for regular monitoring as well as ecological studies since: 1) the time needed for analysis is relatively short; 2) much of the work can be automatized; 3) autotrophic picoplankton is included in the analysis [11–13]. A shortcoming of the pigment-based assessment method is that it does not provide high taxonomic resolution beyond the class level. Therefore it has been suggested to use chemotaxonomy together with quick microscopic screening to gain more specific information about dominant species [14–16].

Despite the fact that chemotaxonomy is well acknowledged and wide-spread in oceanographic studies [8, 17–21], it is still moderately used in freshwater systems [15–16, 22–24]. One of the main difficulties relies upon the fact that CHEMTAX requires specific marker pigment: chlorophyll a (Chl a) ratios to calculate taxonomic composition of algae [11]. It has been shown that these ratios vary for each phytoplankton group depending on the environmental conditions such as light and nutrients and therefore are not constant in time and space [9, 16, 24–26].

There is a relatively large variety of marker pigment:Chl a ratios available for marine ecosystems, but very few for different freshwater ecosystems [16, 24, 27]. To gain accurate assays of phytoplankton composition, more precise ratios are needed [11, 28]. In some studies marine marker pigment:Chl a ratios have effectively been used to describe freshwater phytoplankton (e.g [23]). Still most studies agree that more knowledge about pigment ratios in different types of freshwater systems is needed to improve the CHEMTAX estimations and to use it efficiently in regular monitoring. Although recent studies show significant relationships between CHEMTAX and microscopic counts in different aquatic systems [29–31], validation of marker pigment:Chl a ratios with microscopy data is yet needed [24, 25].

The objectives of this study were (1) to compare the results of direct microscopy and CHEMTAX in describing phytoplankton community composition dynamics in a large, shallow and eutrophic lake; (2) to analyze the effectiveness of pigment-based method to detect changes in phytoplankton seasonal dynamics and during rapid bloom periods; (3) to assess the suitability of available specific marker pigments and published pigment:Chl a ratios for following seasonal changes in a eutrophic freshwater environment. To achieve these objectives, a 5-year (2009–2013) parallel phytoplankton assessment by direct microscopy and by CHEMTAX was conducted with a monthly interval in Lake Võrtsjärv, Estonia.

Fig 1. Sampling location in Lake Võrtsjärv (58°12'40''N, 26°06'20''E).

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Materials and Methods

Study site

Samples were collected from the large (270 km^2) shallow (mean depth of 2.8 m, maximum 6 m) and polymictic Lake Võrtsjärv (58°17'N, 26°03'E) located in a shallow preglacial basin in the southern part of Estonia (Fig. 1). Võrtsjärv is eutrophic with an average total phosphorus concentration of 54 μg/l, total nitrogen concentration of 1.6 mg/l and Chl a concentration of 24 μg/l [32, 33]. Cyanobacterial community forms more than 2/3 of the total phytoplankton biomass and is dominated by two slowly growing shade tolerant species *Limnothrix planktonica* (Wolosz.) Meffert and *L. redekei* (van Goor) Meffert that persistently build up their biomass over the annual cycle reaching the maximum shortly before ice formation [33, 34]. Other common cyanobacteria species in Võrtsjärv are *Planktolyngbya limnetica* (Lemm.) Kom.-Legn. and *Aphanizomenon skujae* Kom.-Legn. and Cronb. The second largest group consists of diatoms and is mostly dominated by centric diatoms from genera *Aulacoseira* and *Cyclotella* [33]. Ice covers the lake for more than 4 months of the year, on average 135 days.

Sampling

Depth-integrated samples were collected monthly in 2009–2012 near the lake's deepest point (58°12'40"N, 26°06'20"E, Fig. 1). During the vegetation period of 2013, we used fortnightly sampling frequency to better record short-term changes in phytoplankton community. Chl a was measured spectrophotometrically by HITACHI U-3010 according to Jeffrey and Humphrey [35]. Secchi depth, water temperature and general meteorological conditions were also recorded. Samples were collected with a 4-L Kemmerer sampler with 0.5 m increments starting from approximately 0.5 m below water surface and finishing half meter above sediment surface. These samples were mixed in a 30-L barrel and processed within 2 hours after collection. Subsamples for water chemistry, spectrophotometric measurements, HPLC and microscopy were taken from the depth-integrated water sample in laboratory. No specific permissions were required for any part of the study and field studies did not involve endangered or protected species.

Microscopic counts

Samples were fixed with 2-% Lugol's iodine solution and stored in cool and dark. Phytoplankton was identified and approximately 400 counting units (cells, colonies, coenobia or trichomes) were counted for each sample under inverted microscope [4]. A transect counting method was used at a 600x magnification. At least one full transect reaching from one edge of the counting chamber to the other along the chamber's diameter was counted and the full chamber area was checked for less abundant large specimen or colonies. Volume for each counting unit was calculated by applying the formulae of the closest geometric shape [36, 37]. Volume was converted into wet biomass assuming a specific gravity of 1 g/cm^3. For purposes of this study, microscopic counts of phytoplankton were summed up by main classes: diatoms, cryptomonads, cyanobacteria, chlorophytes, dinoflagellates and chrysophytes.

HPLC measurements

For pigment analysis integrated lake water samples of 50–200 mL were filtered through Whatman GF/F glass fibre filters (Whatman International Ltd., Maidstone, UK) under gentle vacuum (max. 0.2 bar). Filters were placed in 24-mL plastic tubes and frozen immediately. Samples were stored for not more than 12 months at -70 C° until further analysis. Photosynthetic pigments were extracted from filters with 2 mL mixture of acetone/internal standard and sonicated for 5 minutes with Branson 1210 and kept at -20 C° in darkness for 24 hours. Extracts were filtered through 0.45 μm syringe filters (Millex LCR, Millipore) and stored in darkness at -20 C ° for few hours until HPLC analysis.

Reversed-phase high-performance liquid chromatography (HPLC) was applied, using a Shimadzu Prominence (Japan) series system with a photodiode-array (PDA) detector to separate the phytoplankton pigments. A fluorescence detector with excitation wavelength set at 440 nm and emission at 660 nm was used to confirm correct identification and low concentrations of Chl a. A fluorescence detector with excitation wavelength set at 440 nm and emission at 660 nm was used to confirm correct identification and low concentrations of Chl a. The method was adapted from Airs et al. [38] and slightly modified. As an ion-pairing reagent 0.5 M ammonium acetate was added in a volume ratio of 2:3 to each sample before the injection. To avoid chemical decomposition of pigments, the autosampler was cooled down to +5°C [39]. The sample injection volume was 100 μL. Separations were performed in a reversed-phase mode by using two Waters Spherisorb ODS2 3 μm columns (150 mm × 4.6 mm I.D.) in-line with a pre-column (10 mm × 5 mm I.D.) containing the same phase. A binary gradient elution method (Table 1) was used with isocratic holds between 0–2 and 30–43 min.

Table 1. HPLC elution scheme and solvents used in the separation of phytoplankton pigments.

	Time, min				
	0	2	30	43	50
Solvent A, %	50	50	100	100	50
Solvent B, %	50	50	0	0	50

Solvent A = 80% methanol: 20% 0.5 M ammonium acetate (pH 7.2) (v:v). Solvent B = 80% methanol: 20% acetone (v:v).

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The flow rate of 0.8 mL/min remained constant during the elution. Absorbance was detected at wavelengths from 350 to 700 nm. The software 'LC solution ver. 1.22' (Shimadzu) was applied to collect and analyse the data. The integration of peak areas was made at each pigment absorbance maximum and corrected by internal standard. Commercially available external standards from DHI Water and Environment (Denmark) were used for peak identification and quantification. Standard addition method was used to confirm correct peak identification. Pigments were well separated by this method which was further used for CHEMTAX analysis Fig. 2.

CHEMTAX analysis

HPLC pigment data was processed with matrix factorization program CHEMTAX (version 1.95) which calculates the relative abundance of phytoplankton classes to total Chl a in a water sample [11]. Published marker pigment:Chl a ratios [24, 40, 41], were applied according to

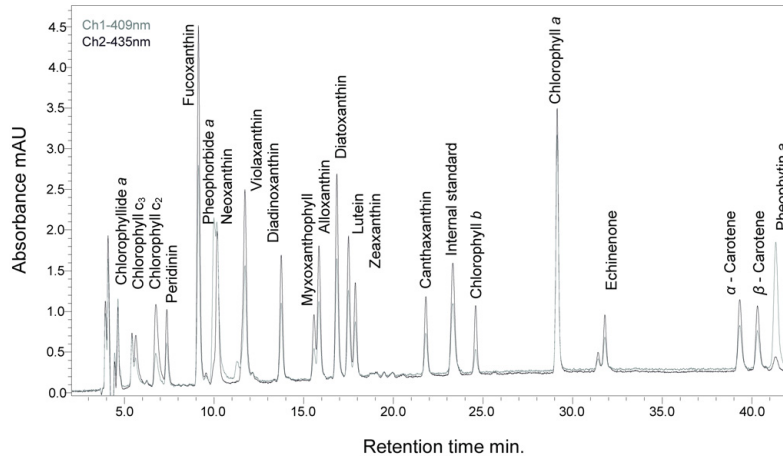

Fig 2. Typical chromatogram of phytoplankton pigment standards. Key pigments, including zeaxanthin and lutein, are well separated.

doi:10.1371/journal.pone.0122526.g002

Table 2. Marker pigment:Chl a ratios used for CHEMTAX calculations: a) initial ratio matrix, b) final ratio matrix.

Class/pigment	Diatoxanthin	Fucoxanthin	Alloxanthin	Lutein	Zeaxanthin	Diadinoxanthin	Chlorophyll-b	Peridinin	Chlorophyll-a
(a) Initial ratio matrix									
Chrysophytes	0.025	0.283							1
Dinoflagellates						0.063		0.21	1
Cryptophytes			0.532						1
Chlorophytes				0.148			0.356		1
Cyanobacteria					0.117				1
Diatoms		0.343							1
(b) Final ratio matrix									
Chrysophytes	0.229	0.553							0.218
Dinoflagellates						0.165		0.045	0.791
Cryptophytes			0.261						0.739
Chlorophytes				0.125			0.23		0.645
Cyanobacteria					0.085				0.915
Diatoms		0.186							0.81

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previous knowledge about phytoplankton classes present in Võrtsjärv. Several initial pigment ratio matrices were constructed and tested to find the best marker pigments and marker pigment:Chl a ratios for each phytoplankton group (diatoms, cyanobacteria, chlorophytes, cryptophytes, dinoflagellates and chrysophytes) according to microscopy. Zeaxanthin was set as marker pigment for dominating cyanobacteria group. Fucoxanthin was used for diatoms, lutein and chlorophyll-b for chlorophytes, alloxanthin for cryptophytes, diadinoxanthin and peridinin for dinoflagellates, diatoxanthin and fucoxanthin for chrysophytes. Same initial ratio matrix (Table 2) was used during all five years. Run configuration settings for CHEMTAX were set according to the suggestions of Mackey et al. [42]. Ratio matrix limit was set to 500, initial step size was 10, step ratio 1.3 and cut-off step 1000. To improve biomass estimations, 10 successive runs of CHEMTAX using the output from each run as the input for the next was used as recommended by Latasa [43]. The final ratio matrix is displayed in Table 2.

Statistical analysis

Statistical analysis was performed using the statistical package R (version 2.15.3, R Development Core Team, 2013). To assess the relationship between microscopy and CHEMTAX methods, linear regression was used and Spearman rank order correlation coefficient (r_s) calculated since the data lacked normal distribution.

Results

Spectrophotometrically measured Chl a in Võrtsjärv had a distinct seasonal dynamics with highest values (50–70 mg/m^3) in summer during cyanobacteria dominance and lowest values (<10 mg/m^3) in winter (Fig. 3). The maximum Chl a was recorded in 2010 and the minimum (0.3 mg/m^3) in February 2011. The overall differences between years under investigation were not remarkable. There was a strong positive correlation (r_s = 0.93, $p<0.001$) between spectrophotometrically measured Chl a and HPLC derived Chl a (Fig. 4). Spectrophotometrically measured Chl a tended to overestimate Chl a concentrations for about 15%. Strong correlation (r_s = 0.88, $p<0.001$) was found also between spectrophotometrically measured Chl a and phytoplankton wet biomass. During the vegetation period, Chl a concentration tended to increase

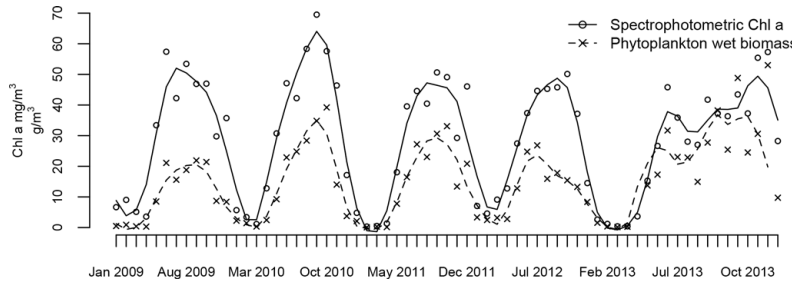

Fig 3. Spectrophotometric chlorophyll-a measurements during years 2009–2013. Loess curve is fit and overlaid on data.

doi:10.1371/journal.pone.0122526.g003

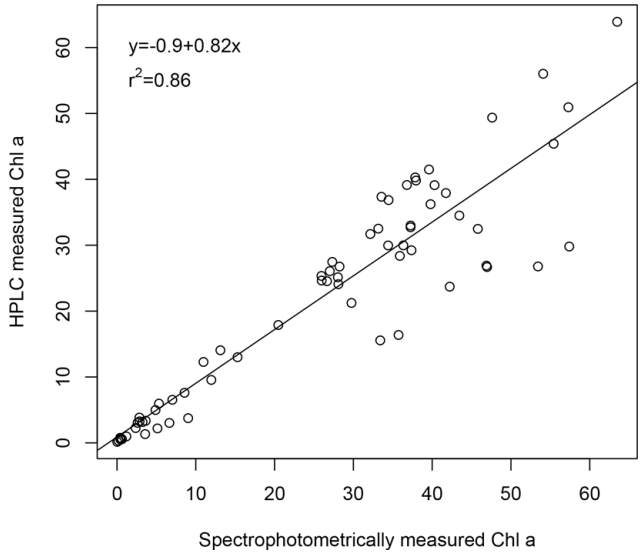

Fig 4. Scatterplot of HPLC measured Chl a and spectrophotometrically measured Chl a in Lake Võrtsjärv (2009–2013).

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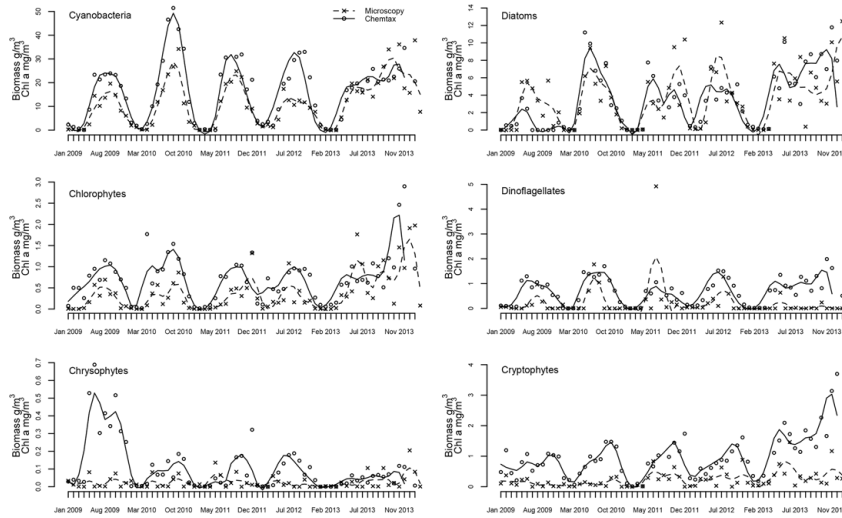

Fig 5. Dynamics of phytoplankton groups in Lake Võrtsjärv (2009–2013) described by microscopy (phytoplankton wet biomass g/m3) and CHEMTAX (Chl a mg/m3). Loess curve is fit and overlaid on data.

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approximately 40% more than the biomass. The average Chl a/biomass ratio was 2.3 in January and 1.5 in July.

Among marker pigments zeaxanthin had the highest concentrations and its abundance patterns followed Chl a dynamics (Figs. 3 and 5). Similarly to Chl a, zeaxanthin values peaked in summer 2010 with the maximum value reaching 4.57 mg/m^3.

Differences between microscopy and CHEMTAX

Overall both techniques had similar outcomes concerning studied phytoplankton class abundances (Fig. 6). Dominant groups (cyanobacteria and diatoms) were detected and well separated from other phytoplankton groups which had minor contribution to total phytoplankton biomass. Best correspondence between the two assessment methods was achieved for cyanobacteria—both methods reflected the summer maxima, winter minima and had similar relative peak sizes (Fig. 5). The scatterplot of microscopy and CHEMTAX results (Fig. 7) also suggests a very good agreement between the methods. Zeaxanthin containing cyanobacteria accounted for about 73% of total Chl a during vegetation periods. Microscopy revealed that the community was nearly always dominated by *Limnothrix planktonica*, on rarer occasions by *L. redekei*.

Seasonal changes were well traced for most groups by both methods though there were inconsistencies for some classes (e.g. diatoms, chrysophytes). Similarly to microscopy, CHEMTAX showed diatoms being the second largest group in phytoplankton community (mostly dominated by genera *Aulacoseira* or *Synedra*), but the relative share of the group varied

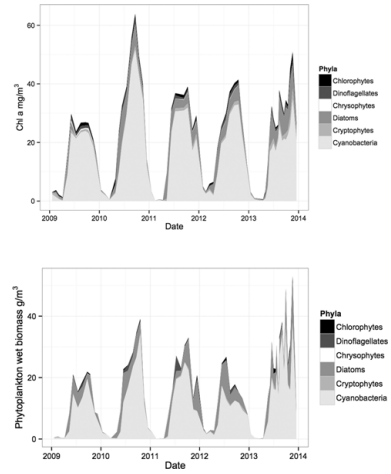

Fig 6. Dynamics of phytoplankton communities in Lake Võrtsjärv (2009–2013) according to CHEMTAX (upper) and microscopy (lower).

doi:10.1371/journal.pone.0122526.g006

considerably in different years depending on the assessment method (Fig. 5). E.g., CHEMTAX calculations suggested significantly ($p<0.01$) lower amount of diatoms in 2009 compared to other years while microscopy did not indicate any significant year-to-year differences. The match between CHEMTAX estimations and microscopy for diatoms was weaker than for cyanobacteria being at about the same level as for chlorophytes (Fig. 7). During the study period, chlorophytes showed no definite dominant species.

Another inconsistency between the methods occurred in chrysophytes (Fig. 5) for which CHEMTAX estimated significantly higher abundance ($p<0.001$) in 2009 whereas microscopy didn't detect any significant differences between the years. The overall diversity of chrysophytes was low and the community was mostly dominated by *Dinobryon* spp. followed by few less abundant species. A similar contradiction between the methods appeared with cryptophytes in year 2013. Alike chrysophytes, the community of cryptophytes was quite homogeneous consisting mostly of *Cryptomonas* spp. and rarely of *Rhodomonas* sp. General agreement between the methods for chrysophytes and cryptophytes was also rather weak (Fig. 5).

Spearman rank order correlations between microscopy and CHEMTAX results were significant for most assessed phytoplankton groups (cyanobacteria, diatoms, chlorophytes, cryptophytes and chrysophytes) except dinoflagellates (Table 3).

Discussion

This study covers a five year period of parallel chemotaxonomic and microscopic analysis of phytoplankton community of Lake Võrtsjärv. Our findings support the hypothesis that pigment-based chemotaxonomy can successfully describe the phytoplankton community

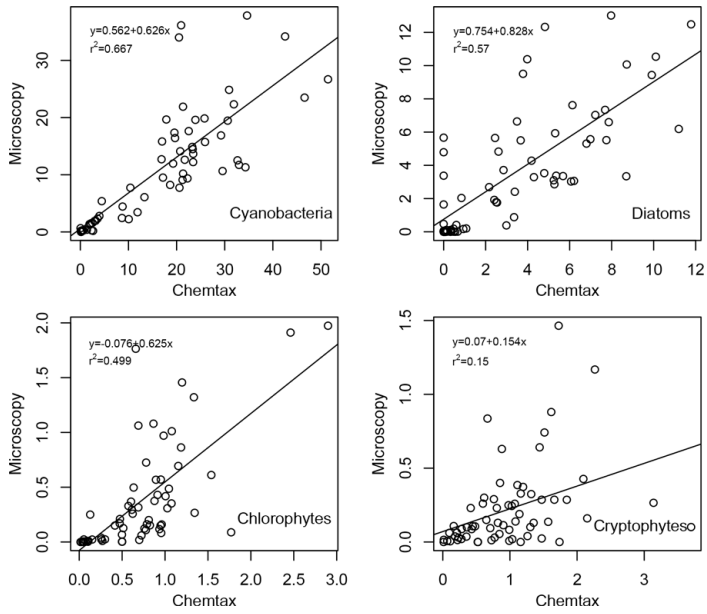

Fig 7. Linear regression of CHEMTAX and microscopy results for cyanobacteria, diatoms, chlorophytes and cryptophytes in Lake Võrtsjärv (2009–2013).

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composition in a large, shallow and eutrophic lake. Good correlations between these two methods were obtained for the more abundant phytoplankton groups—cyanobacteria, diatoms and chlorophytes (Table 3).

The study highlights the need for preliminary knowledge about the phytoplankton community of the lake since this enables selecting the right marker pigments to be used in the initial

Table 3. Spearman rank order correlation coefficients of phytoplankton classes assessment by microscopy and CHEMTAX in Lake Võrtsjärv in 2009–2013.

	r_s	p
Cyanobacteria	0.87	<0.001
Chlorophytes	0.77	<0.001
Diatoms	0.74	<0.001
Cryptophytes	0.52	<0.001
Chrysophytes	0.27	<0.05
Dinoflagellates	0.21	>0.05

doi:10.1371/journal.pone.0122526.t003

ratio matrix for CHEMTAX calculations [26, 44]. For the current study, pigment ratios were acquired from previously published works which were not exclusively done in eutrophic freshwaters [24, 40, 41]. Different matrixes were tried out before constructing the ultimate input matrix which gave most comparable results with phytoplankton microscopy of Võrtsjärv. Same ratios were applied for all years to analyse their capability of responding to seasonal changes.

In addition, phytoplankton wet biomass and Chl a concentrations (HPLC and Spectrophotometry) were compared. Discrepancy between phytoplankton wet biomass and HPLC derived Chl a was most likely caused by photoacclimation to deteriorating light conditions and changes in taxonomic composition of phytoplankton community over summer. A similar shift has been shown in several other studies [45, 46]. Our finding that spectrophotometry gave 15% higher Chl a concentrations than HPLC was well supported by the study of Sørensen et al. [47] in which 15–20% higher spectrophotometric values were observed during an intercomparison exercise. Such disagreement in Võrtsjärv can occur due to the Chl a degradation products (pheopigments) and accessory pigments with overlapping spectra [48, 49]. The HPLC technique measures only the Chl a that matches the commercial standard, while spectrophotometry includes also other Chl a derivatives such as divinyl Chl a, Chl a allomer, and Chl a isomer. Presence of other pigments (e.g. chlorophyll b, c, and the respective degradation products) is known to considerably interfere chlorophyll-a determination [50].

In the present study we observed significant correlations between microscopy and CHEMTAX for most of the phytoplankton groups. Strong correlation between zeaxanthin-derived and microscopically counted cyanobacteria ($r_s = 0.87$, $p<0.001$) are in good agreement with Schlüter et al. [24] who also achieved very high correlation ($r = 0.95$, $p<0.05$) for cyanobacteria in eutrophic lakes using only zeaxanthin as diagnostic pigment. Since zeaxanthin:Chl a ratios are sensitive to light, it is often suggested that the results would benefit from the use of additional pigments—e.g. myxoxanthophyll or echinenone [24, 51]. While this might be inevitable in some cases, our findings show that it is not always required. While testing some of the initial pigment ratios, better consistency was achieved excluding echinenone as additional marker pigment. Same approach was used by Lauridsen et al. [16] in order to avoid errors due to other echinenone-containing algae with high chlorophyll-b levels (e.g. chlorophytes). Using only zeaxanthin as marker pigment for cyanobacteria may avoid distortions that are caused by pigments of other species in phytoplankton community. The good agreement of the two methods tested in Võrtsjärv is likely related to the fact that phytoplankton community was nearly always dominated by same species. This minimised the potential fluctuations of the pigment composition resulting from the variation of algal species.

The correlation between CHEMTAX and microscopy results was also strong for diatoms ($r_s = 0.74$, $p<0.001$) and chlorophytes ($r_s = 0.77$, $p<001$). In eutrophic conditions an even better agreement between the methods ($r = 0.89$) has been previously reported for diatoms [24] whereas in meso- and oligotrophic freshwaters much weaker relationship was achieved by Laurindsen et al. [16] although the same marker pigment was used. This stresses the importance of applying appropriate marker pigment:Chl a ratios.

In the current study the CHEMTAX vs. microscopy correlation coefficient for chlorophytes was significantly higher than found in several other freshwater studies (e.g. [16, 24, 40]). A comparably strong correlation was achieved by Garibotti et al. [20] in Antarctic coastal waters, although only Chl b was used as a marker pigment. Usually additional pigments such as lutein or violaxanthin are used [15, 24].

The agreement between the two methods was generally poorer for less abundant phytoplankton groups (Table 3) such as cryptophytes, dinoflagellates and chrysophytes. These groups never dominated during the vegetation period and the discrepancies were most likely

caused by higher counting errors of these minority groups. Although their biomasses were mostly built up by single genera (by *Cryptomonas* in cryptophytes, *Peridinium* in dinoflagellates, and *Dinobryon* in crysophytes), this small variability of species in certain phytoplankton groups alone could not guarantee good agreement of the different assessment methods.

CHEMTAX generally performs better when the number of marker pigments per class is small or when the cellular content of one of the marker pigments is relatively high as peridinin in dinoflagellates [25]. To the contrary, in our study we did not find significant correspondence of the two methods for dinoflagellates. In our study the CHEMTAX peaks for dinoflagellates tended to be much broader than the microscopy biomass peaks. Since peridinin is an unambiguous marker for dinoflagellates, this refers to the fact that dinoflagellates are present in the phytoplankton community, but are underestimated by microscopy. The biomass of dinoflagellates in Võrtsjärv is very small but is mostly built up by large individuals that both increase the variability of counting results. Moreover, the poor light conditions during summer due to supended particles and high biomass of other phytoplankton species could increase the production of peridinin in light harvesting complexes to sustain the same amount of biomass. This could also add some disagreement to the results achieved by the two methods as seen in Fig. 5.

Cryptophytes were strongly overestimated (especially in 2013) by CHEMTAX (Fig. 5) while good agreement between the methods using same marker pigment and similar marker pigment:Chl a ratios have been achieved by other authors [16, 24]. On one hand, this could imply to specific species composition of phytoplankton community in Võrtsjärv that might cause the difference in alloxanthin:Chl a ratio compared to other lakes. The pigments involved in photoprotection (such as alloxanthin) may increase considerably with high irradiance levels independently from Chl a and can hence cause significant differences in marker pigment:Chl a ratios [9], [24], [26]. Schlüter et al. [24] showed large variability of alloxanthin:Chl a ratios of cryptophyte cultures under different light conditions. This may be another factor causing disagreement between the two methods compared in current study.

We found very weak correlation between CHEMTAX and microscopy results for chrysophytes (Fig. 5, Table 3). Laurindsen et al. [16] also found no significant correspondence for chrysophytes in meso/oligotrophic conditions while, on the contrary, Schlüter et al. [24] had excellent agreement between the two methods for chrysophytes in eutrophic conditions. The weak correlation in our study could be caused by low counting precision of this minority group but also by errors in quantification of minor pigments (such as diatoxanthin in case of chrysophytes) which are shared by several phytoplankton classes and thus may contribute to the discrepancies [22]. Diatoxanthin can also interconvert with diadinoxanthin as was shown in Freiberg et al. [52] that makes the pigment-based chemotaxonomic assessment of chrysophytes and dinoflagellates more difficult. Still it is likely that a separate intercalibration exercise based on high precision counts of the minority groups might yield more consistent results and that the high sensitivity of the CHEMTAX method could prove one of its main advantages lowering the uncertainty of the minority group biomass estimates.

A shortcoming of this study is the fact that we have no microscopy data on picoplankton of Lake Võrtsjärv for the years 2009–2013. Phytoplankton was assessed by both microscopy and CHEMTAX, but picoplankton was included only in the latter. That could account for a considerable amount of variation between the two methods, especially in case of cyanophytes. In future studies, picoplankton counts should always be included.

It is obvious that using pigment-based chemotaxonomy saves time and money. Broader use of this method would enable to expand monitoring networks and increase measurement frequencies as well as precision, especially for the minority groups. Our study confirmed the suggestion by Latasa [43] that generic input of pigment ratio matrixes combined with successive runs of CHEMTAX can give good enough biomass estimates for regular monitoring. Adding

to it a quick microscopic screening of dominant taxa would meet the needs of the WFD [8, 15]. Further studies are needed to determine more precise marker pigment:Chl a ratios in different freshwater environments to improve CHEMTAX estimations and define the main sources of errors.

Author Contributions

Conceived and designed the experiments: MT RF IT. Performed the experiments: MT RF IT. Analyzed the data: MT TN. Contributed reagents/materials/analysis tools: RF IT TN PN. Wrote the paper: MT RF TN PN.

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II

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Parallel assessment of marine autotrophic picoplankton using flow cytometry and chemotaxonomy

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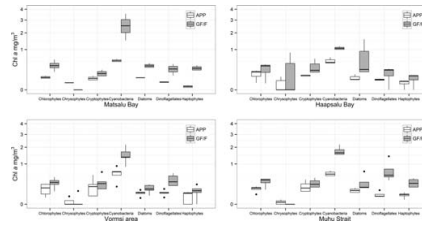
HIGHLIGHTS

- Autotrophic picoplankton was analyzed with CHEMTAX and flow cytometry.
- The study covers area of the West-Estonian Archipelago Sea (Baltic Sea).
- Picoplankton is a significant primary producer in eutrophic coastal areas.
- Picoeukaryotes play an important role in the coastal areas.
- The spatial variability of picoplankton can be considerable even in small regions.

GRAPHICAL ABSTRACT



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ABSTRACT

Autotrophic picoplankton (0.2–2 μm) can be a significant contributor to primary production and hence play an important role in carbon flow. The phytoplankton community structure in the Baltic Sea is very region specific and the understanding of the composition and dynamics of pico-size phytoplankton is generally poor. The main objective of this study was to determine the contribution of picoeukaryotic algae and their taxonomic composition in late summer phytoplankton community of the West-Estonian Archipelago Sea. We found that about 20% of total chlorophyll *a* (Chl *a*) in this area belongs to autotrophic picoplankton. With increasing total Chl *a*, the Chl *a* of autotrophic picoplankton increased while its contribution in total Chl *a* decreased. Picoeukaryotes play an important role in the coastal area of the Baltic Sea where they constituted around 50% of the total autotrophic picoplankton biomass. The most abundant groups of picoeukaryotic algae were cryptophytes (16%), chlorophytes (13%) and diatoms (9%). Picocyanobacteria were clearly dominated by phycoerythrin containing *Synechococcus*. The parallel use of different assessment methods (CHEMTAX and flow cytometry) revealed the share of eukaryotic and prokaryotic part of autotrophic picoplankton.

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1. Introduction

Autotrophic picoplankton (APP) is the smallest fraction of phytoplankton, ranging in size from 0.2 to 2 μm (Sieburth et al., 1978). APP consists of the prokaryotic (picocyanobacteria) and eukaryotic component. The importance of APP was underestimated for a long time but it is now evident that APP can be a considerable contributor to primary production and hence play an important role in carbon flow (Kuosa, 1991; Fogg, 1995; Morán, 2007; Gaulke et al., 2010). APP dominates the autotrophic phytoplankton biomass in most of the world's oceans (Stockner, 1988; Fogg, 1995; Marañón et al., 2001). In oligotrophic

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lakes and oceans the input of APP to the total primary production may reach even 50–90% (Caron et al., 1985; Goericke and Montoya, 1998). In eutrophic conditions the relative contribution of APP to total phytoplankton biomass generally declines (Bell and Kalff, 2001). Picocyanobacteria also constitute an important part of the microbial loop where dissolved organic matter is returned into the food web (Azam et al., 1983; Kuosa, 1991; Worden et al., 2004; Pomeroy et al., 2007).

Being one of the world's largest brackish water basins, the Baltic Sea is exposed to strong anthropogenic pressure. It is one of the most polluted seas worldwide and the long retention time (approximately 25 years) exacerbates many problems that are brought about by organic pollutants and eutrophication. Human-induced climate change is also expected to have a significant impact on the Baltic Sea and the first warming trend is already apparent (Andersson et al., 2015). The large range of salinity (from <1 to ~25 psu) is an important characteristic of the Baltic Sea determining the distribution of phytoplankton species. The projected increase in precipitation resulting in bigger freshwater inflow may decrease the salinity in many areas of the Baltic Sea (Graham, 2004; Kjellström and Ruosteenoja, 2007). Such events have a direct effect on the phytoplankton community leading to changes in the foodweb and hence, the production at higher trophic levels.

The overall phytoplankton community in the Baltic Sea is region specific with no common trends found across all sub-regions (Klais et al., 2011; Wasmund et al., 2011; Klais et al., 2013). The trends in the different regions of the Baltic Sea may even be opposite (Wasmund et al., 2011). Therefore the regional scale studies contribute to revealing the patterns and dynamics of phytoplankton in changing environmental conditions in the Baltic Sea.

During extensive studies of the Baltic Sea, relatively good knowledge has been accumulated on larger phytoplankton. However, the understanding about the composition and dynamics of pico-size phytoplankton is still poor and the focus there tends to be on pico-cyanobacteria. Since the stability of aquatic ecosystems is unquestionably affected by APP, the planktonic microbial communities merit particular attention to improve the understanding of how communities and ecosystems respond to the changing climate (Sánchez-Baracaldo et al., 2008).

Until recently, picocyanobacteria have received more attention than the eukaryotic picoplankton since they have been easier to identify. While the understanding of the significance of picoeukaryotes has improved considerably, most of the data available is still at bulk biomass level. Since epifluorescence microscopy and flow cytometry are deficient in taxonomic resolution, newer molecular and chemotaxonomic methods are starting to fill the knowledge gap in the phylogenetic diversity of picoeukaryotes (Ansotegui et al., 2003; Lovejoy et al., 2006; Not et al., 2007; Massana, 2011; Hugerth et al., 2014).

An interesting hypothesis involving picocyanobacteria in the Baltic Sea proposes that diazotrophic cyanobacterial blooms in the Baltic Sea are controlled by picocyanobacteria that exhaust the formers' nutrient pools (Stal et al., 2003). For further understanding of the controlling mechanisms of cyanobacterial blooms, the information about APP might be more important than we realize now.

This study aims to (1) describe the structure and abundance of late summer APP community in the West-Estonian Archipelago Sea (further denoted by its Estonian name Väinameri for brevity); (2) determine the taxonomic composition of picoeukaryotes and their contribution to the total APP; (3) analyse the agreement between pigment-based chemotaxonomy and flow cytometry in determining the composition of APP.

2. Methods

2.1. Study site and sampling

Sampling stations ($n = 14$) were situated in the Väinameri area (Fig. 1) remaining between the continental Estonia and its western islands in the north-eastern Baltic Sea. This sea area is generally shallow (average depth < 10 m) and the seafloor consists mostly of soft sediments such as mud and sand. Some of the sampling points were located in shallow (average depth 1.5–2 m) and moderately eutrophied bays of Haapsalu and Matsalu which wedge far into the land. Salinity of the region is variable depending on the season and location with higher values occurring in the bay mouth (~5–6.5 psu) and dropping further inside the bays (2.5–4 psu in Haapsalu and down to 0.5 psu in Matsalu) (Kotta et al., 2008). The phytoplankton biomass peaks in late summer when the chlorophyll *a* (Chl *a*) values can reach 17 μg Chl *a* L^{-1} in Haapsalu Bay and 6 μg Chl *a* L^{-1} in Matsalu Bay. The phytoplankton community in Haapsalu Bay has no clear dominant group during summer and different diatom or cyanobacterial species can dominate. In Matsalu bay the abundance of cyanobacteria grows towards the bay mouth with most abundant species being *Pseudanabaena limnetica*, *Merismopedia punctata*, *Aphanizomenon flos-aquae*, and *Nodularia spumigena* (Jaanus, 2003; Kotta et al., 2008).

Sampling took place in July 2013. The surface water samples were collected from the research vessel and kept in a fridge until further onshore processing a few hours later. Secchi depth was measured in all sampling points. Subsamples for spectrophotometric measurements, flow cytometry, and high-performance liquid chromatography (HPLC) were taken from the water sample in the laboratory as soon as possible after the initial water collection.

2.2. Pigment extraction and HPLC analysis

For HPLC measurements of pigment content and composition, seawater samples were vacuum filtered through 47-mm Whatman GF/F (0.7 μm pore size) filters. For the picoplanktonic fraction seawater was vacuum filtered sequentially through the following 47-mm diameter filters with decreasing pore size: 8 μm, 2 μm (both Whatman Nuclepore) and 0.2 μm (Whatman nylon membrane filters). Further we denote the GF/F results as the "conventional" pigment content as this type of filter is used in most standard protocols for pigment analysis (e.g. Chavez et al., 1995; Aminot and Rey, 2002), opposed to the "total" pigment content, i.e. the sum of the results of fractionation filtering that comprises also the 0.2–0.7 μm fraction.

All filters were placed in 5-mL plastic vials, frozen immediately and stored at −70 °C until analysis. Pigments were extracted with acetone containing an internal standard (2 mL) and sonicated (Branson 1210) for 5 min. Samples were kept in dark at −20 °C for 24 h. Extracts were filtered through 0.45 μm syringe filters (Millex LCR, Millipore) before HPLC analysis.

Reversed-phase HPLC (Shimadzu Prominence, Japan) was used with a photodiode-array (PDA) and fluorescence (ex 440 nm, em 660 nm) detectors for the separation of the phytoplankton pigments. The latter assured correct measurements of Chl *a* even at low concentrations. 0.5 M ammonium acetate (ion-pairing reagent) was added in a volume ratio of 2:3 to each sample before the injection. The autosampler was cooled to + 5 °C to prevent chemical decomposition of the pigments (Reuss and Conley, 2005). Pigment separations were carried out in a reversed-phase mode by using two Waters Spherisorb ODS2 3 μm columns (150 mm × 4.6 mm I.D.) in-line with a pre-column (10 mm × 5 mm I.D.). A binary gradient elution method was used (isocratic holds 0–2 and 30–43 min) with a constant flow rate (0.8 mL/min) throughout the elution. Absorbance was detected from 350 to 700 nm. Data analysis was done with the 'LC solution ver. 1.22' (Shimadzu) software. For peak identification and quantification external standards (DHI, Denmark) were used. In respect of accuracy the HPLC Chl *a* data was validated with both spectrophotometric Chl *a* and microscopy biomass data.

2.3. Chemotaxonomic analysis

HPLC pigment data was further used to estimate the algal class abundance with matrix factorization program CHEMTAX (version 1.95).

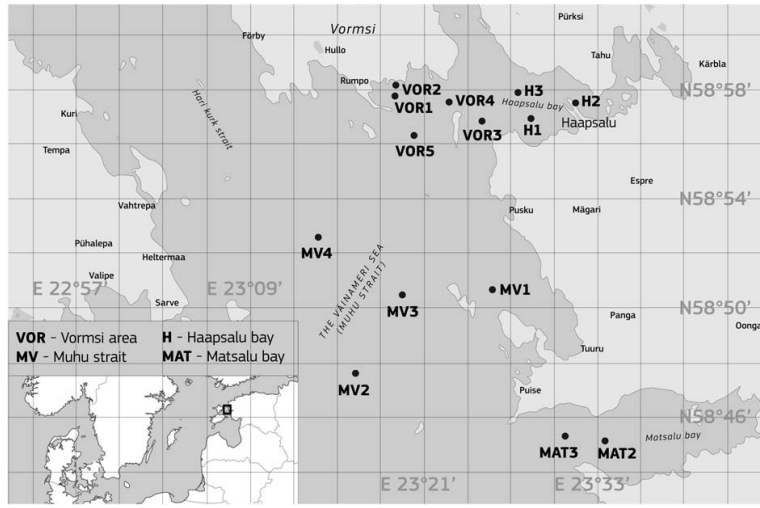

Fig. 1. Study site and measuring stations.

CHEMTAX uses a steepest-descent algorithm to determine the best fit of the data based on an initial estimate of pigment ratio matrix for algal classes. Calculation procedures are further described in Mackey et al. (1996, 1997). The analysis was limited to seven algal groups – chlorophytes (including prasinophytes), dinoflagellates, chrysophytes, diatoms, cryptophytes, cyanobacteria and haptophytes. Contribution of each phytoplankton class to the total Chl *a* was calculated for total (GF/F) phytoplankton community and pico-size algae. Algal groups were determined according to their published marker pigments data (Table 1). Initial pigment ratio matrix used for CHEMTAX calculations was the same in all samples and the CHEMTAX calculation settings were fixed as advised by Mackey et al. (1997). To ensure the precision of CHEMTAX calculations, the pigment data on GF/F filters was verified with microscopy counts (provided in Supplementary Table S1).

2.4. Flow cytometry

Samples for flow cytometry were fixed with paraformaldehyde and glutaraldehyde (final concentrations 1% and 0.05%, respectively; pH = 7.4) and incubated at 4 °C for 30 min. All following flow cytometry analyses were performed on Accuri C6 (Becton Dickinson, USA) equipped with blue (488 nm) and red (640) lasers. Picophytoplankton community was analyzed in unstained samples at flow rate of 66 μL/min and sample volume of 500 μL. The cell morphotypes were discriminated using orange and red fluorescence channels (emission detection ranges of 585 ± 20 nm and 670 nm, respectively). The *Synechococcus* population was identified and gated as in previous flow cytometry studies (Corzo et al., 1999; Veldhuis and Kraay, 2000). Flow-cytometry determined two types of *Synechococcus* – phycoerythrin (PE) containing *Synechococcus* (PE-type *Synechococcus*) and PE-lacking strains of *Synechococcus* that likely are phycocyanin-rich (PC-type) *Synechococcus*. Estimation of carbon biomass of APP was carried out by conversion from cell abundances (Campbell et al., 1994; Buck et al., 1996).

2.5. Statistical analysis

Statistical analysis was performed using the statistical package R (version 2.15.3, R Development Core Team, 2013). Since the data on the Chl *a* of different size fractions was skewed, logarithmic

Table 1
Initial pigment ratio matrix containing marker pigment:Chl *a* ratios used for CHEMTAX calculations.

Class/pigment	Fucoxanthin	Alloxanthin	Lutein	Zeaxanthin	Diadinoxanthin	Chlorophyll-*b*	Peridinin	Chlorophyll-*a*
Chrysophytes	0.63[a]				0.44[a]			1
Dinoflagellates							0.21[d]	1
Cryptophytes		0.41[c]						1
Chlorophytes			0.148[c]			0.356[c]		1
Cyanobacteria				0.223[a]				1
Diatoms	0.343[b]							1
Haptophytes					0.18[a]			1

[a] Mackey et al., 1996.
[b] Marinho and Rodrigues, 2003.
[c] Schlüter et al., 2000.
[d] Greisberger & Teubner, 2007.

transformation was applied before the Pearson correlation coefficient was calculated. Linear regression analysis was conducted with the logarithmic values to examine the relationship between: APP (0.2 to 2 μm) Chl *a* and conventional Chl *a*; relative contribution of APP Chl *a* and conventional Chl *a*; relative contribution of APP Chl *a* and total Chl *a*. Pearson correlation coefficient was also calculated to analyse the relationship between carbon biomass estimations of PE-type *Synechococcus* and chemotaxonomic calculations of picocyanobacteria Chl *a* and between PC-type *Synechococcus* carbon biomass and the difference between conventional Chl *a* and total Chl *a*. To analyse spatial variation, the aggregated data from four studied regions were used.

3. Results

3.1. Chemotaxonomic analysis of APP

The amount of conventional Chl *a* measured from GF/F filters was in the range of 1.5–7.2 mg/m^3 with the average value around 3.4 mg/m^3, Table 2. Highest Chl *a* values exceeding 7 mg/m^3 occurred at station H2 in Haapsalu Bay harbour area while the values near Vormsi Island (on average 2.7 mg/m^3) were slightly lower than elsewhere. In comparison, there was no outstanding peak in APP Chl *a* at station H2 (Table 2), the average amount of this fraction was 1.1 mg/m^3 and the overall variance between the measured stations was quite small. The average contribution of APP Chl *a* to total Chl *a* was about 23% (Fig. 2). The average APP contribution was highest around Vormsi Island (~34%) and lowest in the Haapsalu Bay (~14%).

The conventional Chl *a* values measured on GF/F filters were slightly smaller compared to the total Chl *a* on filters with decreasing pore size (Fig. 2). Only at Station H2 the total exceeded the conventional Chl *a* more than twice (Table 2).

The amount of APP Chl *a* showed a positive correlation ($r = 0.58$, $p < 0.05$) with the conventional Chl *a* (Fig. 3). No significant trend was apparent between the relative contribution of APP and the conventional Chl *a* values (Fig. 4, left). However, the relative contribution of APP showed a significant negative correlation ($r = -0.62$, $p < 0.05$) with the sum of Chl *a* of different size fractions (Fig. 4, right).

CHEMTAX analysis of pigment data (GF/F filters) revealed that in all of the areas of Väinameri cyanobacteria were the main contributors (around 50%) to conventional Chl *a* (Figs. 5, 6). The other half of the phytoplankton biomass generally consisted of a variety of different algal groups without any straightforward spatial patterns or obvious dominants (Figs. 5, 6). Chlorophytes and dinoflagellates were nearly always present and generally more abundant than cryptophytes, diatoms and haptophytes (also mostly present). Station H2 showed slightly distinctive results – no dinoflagellates or haptophytes were detected, the amount of chlorophytes was exceptionally small and there was a large amount of diatoms and chrysophytes (Supplementary Fig. S1, S2). Chrysophytes were altogether detected only at two stations (H2 and VOR1). The pigments of the pico-fraction indicated the relative contribution of cyanobacteria being at about the same level as on GF/F filters in all of the areas of Väinameri (Fig. 6). All other algal groups were usually also present in both fractions, although some differences occurred. For example, in most of the stations the relative amount of cryptophytes in the APP fraction showed a moderate increase while the share of dinoflagellates generally decreased. This trend was more evident in the Muhu Strait and Vormsi area (Fig. 6). Chrysophytes were more often detected in the pico-fraction (7 stations) even though in very small amounts (Supplementary Fig. S2). The average amount of Chl *a* belonging to the group was 0.016 mg/m^3. According to the chemotaxonomic analysis, picoeukaryotes constituted about one half of the APP in the Väinameri (Supplementary Fig. S2).

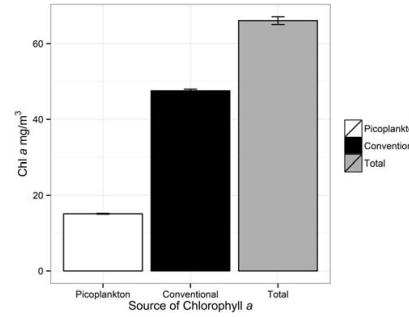

Fig. 2. The sum of autotrophic picoplankton Chl *a* (0.2–2 μm) in all measuring stations compared to the sum of conventional (GF/F) Chl *a* and to the sum of total Chl *a* (on filters with pore sizes 8, 2 and 0.2 μm) in the Väinameri.

3.2. Flow cytometry

Flow cytometry distinguished three major APP groups depending on the size and pigmentation of algae. The carbon biomass estimations of PE-type *Synechococcus* by flow cytometry had positive correlation ($r = 0.6$, $p < 0.05$) with zeaxanthin-based chemotaxonomic calculations of picocyanobacterial Chl *a*. High PE-type *Synechococcus* carbon biomasses appeared in the Muhu Strait (average around 0.045 mg C/L) and Vormsi area (average around 0.038 mg C/L). Low amounts of PE-type *Synechococcus* were detected in Matsalu and Haapsalu bays (Supplementary Fig. S3). Second large APP group consisted of coccoid eukaryotic (Peuk) cells that lack orange pigments. This group was especially abundant at Station H2 and in other parts of Haapsalu Bay

Table 2
The general characteristics observed in measuring stations and Chl *a* values of different size fractions.

Station	N	E	Secchi Depth (m)	Depth to bottom (m)	CDOM 380 (m^{-1})	Conventional Chl *a* (mg/m^3)	Total Chl *a* (mg/m^3)	APP Chl *a* (mg/m^3)
H1	58°57.030′	23°28.399′	2.5	2.5	1.81	1.96	2.05	0.99
H2	58°57.614′	23°31.530′	0.8	2.2	1.75	7.2	17.45	1.11
H3	58°57.988′	23°27.407′	2.6	3.5	5.06	2.46	2.52	0.91
MAT2	58°45.258′	23°33.626′	2.1	2.4	4.61	2.37	3.24	0.73
MAT3	58°45.40980′	23°30.78120′	1.6	3.5	5.16	5.59	6.7	0.99
MV1	58°52.793′	23°50.793′	1.4	3.5	2.28	4.14	5.28	0.96
MV2	58°47.733′	23°15.962′	3.4	5.6	1.67	3.1	3.95	1.25
MV3	58°50.609′	23°19.209	3	6.5	1.69	4.32	5.07	1.59
MV4	58°52.697′	23°13.272′	4	8	1.73	2.85	2.83	0.78
VOR1	58°57.852′	23°18.756′	1.5	1.8	5.92	2.33	3.21	0.82
VOR2	58°58.271′	23°18.757′	3.1	5.2	1.7	4.34	4.39	2.04
VOR3	58°56.956′	23°24.932′	2.4	4	1.69	2.61	3.72	1.33
VOR4	58°57.612′	23°22.576′	2.6	3	1.81	1.53	2.62	0.32
VOR5	58°56.414′	23°20.090′	3	3.2	1.71	2.75	3.07	1.29

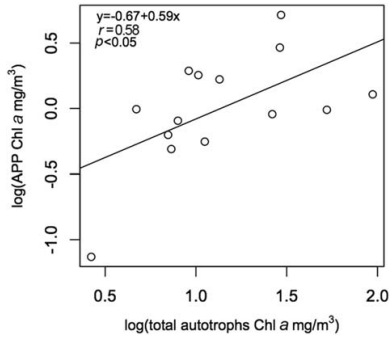

Fig. 3. Relationship between autotrophic picoplankton Chl *a* (0.2–2 μm) and total phytoplankton Chl *a* (mg/m^3) in the Väinameri. Regression line is fitted to data.

(Supplementary Fig. S3). There was no significant correlation between the Peuk group and the amount of picoeukaryote Chl *a* calculated by CHEMTAX.

The relative abundance of the Peuk group was highest (~70%) in the measuring station closest to the coast of Haapsalu Bay (H2), declining in other stations of the area. In Haapsalu bay picoeukaryotes dominated over picocyanobacteria while in Matsalu Bay the relative share of Peuk group was around 50%. The relative share of PE-containing *Synechococcus* increased towards open water areas of Muhu Strait and Vormsi Island (Fig. 7). Flow cytometry indicated relatively small amount of PC-type *Synechococcus* compared to PE-type *Synechococcus* with the exception of H2. This group was slightly more abundant in the bays and less outstanding in other areas (Supplementary Fig. S3).

PC-type *Synechococcus* carbon biomass showed a positive ($r = 0.55$, $p < 0.05$) correlation with the difference between conventional Chl *a* and total Chl *a* of all size fractions (Fig. 8).

4. Discussion

This study demonstrated that APP could play a significant role in the phytoplankton primary productivity – the APP contributed around 20% to the total Chl *a* in the coastal area of Baltic Sea. Pigment analysis and flow-cytometry both suggest that while the cyanobacteria are major players in the APP community, the picoeukaryotes can be also important and exhibit a remarkable taxonomic diversity in this size class.

During our study, the phytoplankton community of the Väinameri most likely had not yet reached its late summer peak as our measured maximum conventional Chl *a* value (around 7 mg/m^3) was much lower than generally registered maximum values around 10–15 mg/^{3} (Jaanus, 2003; Kotta et al., 2008).

Our study found that APP contributed about 20% of total Chl *a* in the Väinameri. Comparing this with the results of other studies, it should be kept in mind that the share of APP could largely depend on: (1) the size limit of APP (<3 or <2 μm); (2) sampling season; (3) properties of the study area. For example, Gaulke et al. (2010) showed that the APP biomass in a eutrophic river-dominated estuary formed about 35–44% of the total biomass during annual cycle, but the cell size limit of APP in this study was set to <3 μm. In a temperate coastal ecosystem Morán (2007) found the APP contribution of 45% in July and 63% in August (APP size limit <2 μm). So far there are no data available for comparisons from the coastal areas of the Baltic Sea.

Another important methodological issue concerns the measurement of total phytoplankton Chl *a*. Conventionally used GF/F filters with 0.7 μm retention could release most of picoplankton and strongly bias the results if the latter is abundant, e.g. in oligotrophic conditions. In our study the total amount of phytoplankton Chl *a* was on average 28% underestimated by using GF/F filters. Even though GF/F filters have been used for a long time to quantify total phytoplankton Chl *a*, the data retrieved can strongly underestimate the overall amount of the primary producers.

The overall difference between total and conventional Chl *a* showed significant correlation ($r = 0.55$, $p < 0.05$) with PC-type *Synechococcus*. This indicates that the difference mainly comes from the fraction between 0.2 and 0.7 μm, given that on average median diameter for the PC-type group was 0.69 μm. Elevated number of virus-like particles were observed via flow cytometry at station H2 (data not shown), hence this group could probably be supplemented with free chloroplasts (0.5–1.0 μm) that might be released by viral lysis (Brown and Bidle, 2014).

The positive correlation ($r = 0.58$, $p < 0.05$, Fig. 3) found between the APP Chl *a* and conventional (GF/F) Chl *a* was assumed since it has been evident in most of the previous studies conducted both in marine and freshwater environments (e.g. Bell and Kalff, 2001; Morán, 2007; Gaulke et al., 2010). When total phytoplankton biomass increases the proportion of APP in it is assumed to decrease (Bell and Kalff, 2001) –

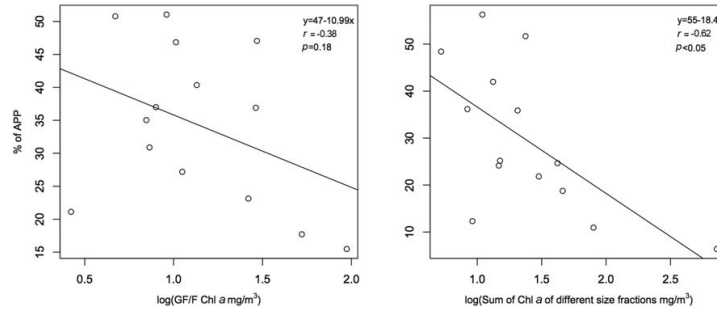

Fig. 4. Relationship between the relative contribution of autotrophic picoplankton Chl *a* (0.2–2 μm) and conventional phytoplankton Chl *a* (mg/m^3) on GF/F filters (left) and the sum of total Chl *a* (mg/m^3) of all size fractions (right) in the Väinameri. Regression line is fitted to data.

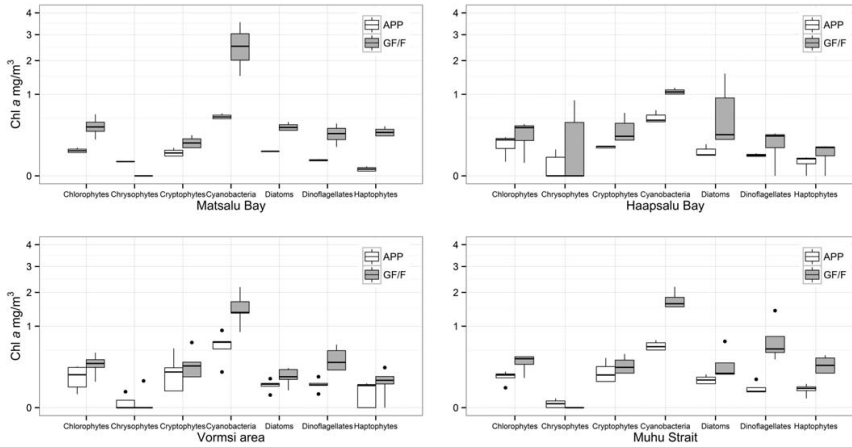

Fig. 5. Phytoplankton community composition based on the results of conventional pigment analysis (GF/F, grey boxplots) and autotrophic picoplankton (0.2–2 μm) size fraction (APP, white boxplots) in different areas of the Väinameri, both calculated by CHEMTAX. Note that on y-axis scale square root transformation is applied (Chl *a* values remain original).

in our study this trend was not significant when we compared conventional Chl *a* and contribution of APP but was apparent when we used the total Chl *a* values (Fig. 4).

The understanding about the taxonomic composition of picoeukaryotic algae is still largely lacking. Our results suggest that eukaryotes might constitute large proportion (around 50% in our study) of total APP biomass and have remarkable taxonomic variety. In summer APP of Väinameri the role of eukaryotes appeared to be bigger than could be expected from published studies, e.g. Morán (2007) found in the southern Bay of Biscay that summer biomass of picoeukaryotes contributed ca 20–30% of the total APP biomass. It has been shown previously that picoeukaryotic algae in the Baltic Sea dominate during colder periods and their importance decreases with higher water temperatures that trigger the summer cyanobacteria blooms (Kuosa, 1991). This study reveals that even during higher water temperatures (18–20 °C) the picoeukaryotes are relevant contributors to

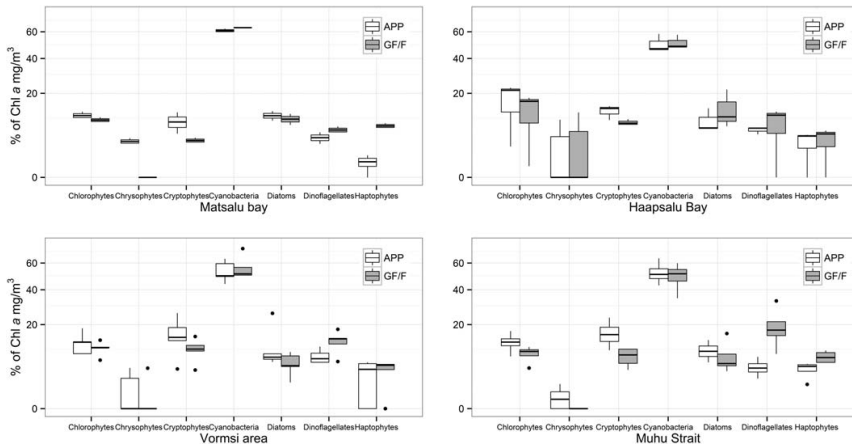

Fig. 6. Relative contribution of phytoplankton groups on GF/F filters (grey boxplots) and in autotrophic picoplankton size fraction (APP, white boxplots) in different areas of the Väinameri, both calculated by CHEMTAX. Note that on y-axis scale square root transformation is applied (% of Chl *a* values remain original).

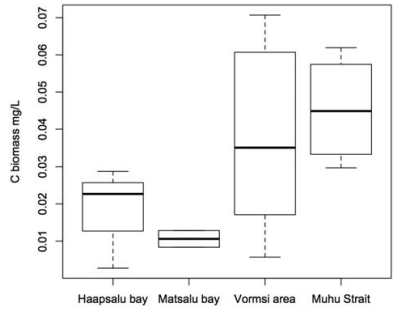

Fig. 7. The PE-type *Synechococcus* carbon biomass in different areas of the Väinameri.

APP community in coastal areas of the Baltic Sea. Moreover, our study gives so far the highest level of taxonomic resolution for the picophytoplankton communities in this region.

Although molecular methods might give the most complete information about picoeukaryote community, pigment-based chemotaxonomy is an efficient and more cost-effective alternative. Our HPLC analysis with CHEMTAX calculations provided a robust estimation of the amounts of different phytoplankton groups including picoplankton. The results gained should still be perceived with some caution. Our study shows that about 6% of picoplankton community consisted of dinoflagellates (Fig. 6). Despite the unambiguous marker pigment peridinin that was present in samples, this result can be doubted since the known minimal size of dinoflagellates remains around 5 μm. The same problem has been noted in previous studies that generally agree that dinoflagellates most likely do not play a part in marine picoplankton communities (Massana et al., 2004; Massana, 2011). Even though the existence of pico-size dinoflagellates cannot totally be excluded, the most likely cause of appearing of the marker pigment in pico size-class is due to the breaking of bigger dinoflagellate cells during filtration. This could contaminate the smallest filters with peridinin indicating dinoflagellate presence in the community. Moreover, some smallest flexible dinoflagellate cells might slip through the 2 μm filters and alter the results (Lovejoy et al., 2006; Vaulot et al., 2008; Massana, 2011).

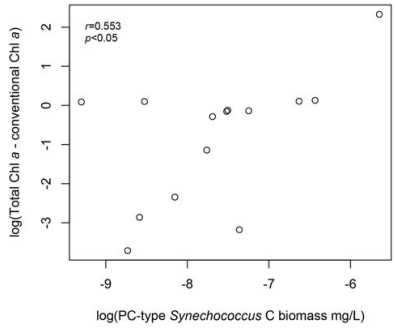

Fig. 8. The relationship between PC-type *Synechococcus* and the difference of total Chl *a* and conventional Chl *a*.

Diatoms and chrysophytes are both known to have pico-size species (Vaulot et al., 2008), but they share the main marker pigment – fucoxanthin. The use of additional marker pigments e.g. diadinoxanthin for chrysophytes should improve the accuracy of the result. It is very likely that chrysophytes are indeed present in small amounts in the picoplankton community of the Väinameri.

Cryptophytes formed the most abundant group (16%) of picoeukaryotic algae followed by chlorophytes (13%) and followed by diatoms (9%). There might still be some overestimation of cryptophytes in picoplankton because some larger elastic cells could pass through the prefilters since many cryptophytes are only slightly bigger than 3 μm (Not et al., 2008; Lepère et al., 2009). Although the occurence of these groups in picoplankton can not be totally excluded (some small, around 2 μm, cryptophytes also exist and they can be abundant – genus *Hillea* sp. (Gracia-Escobar et al., 2015), *Hillea marina* being the only cultured one) the reason for the presence of these specific carotenoid pigments in the sub 2 μm fraction still needs to be clarified. Chlorophytes are known as an important group of small eukaryotes mostly made up by members of the class Prasinophytes (Vaulot et al., 2008). Molecular studies have demonstrated that >90% of the available Prasinophyte sequences belong to the genera Micromonas, Ostreococcus, and Bathycoccus, which are mostly found in temperate coastal waters (Massana et al., 2004; Medlin et al., 2006; Vaulot et al., 2008; Massana, 2011).

We acknowledge that sequential filtration might cause overestimating or subestimating the real concentration values of picoplankton as, respectively, either some large cells could pass through small-pore size filters allowing larger algae cells to stop on 0.2 μm filters or some smaller cells could be trapped in filters with larger pore sizes. Therefore the results of our study should be taken with some caution. However, the method of sequential filtration is nevertheless used and valued in studies of picophytoplankton that involve catching the algal material on filters – e.g. pigment analysis and DNA amplification (Ansotegui et al., 2003; Lovejoy et al., 2006; Gaulke et al., 2010; Collado-Fabbri et al., 2011). A positive example of this approach is a study done by Lepère et al. (2009) about photosynthetic picoeukaryote community structure in the South East Pacific Ocean where pigment analysis revealed Prymnesiophytes as a major component of eukaryotic picoplankton. While almost all described species of this group are larger than 3 μm it was recently confirmed using fluorescent in situ hybridization that there are indeed picoplanktonic size of these cells.

Another problem regarding picoeukaryotic algae is the inconsistent size definition of this group. Three micrometers is often considered the boundary separating micro- and nanoplankton (Morán, 2007; Vaulot et al., 2008; Buitenhuis et al., 2012). In our study we the used same size discriminator for picocyanobacteria and picoeucaryotes – autotrophic cells with a size not bigger than 2 μm. No significant correlation between the Peuk group and any picoeukaryote taxons biomass calculated by CHEMTAX was found, therefore it is probable that the Peuk group consists of several taxa that could not be discriminated via flow cytometry. This can happen when there is no significant difference in cell size and fluorescence profile.

While our HPLC pigment data suggested that about 50% of the APP Chl *a* in the Väinameri belonged to picocyanobacteria, the flow cytometric assessment specified that most of the picocyanobacteria carbon biomass belonged to genus *Synechococcus*. This result was partly expected since *Synechococcus* is one of the most common genera of picocyanobacteria in world's oceans and also in open water areas of the Baltic Sea (Kuosa, 1991; Albertano et al., 1997; Partensky et al., 1999; Stal et al., 2003; Laas et al., 2015). Since *Synechococcus* tends to dominate the picocyanobacteria community in eutrophic conditions, it is likely to be found in nutrient-rich coastal areas of the Väinameri (Partensky et al., 1999).

Throughout the summer period, the Baltic Sea is dominated by picocyanobacteria with a unique novel gene cluster which points to brakish-adapted strains of *Synechococcus* (Larsson et al., 2014). We

separated two picocyanobacterial groups – PE-type *Synechococcus* and PC-type *Synechococcus* along with other picocyanobacterial species. The coexistence of these two pigment types of *Synechococcus* in coastal areas of the Baltic Sea has been shown before (Albertano et al., 1997; Stomp et al., 2007; Haverkamp et al., 2008, 2009). While PE-type and PC-type picocyanobacteria have been noted as equally important players in open water areas of the Baltic Sea, e.g. in the Gulf of Finland (Haverkamp et al., 2009), our results indicate that there is a strong dominance of PE-type *Synechococcus* in the late summer picocyanobacterial community of the Väinameri.

According to sediment records from the Gulf of Finland, *Synechococcus* was a typical taxon already in the Litorina Sea and according to 16S rRNA sequences the same strain of *Synechococcus* is still dominant in the Baltic Sea (Lyra et al., 2013; Laas et al., 2015). The amount of *Synechococcus* in the Väinameri is likely to increase due to climate change since it reaches its maximal growth rates at higher temperatures than other cyanobacteria (Moore et al., 1995; Mackey et al., 2013).

Flow cytometry demonstrated clearly that *Synechococcus* was more dominant in open water areas and less numerous in the bays (Fig. 7). One factor causing this variability in APP community composition is salinity that is considerably lower in the bays due to freshwater inflow from rivers. Similar spatial pattern was not visible in CHEMTAX data (Figs. 5, 6) although there was a significant correlation ($r = 0.6$, $p < 0.05$) between carbon biomass of PE-containing *Synechococcus* and the chemotaxonomic Chl *a* of cyanobacteria. Several factors may have influenced this outcome. Since chemotaxonomy and flow-cytometry have different outputs (Chl *a*; carbon biomass), a linear relationship between the two cannot be expected. Although both reflect the abundance of phytoplankton, the carbon biomass and the Chl *a* ratio strongly depend on complex influences of light, nutrients and temperature (Le Bouteiller et al., 2003; Behrenfeld et al., 2005; Wang et al., 2009). The non-linear relationship between carbon biomass and Chl *a* is especially apparent in case of rapid bloom events when cells reproduce fast but the production of Chl *a* lags behind.

Additionally, there might be some underestimation of *Synechococcus* on picoplankton filters. *Synechococcus* that generally has solitary cells, might also produce extracellular polysaccharides that can aggregate cells (Albertano et al., 1996). These aggregates might be caught on the prefilters instead of the pico-fraction filters.

5. Conclusions

Our study demonstrated that autotrophic picoplankton constitutes an important part of phytoplankton community in the coastal area of the Baltic Sea. The parallel use of different assessment methods (CHEMTAX and flow cytometry) revealed the share of eukaryotic and prokaryotic part of autotrophic picoplankton. Picoeukaryotic algae formed about half of the late summer picoplankton community and consisted of a variety of different algal groups – cryptophytes, chlorophytes, diatoms and small amounts of haptophytes and chrysophytes.

Picocyanobacteria were clearly dominated by phycoerythrin-containing *Synechococcus*. Future studies are needed to investigate the role of APP in the foodweb in the context of eutrophication of the Baltic Sea and changing climate. While chemotaxonomy reveals the picoeukaryotic algae at the class level, the knowledge at species level is still lacking and should be addressed in later studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.234.

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Boosting the monitoring of phytoplankton in optically complex coastal waters by combining pigment-based chemotaxonomy and *in situ* radiometry

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ABSTRACT

Research about the occurrence and extent of the cyanobacterial blooms in the Baltic Sea is critical due to their increased magnitude and frequency. Monitoring of the blooms is complicated due to their spatially and temporally heterogeneous nature. For adequate assessment of the water quality, phytoplankton dynamics needs to be tracked in large areas with high monitoring frequency. The main objectives of this study were (1) to describe phytoplankton community composition by pigment-based chemotaxonomy and validate the results with microscopy; (2) to improve the retrieval of information about phytoplankton community by combining remote sensing with laboratory based approaches (3) to develop a region-specific algorithm to calculate cyanobacteria biomass from reflectance spectra; (4) to detect and quantify potentially toxic bloom-forming cyanobacteria with molecular methods. In our study the reflectance-based chlorophyll *a* (Chl *a*) values overestimated the High-performance Liquid Chromatography (HPLC) values although the correlations with HPLC Chl *a* measurements were very strong ($r_p \sim 0.8$, $p < 0.001$). We found that 709 nm/620 nm reflectance ratio correlated strongly ($r_p = 0.75$, $p < 0.01$) to cyanobacteria wet biomass in CDOM-rich Väinameri even at low cyanobacterial biomass levels. Correlations between pigment-based chemotaxonomy and microscopy were significant in case of cyanobacteria ($r_p = 0.73$, $p < 0.01$), cryptophytes ($r_p = 0.71$, $p < 0.05$) and dinoflagellates ($r_p = 0.64$, $p < 0.05$).

1. Introduction

Phytoplankton is one of the key elements in ecological monitoring of waters. The distribution and dynamics of phytoplankton groups reflect the rate of eutrophication, functioning of food webs and the formation of potentially harmful algal blooms. Since the changes in phytoplankton communities can be rapid and the distribution tends to be uneven in space, it is essential to use appropriate methods to successfully track environmental changes in spatio-temporal scale (Smayda, 1998; Gallegos and Neale, 2015).

Being one of the most polluted seas worldwide, the coastal waters of Baltic Sea suffer of extreme anthropogenic pressure. Despite of the fact that cyanobacterial blooms have been occurring naturally in the Baltic Sea for thousands of years, the human induced growth of primary production has increased the magnitude and frequency of water blooms (Bianchi et al., 2000; Kahru and Elmgren, 2014). Since 1960's, cyanobacterial blooms have become more common (Finni et al., 2001). *Nodularia spumigena* along with *Dolichospermum* (previously *Anabaena*) and *Aphanizomenon* are forming heavy blooms in the Baltic Sea (Sivonen et al., 1989; Halinen et al., 2007). *N. spumigena* and *Dolichospermum* are considered harmful due to their ability to produce hepatotoxic peptides called nodularins (NOD) and microcystins (MC), respectively (Sivonen et al., 1989; Sivonen et al., 2007). *Dolichospermum* is also known to be able to produce neurotoxic alkaloids named anatoxins (ATX) while toxicity of *Aphanizomenon* in the Baltic Sea has not been confirmed (Sivonen et al.,1989; Lehtimäki et al., 1997; Halinen et al., 2007; Koskenniemi et al., 2007; Fewer et al., 2009, Rantala-Ylinen et al., 2011). The research and comprehension about the occurrence and extent of the cyanobacterial blooms in the Baltic Sea is critical but complicated due to their spatially and temporally heterogeneous nature (Kutser, 2004). Comprehensive monitoring networks should therefore aim at tracking the situation and changes in large

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areas with high monitoring frequency to assess the actual water quality in the coastal areas and the processes behind them.

Standard approach in phytoplankton monitoring is inverted microscopy which provides excellent species level information. However, the high cost of time and expertise of the method suppresses the sampling frequency to adequately describe the short-term and spatial changes in algal assemblages. Furthermore, the results vary a lot among analysts depending on their qualification and characteristics of the sample (Jakobsen et al., 2015; Higgins et al., 2011). As toxic and non-toxic cyanobacterial strains are morphologically identical and they can co-exist in the same sample, it is not possible to differentiate them using microscopy (Vezie et al., 1998; Lyra et al., 2005).

Alternative solutions suggest combining microscopy with pigment-based chemotaxonomy and remote sensing for capturing the bigger picture (e.g. Havskum et al., 2004; Pan et al., 2011). In addition, for detection of the potentially toxic cyanobacterial strains, rapid and sensitive qPCR (quantitative polymerase chain reaction) method is recommended to use (Kurmayer and Kutzenberger, 2003; Rantala et al., 2006; Koskenniemi et al., 2007; Pearson and Neilan, 2008).

The concentration of chlorophyll *a* (Chl *a*) is commonly used as a proxy for phytoplankton biomass and as an indicator of ecological status of water-bodies. Satellites can provide detailed information about the distribution of Chl *a* concentrations of large areas in a cost-effective manner (Ferreira et al., 2011; Kahru et al., 2011; Kutser, 2004). In spite of remote sensing being one of the best tools for capturing the bloom events, the quantification of the blooms is still extremely difficult in shallow coastal areas and further complicated with the presence of chromophoric dissolved organic matter (CDOM). In the oceanic waters, the main optically active constituents are typically coming from the life cycle of phytoplankton and are therefore well correlated with Chl *a*. In coastal areas and lacustrine waters, however, the constituents like suspended matter and CDOM can have a variety of sources and therefore they mostly don't correlate with each other, which makes the optical properties of the water in these areas region-specific. Hence, the algorithms for trustful Chl *a* concentration calculations in our study area, the coastal waters of the Baltic Sea, require regional parameters and additional validation (Kutser, 2004; Cui et al., 2010; Harvey et al., 2015; Matthews and Odermatt, 2015). For a quick quantification of different algal groups specific marker pigments should be used. Pigment-based chemotaxonomic approach relies on combining HPLC with matrix factorization program CHEMTAX which uses characteristic marker pigments to calculate the amount of each algal group (Mackey et al., 1997). Advantages of the CHEMTAX method are short analysis time, objectivity, sensitivity and the fact, that picoplankton that is mostly omitted in standard microscopy, is also tracked. In addition, the fragile phytoplankton species that may not preserve in fixatives can be also detected (Jeffrey et al., 1999; Lauridsen et al., 2011; Schlüter et al., 2014).

To convert the pigment data to algal groups, CHEMTAX requires suitable marker pigment:Chl *a* ratios. Since these ratios depend on species composition and their growth rate, light intensity, amount of nutrients etc., the construction of initial pigment ratio matrix can be complicated and needs validation with microscopy depending on the area under investigation (Schlüter et al., 2000; Schlüter et al., 2006; Lauridsen et al., 2011). Although CHEMTAX has been previously used in the Baltic Sea (Schlüter et al., 2004; Eker-Develi et al., 2008), the phytoplankton variability in this water-body is considerable and the literature-based marker pigment:Chl *a* ratios need further verifying to improve the accuracy of the analysis.

During the past decades, molecular methods have become an important tool to detect and quantify potential cyanotoxin producers in environmental samples (Vaitomaa et al., 2003; Rantala et al., 2006; Koskenniemi et al., 2007). As the gene clusters that are involved in the production of nodularins and microcystins are known, several molecular based techniques (PCR and qPCR), targeting those genes have been developed. Those methods provide a sensitive, rapid and cost-effective way to detect and quantify toxic cyanobacteria. Since qPCR can detect the presence of toxin producers in very low concentrations, this method is suitable to evaluate the risk of a toxic bloom formation (Kurmayer and Kutzenberger, 2003; Vaitomaa et al., 2003; Rantala et al., 2006; Koskenniemi et al., 2007; Pearson and Neilan, 2008).

The overall goal of this study was to demonstrate through an example snapshot of the phytoplankton community of the West-Estonian Archipelago Sea (Väinameri) that different pigment-based methods complement each other and their combining by standard phytoplankton monitoring and molecular methods boosts the power of the detection of harmful algal blooms in optically complex coastal areas.

The objectives of the study were (1) to describe phytoplankton community composition by pigment-based chemotaxonomy and validate the results with microscopic counting method; (2) to improve the retrieval of information about phytoplankton community by combining remote sensing with laboratory based approaches (3) to develop a region-specific algorithm to calculate cyanobacteria biomass from reflectance spectra; (4) to detect and quantify potentially toxic bloom-forming cyanobacteria with molecular methods.

2. Materials and methods

2.1. Study site

The study was carried out in optically complex area of the West-Estonian Archipelago Sea (further denoted by its Estonian name Väinameri for brevity) in north-eastern Baltic Sea (Fig. 1). This shallow-water region between continental Estonia and its western islands (average depth < 10 m) has a complex shoreline and many small islets. Seafloor mostly consists of soft sediments such as mud and sand. The study included areas of Haapsalu and Matsalu Bay which extend far into the land. Both of the bays are shallow with the average depth of 1.5–2 m. Salinity in the Väinameri region depends on the season and varies around 5–6.5 psu. Salinity is lower in the eastern parts of Haapsalu (2.5–4 psu) and Matsalu (down to 0.5 psu) bays (Kotta et al., 2008). Water is more transparent in the open water area and the turbidity is increasing in the moderately eutrophicated bays where the range of Secchi depth in summer is 0.2–4.2 m. The optical properties of coastal areas are influenced by several river inflows enriching the water with coloured dissolved organic matter (CDOM) (Kutser et al., 2009). The rapid bloom of phytoplankton occurs in late summer when Chl *a* maximum can reach up to 17 μg L^{-1} in Haapsalu Bay and 6 μg L^{-1} in Matsalu Bay; the amount of phytoplankton decreases up to 20 times towards the open water areas. Haapsalu Bay generally has no clear phytoplankton dominant in summer, several diatoms and cyanobacteria could prevail. In Matsalu Bay the domination of cyanobacteria increases towards the open water area, the most abundant species being *Pseudanabaena limnetica*, *Merismopedia punctata*, *A. flos-aquae*, *N. spumigena* (Kotta et al., 2008). The cyanobacterial blooms in the area patchy and are built up by a variety of species depending on the area.

2.2. Sampling

Sampling took place in July 2013 from 14 stations (Fig. 1). Surface water samples were collected simultaneously with optical measurements and kept in dark and refrigerated until further processing. Secchi depth and general meteorological conditions were determined in all stations. Subsamples for water chemistry, spectrophotometric measurements, HPLC, molecular analysis and microscopy were subtracted from the water sample in laboratory a few hours after the initial water collection.

2.3. HPLC and spectrophotometric measurements

For HPLC pigment analysis water samples of 500 mL were vacuum filtered through Whatman glass fibre filters GF/F (47 mm). Filters were

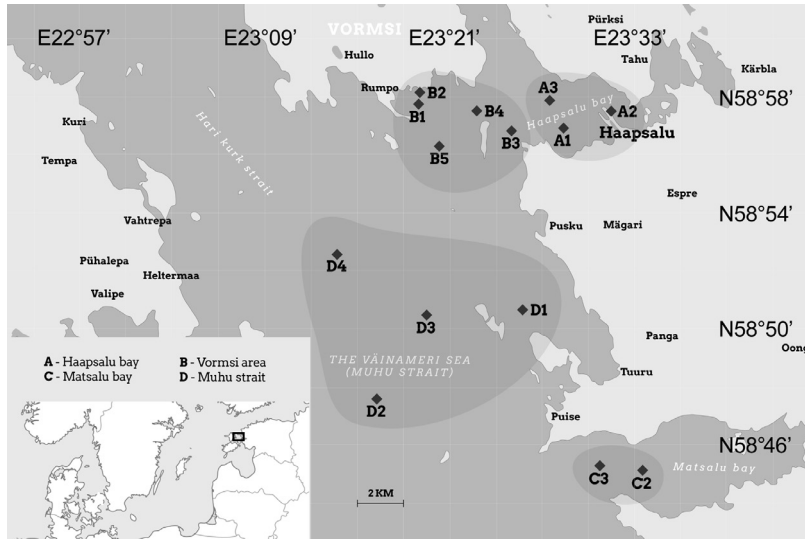

Fig. 1. Study site and measuring stations.

placed in 5-mL plastic vials, frozen immediately and stored at −70 °C until further analysis. Pigments were extracted from filtered phytoplankton with 2 mL mixture of 100% acetone/internal standard, sonicated for 5 min (Branson 1210) and kept 24 h at −20 °C. To remove filter and cell debris extracts were then filtered through 0.45 µm syringe filters (Millex LCR, Millipore) and stored at −20 °C in dark conditions. Pigments in the extracts were analysed by HPLC (for details see Tamm et al., 2015) and the obtained data was processed by CHEMTAX program.

Chl *a* was measured in three different ways: (1) with HPLC; (2) with spectrophotometer (HITACHI U-3010) from HPLC pigment extracts (AChl *a*); (3) with spectrophotometer (BChl *a*) from same water sample but extracted with 96% ethanol. Spectrophotometric measurements were calculated according to Jeffrey and Humphrey (1975).

Absorption by CDOM (aCDOM) was measured with a spectrometer (HITACHI U-3010, in the range of 350–750 nm) in water filtered through Millipore 0.2 µm filter, in a 5 cm cuvette against distilled water and corrected for residual scattering according to Davies-Colley and Vant (1987).

2.4. Optical in situ measurements

Above water reflectance measurements were done using a WISP-3 instrument. This handheld device has three radiometers connected to one cosine corrector to estimate the downwelling sky irradiance [E_d(W/(m^2*nm))], and two Gershun tubes to quantify the downwelling radiance from the sky [Lsky (W/(sr*m^2*nm)] in which the sensor is pointed at 42 degree angle from the zenith and the total upwelling radiance [Lu(W/(sr*m^2*nm)] at 42 degree from the nadir. The viewing angles for the sensors were chosen according to Mobley's (1999) instructions on above-water radiometric measurements (Hommersom et al., 2012). Measurements were taken at an azimuth angle of ~135 deg relative to the sun. In this way the direct reflectance effects (e.g., sun glint), that occur at the surface, are avoided as much as possible (Mobley, 1999). The water leaving reflectance was calculated as: $R = \pi^*(Lu-\rho^*Lsky)/Ed$, where $\rho = 0.0256 + 0.00039^*v + 0.000023^*v2$; v = windspeed (m/s).

To calculate Chl *a* concentrations from reflectance, two approaches were used that have shown good results in Estonian coastal areas (Ligi et al., 2017). As a base, they use the ratio of reflectances (1) at 693.5 nm and 679 nm (Dierberg and Carriker, 1994) and (2) at 706.5 nm and 677.5 nm (Kallio et al., 2001), respectively. Both algorithms take the advantage of the Chl *a* absorption peak and low reflectance values at longer wavelengths. Three sets of factors and offsets for both algorithms were taken from Ligi et al. (2017). One set was obtained by using only *in situ* data. The other two are using modelled data, were the input inherent optical properties (IOPs) for the model were measured (1) during the summer and (2) during the entire vegetation period. The IOPs were collected from bigger research vessel that cannot access the shallow areas investigated in this study. This is why the *in situ* calculation set was used for statistical analyses.

The suitability of 709 nm to 620 nm ratio (Simis et al., 2005) was tested to find a region-specific algorithm to calculate cyanobacteria biomass from the reflectance spectra.

2.5. Microscopy counts

Phytoplankton samples were preserved with acid Lugol solution (0.5 mL per 100 mL sample) at a final concentration of 0.5% and stored in 100 mL plastic bottles. Non of the samples were concentrated with a mesh. Samples were stored in cool and dark until microscopic analyses. Phytoplankton was settled in 10–25 mL tubes and counted under the inverted microscope Leica DM IL using 200-400x magnifications according to Uthermöhl (1958). All taxa were identified to the highest possible level – species level or if not possible to genus level.

Phytoplankton cell sizes were measured and volumes calculated from cell geometry (HELCOM, 1988) or by using standard size-classes (Olenina, 2006). Cell volume was converted into wet biomass assuming a specific gravity of 1 g cm^{-3}. Wet weight biomasses were calculated for the different taxa, for the main classes (diatoms, cryptomonads, cyanobacteria, chlorophytes, dinoflagellates, chrysophytes and haptophytes) and for the total phytoplankton community.

2.6. Chemotaxonomy

To estimate the abundance of different algal classes according to HPLC pigment data, the chemical taxonomy program CHEMTAX was used (version 1.95). The program calculates the relative abundance of phytoplankton classes in reference to total Chl *a* in a water sample using specific marker pigments and marker pigment: Chl *a* ratios (Mackey et al., 1997). Seven algal groups were defined and loaded into CHEMTAX according to our previous knowledge about the phytoplankton groups present in the study area – chlorophytes, dinoflagellates, chrysophytes, diatoms, cryptophytes, cyanobacteria and haptophytes. Chlorophytes and prasinophytes were summed up into the group of chlorophytes. Algal groups were determined according to their published marker pigments data (Table 1). Initial pigment ratio matrix used for CHEMTAX calculations was the same in case of all samples and the CHEMTAX calculation settings were fixed as advised by Mackey et al. (1997).

2.7. Molecular detection of potentially toxic cyanobacteria

In present study, genus specific qPCR was used to detect and quantify potentially hepatotoxic *Nodularia*, *Dolichospermum*, *Microcystis* and *Planktothrix* spp. in environmental samples. To detect potential neurotoxic strains, conventional PCR was used.

For DNA extraction, 50–600 mL of the water (depending on the sampling station) was filtered through 1 µm pore size Whatman Cyclopore Polycarbonate filter (diameter 47 mm) and filters were stored at −70 °C until further analysis. DNA from environmental samples was extracted using the DNeasy PowerWater Kit (Qiagen Inc.) according to manufacturer's instructions. The quality and quantity of extracted DNA was controlled with NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisher Scientific Inc.).

First, to exclude false negative outcome, which might be caused by the presence of PCR inhibitors, a PCR with cyanobacterium-specific 16S rRNA gene primers (Table 2) (Nübel et al., 1997) was performed. PCR reactions were performed in a volume of 20 µL, containing 1x FIREPol® Master Mix Ready to Load (Solis BioDyne, Estonia), 0.5 µM of both primers (Metabion International AG, Germany) and 1 µL of template DNA. PCR program is described in Table 2.

For visualisation, 19 µL of the PCR mixture was loaded to 1.5% agarose gel stained with ethidium bromide (0.25 µg mL^{-1}) and documented with Vilber Bioprint ST4 gel documentation system and VisionCapt software. To detect very faint bands, gels were also checked visually under the UV light.

To detect and quantify potential toxin producing genera, qPCR with standard curve method was used. Microcystis sp. 205, Dolichospermum sp. 315, Planktothrix sp. 49 & Nodularia sp. AV1 from the HAMBI Culture Collection (University of Helsinki) were used as external standards to quantify mcyE/ndaF genes. Standard dilutions of genomic DNA for absolute quantifications were prepared as described by Koskenniemi et al. (2007). Standard curve was determined in each run by dilution series of genomic DNA of external standards. The qPCR amplifications of the *ndaF* and *mcyE* synthase genes (primers used are shown in Table 2) were performed with ESCO Swift™ Spectrum 96 Real Time Thermal Cycler (ESCO, Singapore) in 4 replicates as 10-fold dilutions. As a negative control MilliQ water was used. The reaction mixture (total volume 20 µL) included 1x HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Estonia), 0.35 µM primers (*ndaF*) or 0.3 µM primers (*mcyE*) and 5 µL of diluted DNA. qPCR programs are described in Table 2. Fluorescence was measured in the end of each cycle. To assess the specifity of the amplifications, melting curve analysis with continuous fluorescence measurement was included in the end of cycling. Results were analysed by using ESCO Spectrum PC Software (ESCO, Singapore) and gene copy numbers were calculated and presented as copies per mL of water sample.

To detect potential ATX producers, a conventional PCR with general anaC primers (Table 2) was applied. PCR reactions were performed in a volume of 20 µL, containing 1x FIREPol® Master Mix Ready to Load (Solis BioDyne, Estonia), 0.5 µM of both primers and 1 µL of template DNA. PCR protocol was described earlier by Rantala-Ylinen et al. (2011). Results of PCR were visualised by Vilber Bioprint ST4 gel documentation system.

In current study, molecular markers were used to assess the potential of cyanobacteria to produce cyanotoxins. Analytical methods to analyse toxins directly were not applied.

2.8. Statisitical analysis

Statistical analysis was performed using the statistical package R (version 2.15.3, Team R, 2013). Most of the data visualisation was done with package ggplot2. Since the Chl *a* data retrieved with all methods as well as the microscopic and chemotaxonomic data about phytoplankton groups mostly lacked normal distribution, the logarithmic transformation was applied before the Pearson correlation (r_p) coefficients were calculated and regression analysis performed. In case of BChl *a* and microscopic phytoplankton wet biomass the logarithmic transformation did not provide normally distributed values and Spearman's rank correlation was used. The cyanobacterial biomass calculated from reflectance spectra was normally distributed and therefore no transformation was applied. We analysed the relationship between the CHEMTAX and microscopy counts for each phytoplankton group. To examine the microscopy biomass of *Nodularia* sp. and *Dolichospermum*

Table 1
Initial pigment ratio matrix containing marker pigment: Chl a ratios used for CHEMTAX calculations.

Class/pigment	Fucoxanthin	Alloxanthin	Lutein	Zeaxanthin	Diadinoxanthin	Chlorophyll-b	Peridinin	Chlorophyll-a
Chrysophytes	0.63[1]				0.44[1]			1
Dinoflagellates							0.21[4]	1
Cryptophytes		0.41[3]						1
Chlorophytes			0.148[2]			0.356[3]		1
Cyanobacteria				0.223[1]				1
Diatoms	0.343[2]							1
Haptophytes					0.18[1]			1

[1] Mackey et al., 1997.
[2] Marinho and Rodrigues, 2003.
[3] Schlüter et al., 2000.
[4] Greisberger and Teubner, 2007.

Table 2
Primers used to detect potentially toxic cyanobacteria in the Baltic Sea.

Target gene	Primer	Primer reference	PCR program
Cyanobacterial 16S rRNA	CYA359F CYA781R	Nübel et al., 1997	94 °C 5 min; 30 cycles: 94 °C 60 s; 60 °C 60 s; 72 °C 60 s and 72 °C 10 min
General anaC	anaC-genF anaC-genR	Rantala-Ylinen et al., 2011	94 °C 5 min; 35 cycles: 94 °C 30 s; 58 °C 30 s; 72 °C 30 s and 72 °C 10 min
Microcystis mcyE	127F 247R	Sipari et al., 2010	95 °C 15 min; 40 cycles: 95 °C 15 s; 62 °C 60 s; *
Dolichospermum mcyE	611F 737R	Sipari et al., 2010	95 °C 15 min; 40 cycles: 95 °C 15 s; 62 °C 60 s; *
Planktothrix mcyE	664F 744R	Rantala-Ylinen et al., unpublished	95 °C 15 min; 40 cycles: 95 °C 15 s; 60 °C 60 s; *
Nodularia ndaF	ndaF8452 ndaF8640	Koskenniemi et al., 2007	95 °C 15 min; 40 cycles: 90 °C 15 s, 62 °C 45 s, *

* To assess the specifity of the amplification, cycling was followed by melting curve analysis (58 °C–95 °C).

spp. in relation to qPCR data, Spearman's rank correlation coefficient was calculated since logarithmic transformation of these data did not provide normal distribution.

3. Results

3.1. Secchi depth and CDOM

Water transparency by Secchi depth was lowest (average around 1.9 m) in the bays and near Vormsi island, increasing toward deeper open water areas (average around 3 m). The smallest measured Secchi depth was 0.8 m in Haapsalu Bay and the highest value (4 m) was registered in Muhu Strait (Table 3). The Secchi depth was positively correlated ($r_p = 0.77$, $p < 0.01$) to overall depth of the measuring stations. The absorption at 380 nm characterising CDOM concentration ($aCDOM_{380}$) was lowest near Muhu Strait (min 1.7 m^{-1}) and peaked near the coast of Vormsi Island (max 5.9 m^{-1}, Table 3). $aCDOM_{380}$ values in Haapsalu Bay were rather moderate and on about the same level as in open water areas with the exception of station A3 where $aCDOM_{380}$ was much higher (5.1 m^{-1}). Highest average $aCDOM_{380}$ values (~4.9) appeared in Matsalu Bay.

3.2. Chl a by different methods

The concentration of HPLC-Chl *a* in the study area varied generally in the range of 1.5–5.6 µg L^{-1}, the highest value (7.2 µg L^{-1}) was registered in Haapsalu Bay, close to the harbour (station A2). There was a strong correlation between Chl *a* measured by spectrophotometry and HPLC (AChl *a*: $r_p = 0.85$, BChl *a*: $r_s = 0.79$; $p < 0.001$) but weaker between the two spectrophotometric values AChl *a* and BChl *a* ($r_s = 0.75$, $p < 0.01$). Ethanol- extracted BChl *a* values were about 30% higher than those extracted by acetone (AChl *a* and HPLC-Chl *a;* Fig. 2). Most severe difference occurred in station A2, where BChl *a* value exceeded the HPLC-Chl *a* almost three times. Correlation between the microscopic phytoplankton wet biomass and HPLC-Chl *a* was strong ($r_s = 0.71$, $p < 0.01$). The correlation between phytoplankton wet biomass and spectrophotometric values of Chl *a* was stronger ($r_p = 0.78$, $p < 0.01$) for acetone- extracted AChl *a* than for BChl *a* ($r_s = 0.6$, $p < 0.05$) that was extracted by ethanol. The average values of Chl *a* degradation products pheophytin *a* and chlorophyllide *a* measured with HPLC were 0.06 and 0.33 µg L^{-1}, respectively.

Table 3
Ranges of the measured general indices in the Väinameri.

Variable	Min	Max	Mean
Secchi depth (m)	0.8	4	2.43
Depth (m)	1.8	8	3.92
$aCDOM_{380}$ (m^{-1})	1.67	5.92	2.76
HPLC Chl *a* (µg L^{-1})	1.53	7.2	3.4

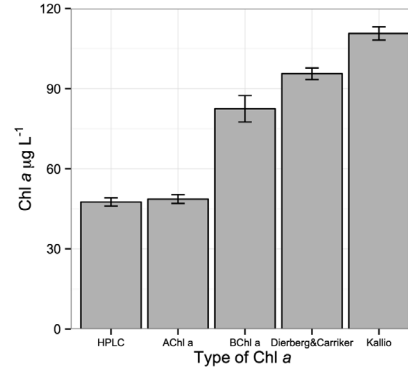

Fig. 2. The sum of Chl *a* in the Väinameri obtained with different methods: High-performance Liquid Chromatography (HPLC), Spectrophotometry (AChl *a*, BChl *a*) and concentrations calculated from reflectance spectra (Dierberg and Carriker, 1994; Kallio et al., 2001) in Väinameri. Error bars represent standard deviation.

3.3. Optical measurements vs pigment data

The measured reflectance values in Haapsalu and Matsalu Bays were rather similar and higher than in the open water regions (Vormsi area and Muhu Strait, Fig. 3).

The Chl *a* concentrations calculated from the reflectance spectra were more than twice higher than HPLC-Chl *a* values (Fig. 2) while strongly correlated ($r_p \sim 0.8$, $p < 0.001$; Fig. 4). The absolute values of reflectance-based Chl *a* were rather consistent with spectrophotometric BChl *a* (Fig. 2) while their correlation was weaker ($r_s \sim 0.7$, $p < 0.05$) than those with HPLC-Chl *a*. The correlations between reflectance-based Chl *a* and microscopy total phytoplankton biomass were also strong ($r_s \sim 0.8$, $p < 0.001$).

The ratio of reflectances at 709 nm and 620 nm was strongly correlated with cyanobacteria wet biomass based on microscopy ($r_p = 0.75$, $p < 0.01$; Fig. 5) and slightly weaker with chemotaxonomic Chl *a* of cyanobacteria ($r_p = 0.71$, $p < 0.05$).

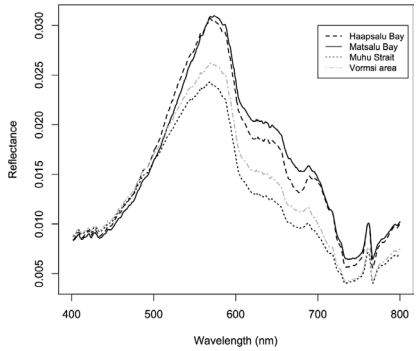

Fig. 3. Regionally averaged reflectance spectra measured with TriOS RAMSES radiometers.

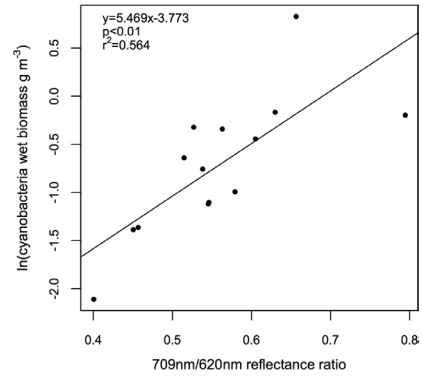

Fig. 5. Correspondence of cyanobacteria wet biomass based on microscopic counts to the reflectance spectra ratio of 709 nm/620 nm (Simis et al. 2005).

3.4. Phytoplankton cell counts and chemotaxonomy

Phytoplankton community in studied areas was nearly always dominated by the cyanobacteria and both methods detected this fact successfully (Figs. 6 and 7). The cyanobacterial community consisted mostly of *A. flos-aquae* (around 85% of all cyanobacteria) except of the Matsalu Bay where the relative share of this species was smaller (around 50%) and was co-dominated by another filamentous species *Dolichospermum flos-aquae* (25%, full species list is provided in Supplementary Table S1).

Even though the overall pictures that the two methods drew were quite similar, there were several inconsistencies between them. E.g., in Haapsalu Bay and Vormsi area (stations A1 and B5) microscopy indicated the dominance of dinoflagellates while cyanobacteria prevailed by CHEMTAX. In Haapsalu Bay station A2 microscopic counts detected large amount of a diatom *Nitzschia longissima,* which made up more than half of the total algal biomass while CHEMTAX showed the overall dominance of cyanobacteria, still detecting also rather high biomass of diatoms.

According to microscopy the dinoflagellate community was nearly always dominated by *Heterocapsa triquetra* which built up almost 90% of the total biomass of the phyla. It was the second most abundant species after *A. flos-aquae* with the exception of Matsalu Bay. In whole study area microscopy showed rare findings of a small variety of diatom species (e.g. *Navicula sp., Cyclotella spp., Rhoicosphenia abbreviata, N. longissima, Cylindrotheca closterium*) with no clear dominant species. Neither of the methods indicated any clear differences of community

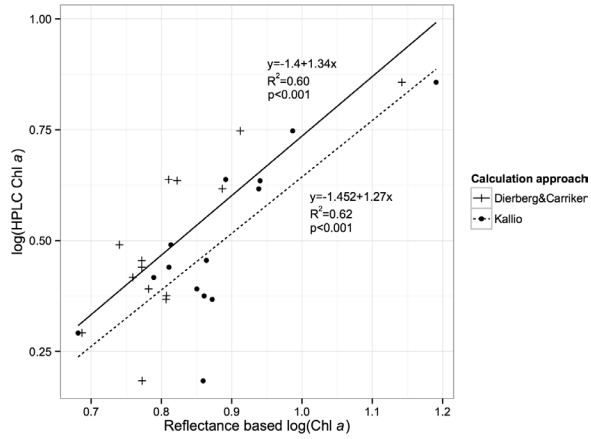

Fig. 4. Correspondence of lab-measured (HPLC) Chl *a* concentrations to the reflectance-based Chl *a* values calculated according to Dierberg and Carriker (1994) and Kallio et al. (2001).

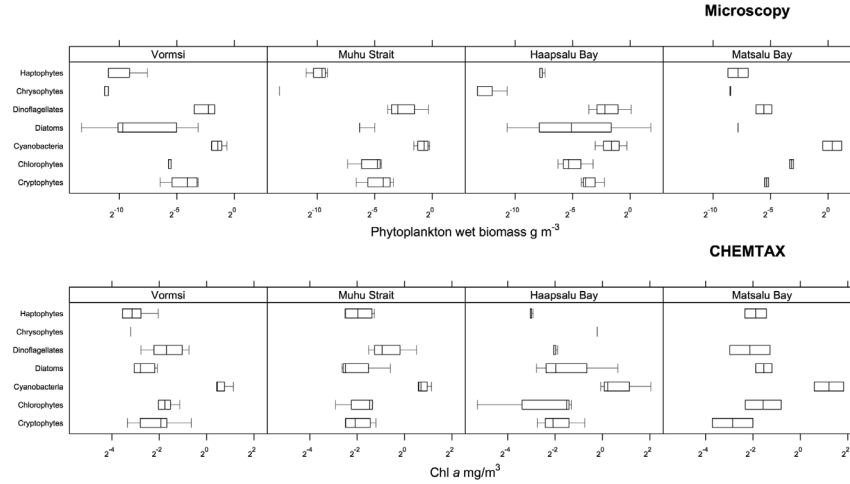

Fig. 6. Phytoplankton class abundances in different areas of the Väinameri according to microscopy (upper) and CHEMTAX (lower). Note that on x-axis scale logarithmic transformation is applied (Chl *a* values remain original).

composition in the spatial scale.

Generally, the relative share of chlorophytes was slightly bigger according to CHEMTAX than counted by microscopy (Fig. 7). Chrysophytes were detected in two stations (Haapsalu Bay and Vormsi area) by CHEMTAX and in nine stations according to microscopy. Quantities of this group were extremely small. Haptophytes were present in almost all areas (11–12 stations) by both CHEMTAX and microscopy (mainly *Chrysochromulina* sp.), although in very small amounts (Figs. 6 and 7).

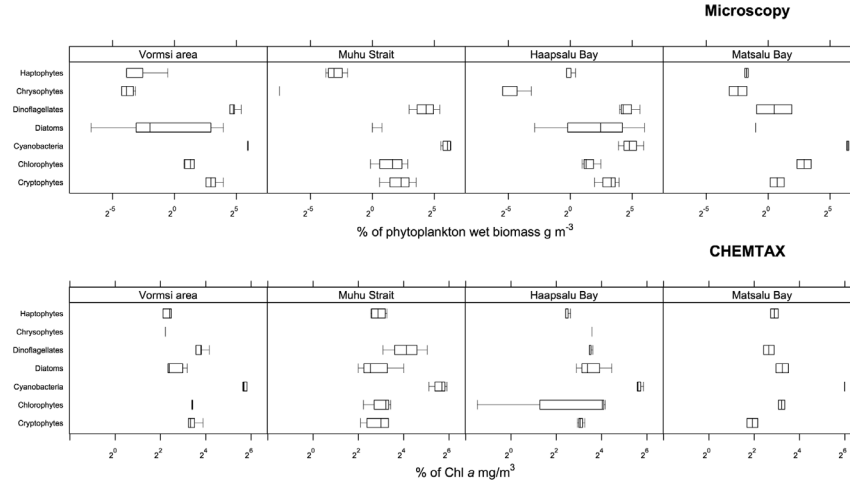

Fig. 7. The relative share of phytoplankton groups in the Väinameri according to microscopy (upper) and CHEMTAX (lower). Note that on x-axis scale logarithmic transformation is applied (relative Chl *a* values remain original).

Table 4
Pearson product-moment correlation coefficients and regression equations of phytoplankton classes assessment by microscopy (y) and CHEMTAX (x) in the Väinameri. Since CHEMTAX detected chrysophytes only in two stations, r_P was not calculated.

	r_P	p	y = a + bx x = ln(Chemtax Chl *a*); y = ln(Microscopy biomass)
Cyanobacteria	0.73	< 0.01	y = −1.319 + 1.169×
Cryptophytes	0.71	< 0.05	y = −1.576 + 1.017×
Dinoflagellates*	0.64	< 0.05	y = −0.866 + 1.437×
Haptophytes	0.46	> 0.05	–
Diatoms	0.45	> 0.05	–
Chlorophytes	−0.15	> 0.05	–

* Due to a technical error with software at station A2, it was removed from analysis in case of dinoflagellates.

Correlations between CHEMTAX and microscopy were significant in case of three phytoplankton groups – cyanobacteria, cryptophytes and dinoflagellates (Table 4).

3.5. Detection and quantification of potential toxin producers in the Baltic Sea

Potentially toxic *Nodularia* sp. was detected in all sampled stations, but *ndaF* gene copy numbers were relatively low (varied from 50 to 824 mL^{-1}) (Fig. 8, left). The highest value was observed in Matsalu Bay (station C2). Detection limit was set to 10 *ndaF* gene copies mL^{-1}. Potentially MC producing *Dolichospermum* sp. was also detected in all stations, but gene copy numbers exeeded the reliable detection limit (50 *mcyE* gene copies mL^{-1}) only in 6 stations out of 14 (Fig. 8, right). Highest value (352 *mcy*E gene copies mL^{-1}) was observed also in Matsalu Bay area (C3). Potentially toxic *Microcystis* sp. was detected in 5 stations (A2, A3, B3, D1, C3) and potentially toxic *Planktothrix* sp. was detected in two stations in Vormsi area (B2; B3), but the gene copy numbers were too low for reliable quantification (detection limit was set to 50 *mcy*E gene copies mL^{-1}). Potential ATX producers were not detected from the analysed samples.

Nodularia ndaF gene copies correlated positively with CHEMTAX retrieved cyanobacteria Chl *a* ($r_s = 0.74$, $p < 0.01$) while insignificant with microscopic counts of *Nodularia* sp. *Dolichospermum* spp. *mcy*E gene copies were correlated to cyanobacteria Chl *a* concentrations ($r_s = 0.55$, $p < 0.05$) and with microscopic *Dolichospermum* spp. wet biomass ($r_s = 0.71$, $p < 0.01$).

4. Discussion

This study demonstrated that the conventional methods in phytoplankton monitoring that often lack the ability to reflect rapid spatiotemporal processes in phytoplankton communities could benefit from integrated approach with pigment-based chemotaxonomy, remote sensing and qPCR. Combination of these methods allows researchers to avoid the shortcomings that are prone to occur by using them separately. This paper provides a region-specific algorithm to calculate cyanobacterial biomass from reflectance spectra and boost the monitoring of cyanobacterial populations in eutrophic CDOM-rich coastal waters of the Baltic Sea.

4.1. Choice of the best Chl a method for remote sensing calibration

In our study in the Väinameri acetone- and ethanol-extracted Chl *a* values differed in a rather large extent – ethanol extraction resulting in about 30% higher Chl *a* concentrations than acetone extraction (Fig. 2). This outcome was strongly influenced by the measuring station A2 where all used methods indicated the highest Chl *a* concentration.

While acetone extraction is most commonly used for Chl *a* analysis in marine research, choice of the proper solvent still remains under discussion (Aminot and Rey, 2002; Wasmund et al., 2006). HELCOM (2017) encourages the use of ethanol since acetone may poorly extract some of the frequent phytoplankton species in the Baltic Sea. Although some studies have demonstrated that acetone may be a poor extractant of chlorophyll from green algae, some cyanobacteria and diatoms (Pápista et al., 2002; Wasmund et al., 2006; Ritchie, 2008), the underestimation should occur in environments dominated by these groups. Overall acetone prevents the increase of the chlorophyll degradation products and tends to be the best choice when there is a lack of information about the phytoplankton community (Mantoura and Llewellyn, 1983; Aminot and Rey, 2002). Generally the pigment extraction from the natural phytoplankton samples is not limited by pigment solubility in commonly used solvents (ethanol, acetone, methanol) while at extremely high phytoplankton biomass this limitation may occur and the extraction process could depend on the used solvent more strongly (Pinckney et al., 2011).

Since the validation of remote sensing algorithms is often done only by spectrophotometry these possible methodological errors need further investigation. Intercomparison excercise of Sørensen et al. (2007) showed that generally spectrophotometric Chl *a* values were about 15–20% higher than HPLC values, which was not a case in our study if same extraction method was used. Although it is widely acknowledged

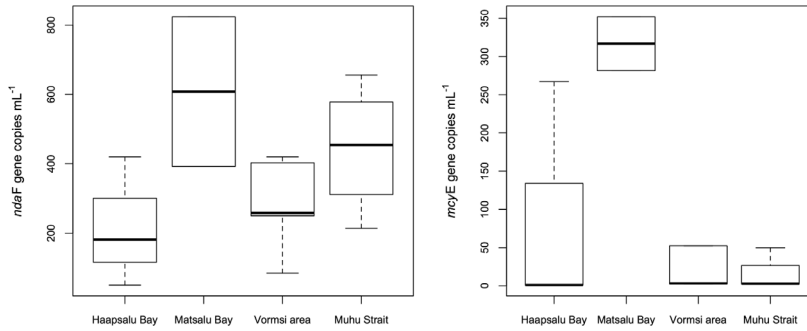

Fig. 8. *Nodularia* sp. *nda*F (left) and *Dolichospermum* sp. *mcy*E (right) gene copy numbers in different areas of the Väinameri.

that the most precise Chl *a* estimations are obtained by HPLC, the use of inefficient extraction solvent could also cause the underestimation of the concentrations. However, for proper determination of the different phytoplankton pigments, HPLC measurements are inevitable. In the Väinameri, ethanol- extracted spectrophotometric Chl *a* should be measured in parallel with HPLC assessment for the calibration of remote sensing algorithms.

In most remote sensing approaches, Chl *a* is calculated using the peaks in blue or green parts of the spectrum and another band in the red region as a base. This is not reliable at the Estonian coastal areas due to the high input from CDOM that absorbs most of the signal in the shorter wavelengths. Blue part of the spectrum is also strongly influenced by atmosphere and is more likely to suffer from glint effects. Therefore it is recommended to use the Chl *a* peak around 700 nm together with low reflectance values at red wavelengths for computationally cost-effective calculations. This approach gives also a good correlation with laboratory Chl *a* measurements. For applying these algorithms to the satellite data, suitable region-specific atmospheric correction factors and offsets need to be found. In our study the reflectance-based Chl *a* values overestimated the laboratory values (Fig. 2), although the calculation algoritms were estimated for Estonian coastal waters (Ligi et al., 2017) and also the correlations with lab-Chl *a* were similar (r_p remained between 0.73 and 0.8 in both studies) to those in Ligi et al. (2017).

One of the possible causes for the seemingly bigger overestimation in our study might come from the fact that in Ligi et al. (2017) the Chl *a* measurements were done only with spectrophotometer (ethanol extraction) which can generate notably bigger measurement errors as described above. Additionally, the *in situ* optical dataset used in the study of Ligi et al. (2017) used different radiometers that also have different measuring geometry. The number of measurements in this study was too low to retrieve factor and offset parameters for WISP-3. It is also important to point out, that some error always stays as the signal measured by the sensor comes from different area and depth (integrated) than the water sample analysed in the laboratory.

Unfortunately our Chl *a* concentrations could not be compared to any satellite remote sensing data as no suitable satellite sensor measurements were available during the time of the fieldwork. However the Sentinels 2 and 3 data are now available for free and the understanding how Chl *a* concentrations measured in the lab compare to the ones calculated from optical field data is a first step of retrieving Chl *a* concentrations and spatial distribution from Sentinel data.

4.2. Applicability of remote sensing and CHEMTAX for detecting phytoplankton groups

The correlation between cyanobacteria wet biomass and the 709 nm/620 nm reflectance ratio was suprisingly good ($r_p = 0.75$, $p < 0.01$, Fig. 5). It was anticipated that the biomass was too low and the signal would be influenced too much by the noise. During our study, the phytoplankton community of the Väinameri had not reached its late summer peak mainly consisting of cyanobacteria. Nevertheless, our results indicate that the longer wavelengths provide sufficient information even at lower cyanobacterial biomass. The monitoring of CDOM-rich coastal areas dominated by filamentous species like *A. flos-aquae* and *D. flos-aquae* benefit a great deal from this algorithm since the cyanobacterial phycocyanin can be detected and tracked even before the formation of the heavy blooms and may significantly improve our understanding about bloom formation and termination. To calculate cyanobacterial biomass from reflectance spectra in Estonian coastal waters we suggest using the formula seen on Fig. 5.

The HPLC data indicating different phytoplankton groups according to marker pigments was quite consistent with microscopy and the general picture in most areas was much the same. The results were especially similar in tracking the major phytoplankton groups and their proportions in the community.

Agreement between the methods was very good in case of cyanobacteria ($r_p = 0.73$, $p < 0.01$), which was the dominant group in nearly all areas of the Väinameri. The mismatch was biggest in case of Haapsalu Bay (Figs. 6 and 7), where microscopy did not indicate cyanobacteria as the distinct dominant group. Unlike other areas, in Haapsalu Bay *A. flos-aquae* was not among the top three species with the highest biomass. While big chain-forming cyanobacteria are easy to detect with microscopy, the small cyanobacteria cells can be strongly underestimated by microscopy (Schlüter et al., 2000; Wänstrand and Snoeijs, 2006). Recent study by Tamm et al. (2018) shows the presence of picocyanobacteria (mainly *Synechococcus sp.*) in the whole Väinameri. The pico-size algae, the pigments of which are detected by chemotaxonomy could be only partly tracked by microscopic counts and cause serious underestimations of phytoplankton biomass by microscopy.

Strong correlation between CHEMTAX and microscopy occurred also in case of cryptophytes ($r_p = 0.71$, $p < 0.05$). Microscopy data indicated that additionally to cryptophytes (*Plagioselmis prolonga* and *Teleaulax* spp.), kleptochloroplastidic ciliate (*Mesodinium rubrum* = *Myrionecta rubra*) that steals pigmented organelles from cryptophyte prey was present in most of the Väinameri (Gustafson et al., 2000). Since these ciliates retain the alloxanthin-containing cryptophyte plastids, they are often included in the cryptophyte wet biomass counts in this type of comparisions (Eker-Develi et al., 2008; Schlüter et al., 2014). Due to relatively low counts, they were not integrated in cryptophyte biomass and we assumed that most of the alloxanthin in the area belonged to cryptophytes.

Diatoms, chrysophytes and haptophytes all share the marker pigment fucoxanthin which was present in the whole Väinameri area. The shared marker pigments are acknowledged as a weakspot of the chemotaxonomic analysis that most often causes the poor detection of diatoms and chrysophytes (Schlüter and Møhlenberg, 2003; Simmons et al., 2016). Usually the discrepancies between different methods are more strongly expressed in detecting minor phytoplankton groups (Eker-Develi et al., 2008; Tamm et al., 2015). In our study diadinoxanthin was used as the only marker pigment for the minor group haptophytes. The poor ($r_p = 0.46$) and insignificant correlation between CHEMTAX and microscopy for detecting haptophytes has several possible reasons. First, when the amount of the algal cells counted by microscopy is small, the accuracy is low and could considerably over- or underestimate the real concentration. On the other hand, while the Chl *a* concentrations of a minor group could be correctly quantified by CHEMTAX if there is a single and unambiguous marker pigment (Latasa, 2007), this was not a case with haptophytes in this study – diadinoxanthin is also present in diatoms and in some dinoflagellates e.g. *Heterocapsa triquetra* (Latasa and Berdalet, 1994). Acknowledging all these problems, we could still assume that most likely this group was indeed present in the Väinameri in very small amounts.

Diatom and chrysophyte pigments both showed low and statistically insignificant correlations with microscopy counts. Microscopy detected diatoms in very small amounts in all areas of the Väinameri and very abundantly in one station in Haapsalu Bay (Figs. 6 and 7). Same markerpigment:Chl *a* ratio has showed good results in eutrophic freshwater environment (Tamm et al., 2015), where diatoms were the second largest phytoplankton group during the study period. Microscopy showed in Väinameri that diatoms were a minority group of mainly large species (e.g. *Navicula sp.*, *Rhoicosphenia abbreviata*, *Nitzschia longissima*), without distinct dominants. Presence of large cells might notably distort the conformity of microscopy and CHEMTAX since they tend to have smaller Chl *a* content (Rodriguez et al., 2002; Schlüter and Møhlenberg, 2003). The exclusion of large cells from the analysis may significantly improve the correlation between these two methods. Moreover, dead and living diatom cells are often difficult to distinguish via inverted microscopy and that enhances the counting error probability (Schmid et al., 1998; Schlüter and Møhlenberg, 2003).

Microscopy of chlorophytes, which have rather easily distinguishable marker pigments (Chl *b* and lutein) did not show up significant

correlation with CHEMTAX. Poor correlations of CHEMTAX and microscopy for minor phytoplankton groups have been reported in previous studies (Tamm et al., 2015; Eker-Develi et al., 2008; Schlüter et al., 2016) and this is not always related to shared marker pigments. At least partly the discrepancy in case of the minority groups is related to the sample volume. E.g. for microscopy counts the sample volumes are commonly not bigger than 50 mL (Paxinos and Mitchell, 2000), in our study it was 10–25 mL. Filtered water volumes for chemotaxonomy are usually bigger, in our study the amount of water filtered was 500 mL except for station A2 where the amount was only 250 due to fast filter clogging. Bigger sample volume increases the likelihood for the less abundant phytoplankton species to be included into the analysis (Irigoien et al., 2004; Rodríguez-Ramos et al., 2014). Rodríguez-Ramos et al. (2014) showed on synthetic communities that roughly 20–40% of the species are missed by the conventional small volume samples and therefore the sampling methods require considerable improving to adequately represent the species with low population abundances in phytoplankton communities.

Nevertheless, problems related to the quantification of minor pigments with HPLC and the uncertainty of CHEMTAX treating these pigments may further increase the inconsistencies in case of minor groups (Tamm et al., 2015; Eker-Develi et al., 2008). Consequently, several authors (Havskum et al., 2004; Irigoien et al., 2004; Schlüter et al., 2014) suggest combining CHEMTAX and microscopy to reduce their limitations. Development of region-specific calculation matrices for CHEMTAX will further improve the estimations of phytoplankton groups.

4.3. Detection of toxic cyanobacteria

Since neither CHEMTAX nor microscopy are able to separate the toxic and non-toxic strains of algae, highly sensitive qPCR was used to provide additional information about the toxic species in the Väinameri. Potentially hepatotoxic *Dolichospermum* and *Nodularia* were both found in all areas of the Väinameri. Few previous studies have found microcystin producing *Dolichospermum* in the Gulf of Finland (Halinen et al., 2007; Fewer et al., 2009), but since there are no similar studies done in coastal areas, this is the first finding of microcystin producing *Dolichospermum* in a low salinity coastal area of the Baltic Sea. However, nodularin producing *Nodularia* has been common in the entire Baltic Sea area (Mazur and Plinski, 2003; Sivonen et al., 2007).

In case of *Nodularia*, strong correlation ($r_s = 0.74$, $p < 0.01$) was found between molecular detection methods and CHEMTAX while the relationship with microscopy was not apparent. Insignificant correlation might be related to the bigger sample volume used in the analysis and higher sensitivity of the molecular method (10 *nda*F gene copies mL^{-1}). Similarly to chemotaxonomy, in molecular analysis the sample volumes (50–600 mL) largely exceed those for the microscopy (10–25 mL) and if potential toxin producing cyanobacteria are present in smaller concentrations, qPCR might have an advantage over microscopy because of its higher sensitivity (Pacheco et al., 2016). The significant positive correlation ($r_s = 0.71$, $p < 0.01$) for *Dolichospermum*, *mcy*E gene copy numbers and wet biomass presumably occurrs due to the higher porpotion of potentially microcystin-producing *Dolichospermum* population in the Väinameri area.

In the current study, cyanotoxins in the samples were not measured directly. Earlier study by Vaitomaa et al. (2003) has shown a strong positive correlation between *nda*F gene copy numbers and nodularin concentrations, which refers to a continuous production of nodularin by *Nodularia* in the Baltic Sea. In current study, *nda*F gene copy numbers were relatively low (varied from 50 to 824 *ndaF* mL^{-1}) and accordingly we might presume that nodularin concentrations were also rather low. Ngwa et al. (2014) found that *mcy*E copy numbers in environmental water samples were the best predictors of microcystin concentrations, which make qPCR a useful complementary tool for risk assessment of cyanobacterial blooms in the Baltic Sea.

5. Conclusions

Our study demonstrated that the integrated monitoring approach improves the understanding of phytoplankton communities. Combining the methods in economically efficient way, e.g. quick microscopy screening paired with pigment analysis might be the best solution for monitoring of optically complex nutrient rich coastal waters. Quantification of the cyanotoxin-synthesis genes could provide crucial information about the water quality and safety and assist local authorities to avoid human and animal exposure to toxins. Our detection of microcystin-producing *Dolichospermum,* not previously found in low-salinity coastal areas of the Baltic Sea, underlines the usefulness of merging different approaches for identifying the hazardousness of cyanobacterial blooms. Promisingly efficient remote sensing detection of even low cyanobacterial biomass in CDOM-rich waters encourages the joint employment of remote sensing and pigment-based chemotaxonomy. Though in specific regions further investigation with larger datasets are needed, some guidelines are provided in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2018.10.024.

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Popular science articles:

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Tanika Ojasild, magistrikraad, 2016, Pigmentidel põhineva kemotaksonoomia rakendamine erineva troofsustasemega järvede fütoplanktoni uurimisel, Eesti Maaülikool.

Populaarteaduslikud artiklid:

Tamm, Marju (2018). Vetikaviljelus tõstab pead. Horisont 3, 28–33.

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