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Comparison of epifluorescence microscopy and flow cytometry in counting freshwater picophytoplankton

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The smaller the phytoplankton, the greater effort is required to distinguish individual cells by optics-based methods. Flow cytometry is widely applied in marine picophytoplankton research, but in freshwater research its role has remained minor. We compared epifluorescence microscopy and flow cytometry in assessing the composition, abundance and cell sizes of autofluorescent picophytoplankton in epilimnia of 46 Finnish lakes. Phycocyanin-rich picocyanobacteria were the most dominant. The two methods yielded comparable total picophytoplankton abundances, but the determination of cell sizes, and thus total biomasses, were on average an order of magnitude higher in the microscopy results. However, flow cytometry yielded higher cell sizes when applied on small-celled cultured algae. Our study demonstrated that both epifluorescence microscopy and flow cytometry are useful methods in assessing abundances of phycocyanin-rich and phycoerythrin-rich picocyanobacteria and eukaryotic picophytoplankton in lakes. However, accurate determination of cell size and biomass remain challenges for microscopy and especially for flow cytometry.

Introduction

Epifluorescence microscopy and flow cytometry are the most commonly used methods to count picophytoplankton — the smallest (cell diameter < 2 µm) photosynthetic primary producers of open waters that are difficult to observe with traditional light microscopy. Both epifluorescence microscopy and flow cytometry are based on distinguishing autofluorescence of light harvesting pigments in picophytoplankton cells. Based on the autofluorescence profiles, picophytoplankton can be divided into phycocyanin-rich picocyanobacteria, phycoerythrin-rich picocyanobacteria and eukaryotic picophytoplankton — three groups that thrive in slightly different environments (MacIsaac and Stockner 1993, Callieri 2008).

Flow cytometry is routinely applied in studies of marine environments where the existence of small-celled autotrophs is pronounced, and parallel microscopic observations are made if necessary (Collier 2000, Veldhuis and Kraay 2000, Johnson and Martiny 2015). Flow cytometry is also highly useful in enumerating unicellular phytoplankton in laboratory cultures

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(Thomas *et al.* 2018). Instead, the possibilities of automated fluorescence detection and cell counting remain rather underexploited in freshwater studies (Crosbie *et al.* 2003, Cellamare *et al.* 2010). The fact that microscopy is applied more commonly in freshwater research than flow cytometry is probably due to the better visualization properties of microscopy, which enable the assessment of more diverse communities with multicellular and filamentous taxa (Peeters *et al.* 1989, Toepel *et al.* 2004).

Picophytoplankton abundances based on either epifluorescence microscopy or flow cytometry can be converted to biomasses by assuming a fixed cell volume (Bergkemper and Weisse 2018). With epifluorescence microscopy, cell sizes can be estimated by measurements with an eyepiece graticule or image analysis (Callieri 2008). Scattering data yielded by flow cytometry of marine picophytoplankton have also been utilized in quantifying the variation within cell sizes by establishing a conversion between diameters of reference particles and forward or side scattering values (Charpy and Blanchot 1998, Veldhuis and Kraay 2000).

Bergkemper and Weisse (2018) stated that the exclusion of pico-sized algae is a weak spot in European lake monitoring programmes where phytoplankton is counted using light microscopy. However, flow cytometry of freshwater picophytoplankton has shown good conformity with other quantification methods, such as the traditional epifluorescence microscopy (Crosbie *et al.* 2003, Bergkemper and Weisse 2018), DNA-based quantification and analysis of photosynthetic pigment concentration (Veldhuis and Kraay 2000). Since instrumentation for epifluorescence microscopy and flow cytometry is commonly available, and one of these two methods is routinely applied, their conformity deserves closer inspection.

In this study, we tested the applicability of flow cytometry to assess picophytoplankton in 46 boreal lakes. In addition to abundances, we compared biomass estimates determined by the flow cytometry and epifluorescence microscopy. In microscopy, we used eyepiece graticule to measure cell sizes; and in flow cytometry, we used latex beads of a known size as references. To determine the accuracy of the cell size estimates, we measured cell diameters of cultured small-celled cyanobacteria by scanning electron microscopy (SEM), epifluorescence microscopy and flow cytometry. We hypothesised that epifluorescence microscopy and flow cytometry should show coherent abundances and biomasses of the three picophytoplankton pigment groups. We anticipated SEM, epifluorescence microscopy and flow cytometry to yield consistent cell diameter estimates.

Material and methods

Sampling and sample processing

Samples were taken from epilimnia of 46 lakes in southern, central and eastern Finland between June and August 2015 (Table 1). A water sampler (volumes: 2.0 L, 2.6 L or 3.5 L; Limnos, Limnos.pl, Poland) was used to integrate the water column from the surface to maximum 2 m depth or until a thermocline (temperature change > 1°C per meter) was met. Temperature was measured with a thermometer attached to the sampler. In the laboratory, water for picophytoplankton samples was pre-sieved through a 250 µm mesh and then through a sterile 5 µm pore size syringe filter to remove larger phytoplankton and other large particles. For epifluorescence microscopy, unpreserved picophytoplankton were collected onto black polycarbonate filters (pore size: 0.22 µm, Merck Millipore, Germany). The black filters were wet mounted with glycerol and stored frozen at –20°C (e.g. Booth 1993, Salmi *et al.* 2014) until counted with an epifluorescence microscope within 6–8 months. For flow cytometry, 2.5–4 mL of pre-filtered water was stored in 4.5 mL cryovials. To enhance the preservation, paraformaldehyde (16%) was added to a final concentration of 1% (MacIsaac and Stockner 1993). Cryovials were stored frozen at –80°C until counted with flow cytometry within 8–10 months.

Epifluorescence microscopy

An Axio Vert.A1 epifluorescence microscope (Carl Zeiss, Germany) equipped with blue (470 nm) and green (530 nm) LED light sources

Table 1. List of sampling dates, sampled lakes and coordinates of the sampling sites.

ID	Sampling Date (*dd.mm.yyyy*)	Lake	Coordinates North	Coordinates East
1	03.08.2015	Ahveninen	62.94957	26.86753
2	08.07.2015	Ala-Keitele	62.67649	25.86500
3	01.07.2015	Aurejärvi	62.04546	23.36944
4	06.08.2015	Hiidenvesi	60.39003	24.16344
5	03.08.2015	Hirvijärvi	62.95176	26.91284
6	20.07.2015	Ilomantsinjärvi	62.68340	30.90113
7	29.06.2015	Iso Rautavesi	62.06703	25.04697
8	29.07.2015	Juojärvi	62.76788	28.56303
9	27.07.2015	Karankajärvi	62.71111	24.81972
10	29.07.2015	Kermajärvi	62.44644	28.67387
11	08.07.2015	Keski-Keitele	62.83782	26.02901
12	20.07.2015	Koitere	62.94894	30.62775
13	20.07.2015	Konnivesi	61.13990	26.14805
14	03.08.2015	Koskelovesi	62.67619	26.86563
15	08.07.2015	Kuhnamo	62.60993	25.67405
16	29.07.2015	Lannevesi	62.55910	25.44299
17	07.07.2015	Leppävesi	62.23902	25.95099
18	10.08.2015	Lohjanjärvi	60.24951	24.03428
19	27.07.2015	Mahlunjärvi	62.67585	25.08897
20	13.07.2015	Muuruejärvi	63.08189	25.51459
21	03.08.2015	Niinivesi	62.73920	26.85005
22	20.07.2015	Nuorajärvi	62.68001	31.14093
23	01.07.2015	Palovesi	61.89591	23.93470
24	31.07.2015	Pankajärvi	63.37140	30.20379
25	06.07.2015	Peurunka	62.44579	25.85154
26	24.07.2015	Pieksänjärvi	62.32546	27.13805
27	26.07.2015	Pielinen	63.10321	29.98289
28	10.08.2015	Pihlajavesi	62.35952	24.32613
29	29.07.2015	Pyhäjärvi	62.72323	25.44316
30	22.07.2015	Retunen	62.94876	28.65307
31	22.07.2015	Rikkavesi	62.80724	28.74926
32	01.07.2015	Ruovesi	62.00598	24.10244
33	05.08.2015	Rutajärvi	61.94827	26.07683
34	27.07.2015	Saarijärvi	62.72306	25.18175
35	29.07.2015	Summasjärvi	62.64539	25.38419
36	29.07.2015	Suvasvesi	62.48378	28.22890
37	19.07.2015	Sääksjärvi	61.39501	22.40007
38	15.07.2015	Tarjanne	62.13989	24.03638
39	15.07.2015	Toisvesi	62.28604	23.73901
40	24.07.2015	Unnukka	62.38304	28.03689
41	06.07.2015	Uurainen	62.48408	26.13479
42	15.07.2015	Vaskivesi	62.13944	23.77312
43	06.07.2015	Vatianjärvi	62.48346	25.89432
44	01.08.2015	Viekijärvi	63.38422	29.73474
45	13.07.2015	Vuosjärvi	62.99522	25.52393
46	13.07.2015	Ylä-Keitele	63.07631	25.77570

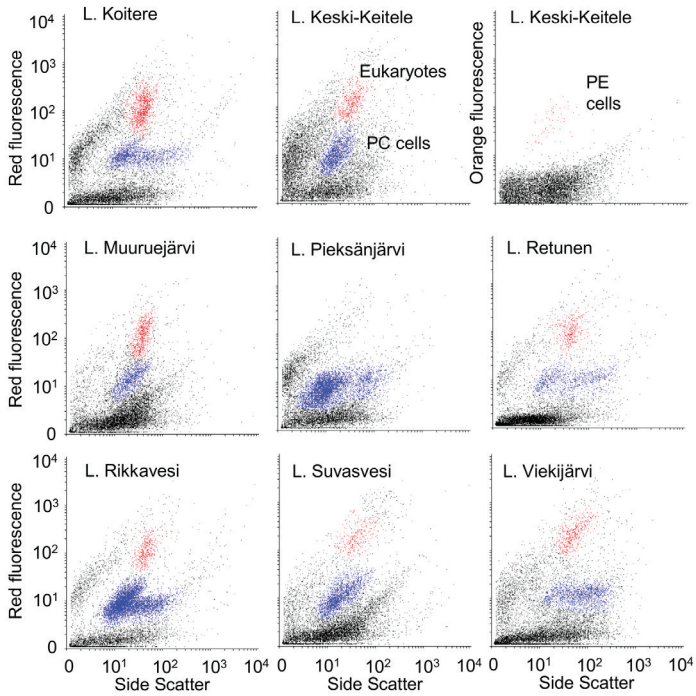

Fig 1. Picophytoplankton cytograms selected to illustrate the variation in lakes Koitere, Keski-Keitele, Muuruejärvi, Pieksänjärvi, Retunen, Rikkavesi, Suvasjärvi and Viekijärvi. Phycocyanin-rich (PC) cells are indicated by blue dots and eukaryotic cells by red dots. The second plot of Lake Keski-Keitele describes the location of phycoerythrin-rich (PE) cells represented by orange dots. Note that both axes are on a logarithmic scale.

was used for counting picophytoplankton on the black filters. The blue LED was connected to filter set 09 (EX: BP 450-490, beamsplitter: 510, EM: LP 515, Carl Zeiss, Germany) and the green LED to filter set 14 (EX: BP 510-560, beamsplitter: 580, EM: LP 590, Carl Zeiss, Germany). Phycocyanin-rich (hereafter PC cells) showed rather weak deep red autofluorescence with the blue set. Instead, with the green set they showed brighter red autofluorescence. With the blue set, autofluorescence of phycoerythrin-rich picocyanobacteria (hereafter PE cells) was distinguished as light orange and that of eukaryotic picophytoplankton as red. PE cells showed bright orange and eukaryotic picophytoplankton only weak red autofluorescence with the green set (MacIsaac and Stockner 1993). Picophytoplankton were counted with 1000× total magnification from at least ten randomly chosen fields of view across the black membrane filter.

Flow cytometry

A FACSCalibur flow cytometer (Becton-Dickinson, USA) equipped with 488 nm laser excitation, detectors for forward (FSC) and side (SSC) scatters and three channels for fluorescence detection: green (FL1, 530/30 nm), orange (FL2, 585/42 nm) and red (FL3, 650 nm, LP) was used in this study. Picophytoplankton were divided into PC, PE and eukaryotic cells according to the intensities of their orange and red fluorescent signals (Fig. 1). The applied settings for photomultipliers were FSC-H E01, SSC-H 350, FL1 600, FL2 600 and FL3 600. All channels were deployed in logarithmic mode. The primary parameter for recording counted events was side scatter and secondary parameter red fluorescence. The threshold value for both was 25. A low flow rate was used for all samples and 10 000 events were recorded. The flow rate was verified twice every day (before and after samples) by weighting 1 mL of water with an analytical balance (AT21, Mettler-Toledo, Australia, readability 1 µg), running it on flow cytometer for 5–10 minutes and weighing it again to measure the volume of the water that went through the flow cell in a specified time. Daily specific flow rates (mean 10 µL min^{-1}, SD = 0.9, number of working days = 9) were used to convert the counted events into cell

abundances. Cell size estimates were obtained by establishing a regression model between forward scatter and diameters of reference monodispersal latex beads (Phinney and Cucci 1989). Diameters of the used latex beads were 0.3 µm, 0.6 µm (Sigma-Aldrich, USA), 1.0 µm (Beckman Coulter, USA), 3.0 µm (Sigma-Aldrich, USA) and 6.0 µm (Thermo Fisher Scientific, USA) and their forward scatters were recorded by the flow cytometer on 2–5 separate working days with the same FSC and SSC settings as for the picophytoplankton (Fig. 2).

Flowing Software ver. 2.5.1 (University of Turku, Finland) was used for processing the flow cytometry data. Picophytoplankton populations were delimited from the scatter plots without first scrutinizing the microscopy results to avoid bias in the interpretation. The FSC of each cell was converted to cell diameters before the average cell diameter in the population was calculated.

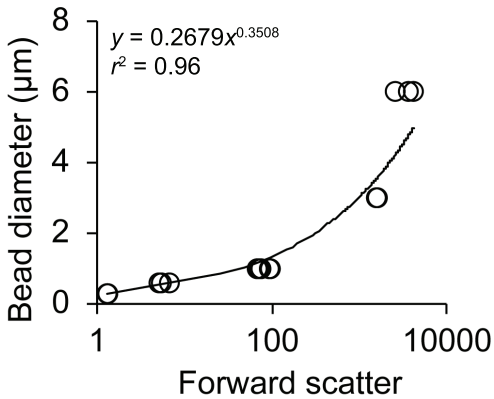

Fig 2. Comparison of mean forward scattering (FSC) and nominal diameter of latex beads, regression equation and degree of explanation.

Cell size measurements of cultured cyanobacteria

Fresh clonal cultures of *Chroococcus*, *Snowella* and *Synechococcus* sp. in liquid Z8 medium were filtered through 5 µm pore size syringe filters similarly to lake water samples. Samples for epifluorescence microscopy and flow cytometry were prepared as described above and examined with the same instruments as the lake samples. Using epifluorescence microscopy, the diameters of cultured cyanobacteria were measured with the green excitation filter set. Within flow cytometry, same samples of each culture were run on three consecutive days.

For scanning electron microscopy (SEM), 1–3 mL of each culture was collected on a Whatman GF/F filter and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After washing with phosphate buffer and rinsing with water, samples were dehydrated with a graded ethanol series. Drying was done using a critical point dryer (K850, Quorum Technologies, UK) and afterwards, samples were attached to specimen stubs, they were sputter coated (Q150 T ES, Quorum Technologies, UK) with a thin layer of platinum. Samples were imaged with field-emission scanning electron microscope (Sigma HD VP, Carl Zeiss AG, Germany). The preparation and imaging of samples was done in the Electron Microscopy Core Facility of Biocenter Oulu, University of Oulu.

Statistical methods

To count picophytoplankton with the epifluorescence microscope (see Epifluorescence microscopy section), a proprietary computer programme was used to calculate 95% confidence intervals for mean abundances and biomasses in real time to optimize counting effort (e.g. Salmi *et al.* 2014). Confidence intervals for mean abundance were calculated as:

$$\mathrm{cfl\%} = \frac{100 \times t_{0.025} \times \sqrt{s^2 / n}}{\mathrm{mean}}, \qquad (1)$$

where $t_{0.025}$ is the 97.5% percentile of the t distribution with $n-1$ degrees of freedom, s^2 is the sample variance and n is the number of replicate microscope views.

Shapes (sphere, rotational ellipsoid, cylinder) of picophytoplankton cells were estimated and the main dimensions measured with an eyepiece graticule at a scale of 1 µm. The biomass of picophytoplankton was calculated by assuming a cell density equal to that of water.

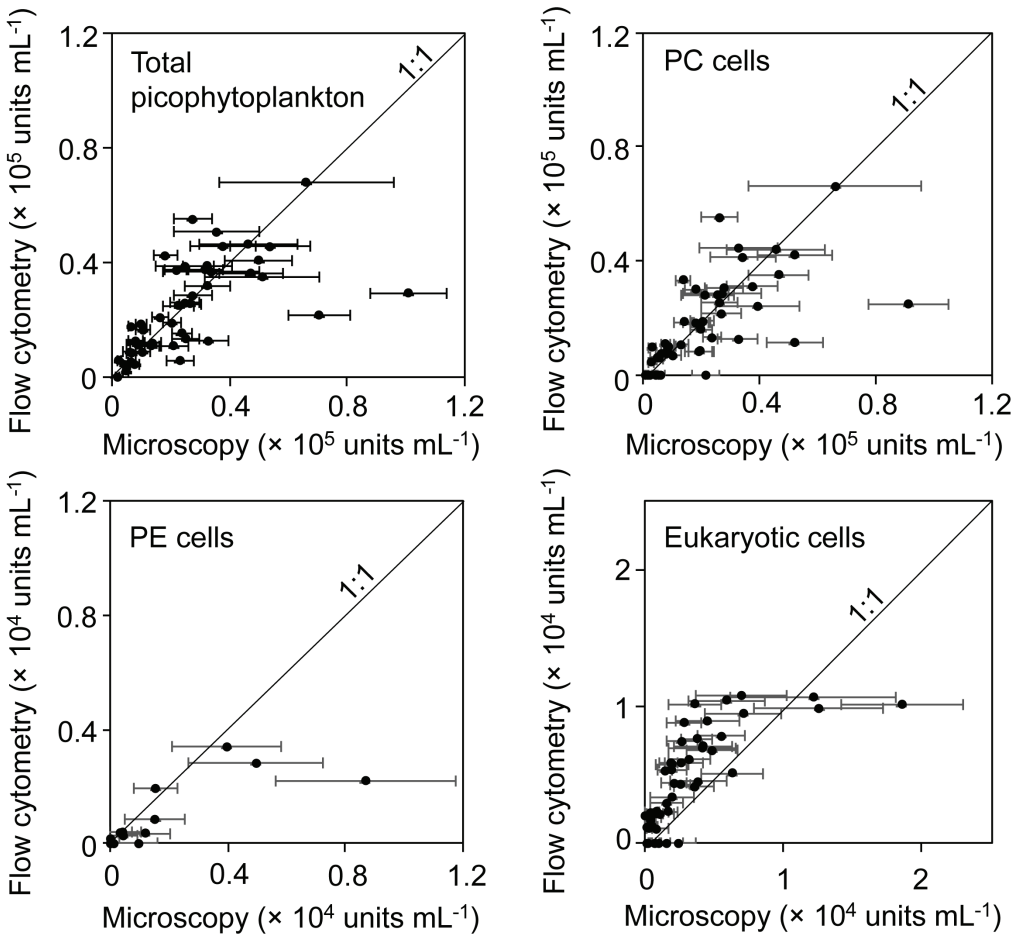

Fig. 3. Picophytoplankton abundance ratios determined by epifluorescence microscopy and flow cytometry for all picophytoplankton cells (Total picophytoplankton,upper left panel), phycocyanin-rich cells (PC cells, upper right panel), phytoerythrin-rich cells (PE cells, lower left panel), and eukaryotic picophytoplankton (Eukaryotic cells, lower right panel). The horizontal error bars represent the 95% confidence interval for the average abundance in the sample counted using an epifluorescence microscope. Note the different axis scales in the upper and lower panels.

Confidence intervals for mean biomass of picophytoplankton were calculated as:

$$\text{cfl\%} = \frac{100 \times t_{0.025} \times \sqrt{\sum s^2 / n}}{\text{mean}}, \qquad (2)$$

where $t_{0.025}$ is the 97.5% percentile of the t distribution with n−1 degrees of freedom and, deviating from the equation 1, $\sum s^2$ is sum of the variances of biomasses of different size classes and n is the number of replicates. The required number of counted views was estimated so that cfl% ≤ 30 for total biomass was reached, but at least 10 views were counted.

Flow cytometry-based abundances were compared against microscopy-based abundances and biomasses by scrutinizing if the flow cytometry results fall inside the confidence intervals of the microscopy assessments. Correlations between the two methods were scrutinized using Spearman's correlation and the significance of the differences were tested with a related-samples Wilcoxon signed-rank test. The non-parametric approaches were chosen because of the relatively low number of the samples (46 lakes). The statistical analyses were made using SPSS Statistics ver. 26 (IBM, USA).

Results

Our dataset from 46 Finnish lakes offered a good basis to evaluate the consistency of picophytoplankton abundance and biomass measurements by epifluorescence microscopy and flow cytometry in natural conditions. Here, we report the quantitative results yielded by the two methods as well as the comparisons of cell size measurements based on epifluorescence microcopy, flow cytometry and SEM.

Abundance

According to microscopic observations of the lake samples, most (mean: 77%, SD = 7) of the picophytoplankton units were solitary cells and only a few dividing cells or small colonies were observed in the lake samples. Some of the individuals, however, might have originated from colonies, destroyed during pre-filtrations. Total picophytoplankton abundances in microscopically counted samples varied from 2.0×10^3 to 1.0×10^5 units mL^{-1} (Fig. 3). PC cells covered most (mean: 80%, SD = 17) of that. The proportion of eukaryotic units was notably lower (mean: 18%, SD = 17) and only a few samples contained PE cells (Fig. 3).

Consistent with the fluorescence microscopy samples, PC cells also accounted for the majority of flow cytometry samples, and eukaryotic and PE cells were present in small numbers (Fig. 3). For total picophytoplankton abundance, the median microscopy/flow cytometry ratio was 1.0 (mean: 1.2, SD = 0.81). The difference between total abundances was not significant (related-samples Wilcoxon signed-rank test, $p = 0.9$) and 46% of the flow cytometrically determined total abundances fell inside the 95% confidence intervals of corresponding microscopic results (Fig. 3; Spearman's correlation, $\rho = 0.79$, $p < 0.001$). When PC cells, PE cells and eukaryotic cells were analysed separately, the median microscopy/flow cytometry ratios were 0.92, 1.01 and 0.46, respectively (means: 1.0, SD = 0.89; 1.2, SD = 1.22 and 0.49, SD = 0.37, respectively). The differences for picocyanobacteria were not significant (related-samples Wilcoxon signed-rank test, $p = 0.2$ for PC and $p = 0.06$ for PE). Additionally, 50% of the flow cytometrically determined PC and 75% of the PE cell abundances were inside the 95% confidence intervals of microscopic cell counts. For eukaryotic picophytoplankton, flow cytometry gave significantly higher counts than microscopy did (related-samples Wilcoxon signed-rank test, $p < 0.001$), and 22% of the flow cytometry assessments were inside the 95% confidence intervals of microscopy-based abundance assessments.

The average time used for counting by flow cytometer was relatively long (mean: 9 minutes SD = 5), since the natural samples were rather sparse for flow cytometry. Compared with microscopy where the counting effort was adjusted to reach the 95% confidence intervals for the mean biomass, flow cytometry was approximately tenfold faster. The total number of counted picophytoplankton units was notably higher in flow cytometric samples (average 1885, SD = 1280) compared with microscopic counts (mean: 162, SD = 66). Thus, timewise flow cytometry was a more cost-effective way to count picophytoplankton.

Cell size and biomass

In microscopic estimations, the most common cell diameter in the lake samples was 1 µm (70%, SD = 18). Instead, average diameter based on reference beads and forward scatter (FSC) was strikingly different: 0.56 µm (SD = 0.39). Therefore, biomass estimates were, on average, 15-fold higher (SD = 23) using microscopy, and correlation between FSC and microscopy-based total biomass estimates was rather weak (Spearman's correlation, $\rho = 0.51$, $p < 0.001$, Fig. 4).

Cell size measurements of cultured cyanobacteria

Scanning electron microscopy (SEM), epifluorescence microscopy and flow cytometry yielded similar cell diameters for picosized *Synechococcus* (Table 2). However, for *Chroococcus* and *Snowella*, flow cytometry gave notably wider diameters than SEM or epifluorescence micros-

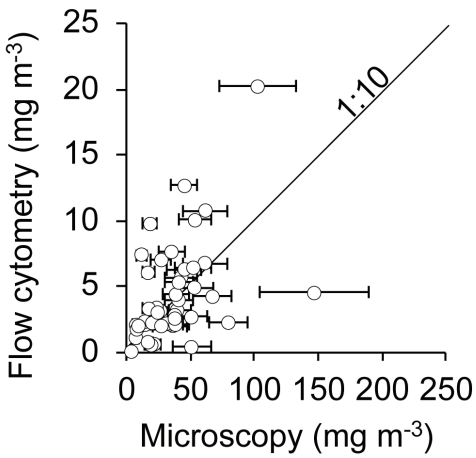

Fig 4. Relationships between total picophytoplankton biomasses (mg m^{-3}) determined by epifluorescence microscopy and flow cytometry.

copy. Additionally, the ratio of epifluorescence microscopy-based cell abundances to flow cytometry-based unit counts was less than 1 (Table 2). Thus, the wider diameter estimates were likely partly due to the appearance of *Chroococcus* and *Snowella* as dividing cells and microcolonies rather than as individual cells; in flow cytometry particles position themselves along their longest diameters. The presence of dividing cells was confirmed by the microscopy methods.

Discussion

To scrutinize the overall congruence of the abundances and biomasses assessed by epifluorescence microscopy and flow cytometry in our study, several sources of variation need to be considered. Variation accumulates from (a) sampling and sample processing; (b) storage of the samples; and the (c) counting protocols themselves. Since these steps are rather well outlined in literature for different types of microscopy and flow cytometry samples, we emphasise in the discussion the counting procedures and accumulation of total variation.

Abundance

Crosbie *et al.* (2003) compared flow cytometry and epifluorescence microscopy in counting picophytoplankton from Lake Mondsee. Using a FACSCalibur instrument, similar to the one we used here, they reported strong regression between the two methods when counting solitary picophytoplankton (r^2 = 0.95) or microcolonies (r^2 = 0.88). In our study, correlation between the methods remained lower (Spearman's correlation, $\rho = 0.79$, $p < 0.001$). In our study, samples for microscopy and flow cytometry were parallel subsamples that were processed and stored differently, but according to the general outlines of the discipline. Additionally, the success of microscopic counting from filters depends highly on the distribution of counted units on the filter. The distribution is often expected to be random, which is seldom the case due to the properties of the filtering system and interactions between cells (e.g. McNabb 1960, Sanford *et al.* 1969). The aggregation of cells was likely a major reason for the sometimes relatively wide confidence intervals of the mean total abundance (up to 49% of the mean abundance; Fig. 3). In flow cytometry, in addition to the distribution of cells in the sample water, error might accumulate from inconsistent flow rate. To overcome this, reference beads with known concentrations

Table 2. Mean cell diameter of cultured cyanobacterial cells estimated by different methods, and the abundance ratio assessed by epifluorescence microscopy (cells) and flow cytometry (units). Standard deviations for scanning electron microscopy (SEM) and flow cytometry (Flow) are indicated in parentheses. In epifluorescence microscopy, standard deviations are not given due to separate, eyepiece graticule-based measurements.

Cell diameters	SEM (µm)	Epifluorescence (µm)	Flow (µm)
Chroococcus	2.71 (0.27)	3	5.0 (4.4)
Snowella	2.36 (0.31)	3	4.4 (0.6)
Synechococcus	0.73 (0.13)	1	1.0 (0.3)

could be added to each cytometry sample as internal standards to calculate the cell abundance (Olson *et al.* 1993). However, the use of beads might cause problems due to their tendency to settle and aggregate to each other. On the other hand, Gasol and Del Giorgio (2000) reported congruent abundances of stained bacteria assessed with bead-based and volumetric calibration-based methods. The flow rate of our FACSCalibur was rather stable (coefficient of variation = 10%) as measured every working day, yet by relying on volumetric calibration we could not detect possible occasional inconsistencies in the flow rate or in the electronics during counts of the actual samples.

The processing of cytometry data could be a notable source of variation; subjective decisions must be made on how to delimit the populations of interest to the cytograms. In the dataset of several lakes, populations were cropped by eyeballing cytograms of each sample individually, because fluorescence as well as side and forward scattering and amount of background noise varied between samples. In conformity with the cytograms of lake picophytoplankton published by Metz *et al.* (2019), our cytograms contained lots of background noise from non-target fluorescent particles and structures of broken-down cells originating from the pre-filtration process or naturally from the lake water (Fig. 1). In some samples, the picophytoplankton populations were not clearly distinguished from the noise, which led to occasional cases of zero abundances and further to zero biomasses (Figs. 3 and 4). In contrast, epifluorescence microscopy never yielded zero abundances. However, our results demonstrated that picophytoplankton abundances in summer epilimnia of boreal lakes were generally well within the detection range of a flow cytometer.

Cell size

Very few studies have compared picophytoplankton cell sizes measured microscopically and flow cytometrically. Olson *et al.* 1989 reported good predictability between Coulter volumes and vertically polarized scattering of larger phytoplankton, excluding pennate diatoms. Chrisholm (1992) reported a calibration curve between Coulter volume and flow cytometry forward scatter of *Synechococcus*. In addition, Moreira-Turcq *et al.* (2001) reported a linear relationship between FSC and coulter counter-based cell diameters using cultured phytoplankton with a cell diameter of 1–3.5 µm. Chapry and Blanchot (1998) determined *Synechococcus* population microscopically and then related the known cell size to cytometer reference beads to establish a conversion factor between FSC and cell diameters of natural populations.

Although latex beads are successfully used as internal standards for abundances and sizes (Koch *et al.* 1996), the FSC of latex beads might not be the most optimal reference for the cells in our study. Our dataset from different lakes consists of populations likely displaying a variety of cell morphologies and fine structures and thus the refractive indices of cells probably deviate from each other and that of the reference. Silica beads with refractive index closer to small living cells, as used by Foladori *et al.* (2008) in studies of heterotrophic bacteria, might be an option worth testing in picophytoplankton studies as well. Microscopic measurements of cell dimensions no doubt contain variation. The diffraction of autofluorescence, which can be further enhanced by a coating of glycerol as observed by Callieri *et al.* (1996), might have disturbed the microscopic measurements of cell dimensions. Additionally, the 1000× total magnification and 1 µm division in the eyepiece graticule used in this study were rather coarse for the small cells. Lastly, fixatives and freezing have been shown to affect many cell types (e.g. Troussellier *et al.* 1995).

In flow cytometry, the typical way of recording FSC with logarithmic scale possibly reduces the accuracy of diameter estimates of small targets (Herzenberg *et al.* 2006), and generally, the logarithmic scale compresses the digital information in the upper end of the scale. Thus, it is challenging to apply flow cytometry with the same settings to analyse a broad scale of different sized targets. Additionally, the observed cell duplicates and microcolonies of *Chroococcus* and *Snowella* cultures might have affected the wider flow cytometry-based cell diameter estimates. Overall, due to the multiple sources

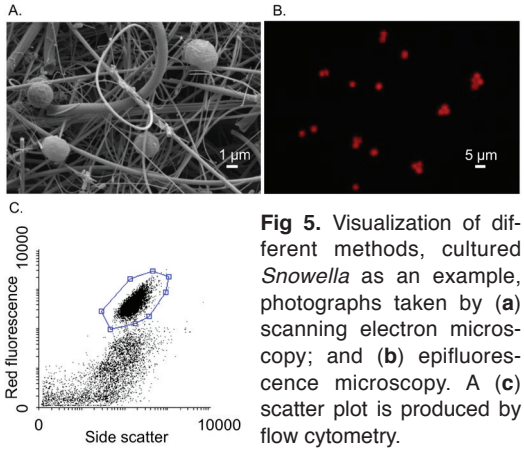

Fig 5. Visualization of different methods, cultured *Snowella* as an example, photographs taken by (**a**) scanning electron microscopy; and (**b**) epifluorescence microscopy. A (**c**) scatter plot is produced by flow cytometry.

of variation discussed above, the biomass assessments can be considered rather crude, by both epifluorescence microscopy and flow cytometry. However, as demonstrated by the comparison of SEM, epifluorescence microscopy and flow cytometry, the tested methods have their own strengths in visualization (Fig. 5).

Conclusions

Despite the variation and substantial subjectivity related to both microscopy and flow cytometry, both methods revealed the abundances of the three picophytoplankton pigment groups in a rather congruent manner. However, cell sizes, estimated by the two methods, differed notably from each other and the coherence between biomasses assessed by the two methods was weak. Therefore, assessment of cell sizes requires further scrutinization. The main outcome of this study was that both methods were practical to use to assess total picophytoplankton abundances and relationship of classes in pre-filtered lake water samples, but not for exact biomass estimates. The major advantage of the flow cytometry was its counting speed. Microscopy, in turn, yielded better visualization of the research material.

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