**Trait-based analysis of subpolar North Atlantic phytoplankton and plastidic ciliate communities using automated flow cytometer**

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Running head: Automated analyses of plankton traits

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**Abstract**

Plankton are an extremely diverse and polyphyletic group, exhibiting a large range in morphological and physiological traits. Here, we apply automated optical techniques, provided by the pulse-shape recording automated flow cytometer - CytoSense - to investigate trait variability of phytoplankton and plastidic ciliates in Arctic and Atlantic waters of the subpolar North Atlantic. We used the bio-optical descriptors derived from the CytoSense (light scattering (forward and sideward) and fluorescence (red, yellow and orange from chlorophyll *a*, degraded pigments and phycobiliproteins, respectively)) and translated them into functional traits to demonstrate ecological trait variability along an environmental gradient. Cell size was the master trait varying in this study, with large micro-plankton (> 20 *µ*m in cell diameter), including diatoms as single cells and chains, as well as plastidic ciliates found in Arctic waters, whilst small-sized plankton groups, such as the pico-eukaryotes (< 2 *µ*m) and the cyanobacteria *Synechococcus* were dominant in Atlantic waters. Morphological traits, such as chain/colony formation and structural complexity (i.e. cellular processes, setae and internal vacuoles) appear to favour buoyancy in highly illuminated and stratified Arctic waters. In Atlantic waters, small cell size and spherical cell shape, in addition to photo-physiological traits, such as high internal pigmentation, offer chromatic adaptation for survival in the low nutrient and dynamic mixing waters of the Atlantic Ocean. The use of automated techniques that quantify ecological traits holds exciting new opportunities to unravel linkages between the structure and function of plankton communities and marine ecosystems.

**Introduction**

Marine plankton are an extremely diverse and polyphyletic group, revealing a large range in morphological and physiological characteristics, nutritional and light requirements, life-cycle and predation-avoidance strategies (Falkowski 2004). The overall fitness of individuals within mixed species assemblages is an evolutionary response to the spatial and temporal heterogeneity of pelagic ecosystems, in addition to biotic interactions (e.g. competition, mutualism, predation) (Litchman et al. 2010). Over global spatial scales, marine plankton communities are biogeographically structured, revealing contrasting patterns of distribution (Follows et al. 2007; Bibby et al. 2009; Cermeño et al. 2010).

Functional traits, which define species in terms of their ecological, physiological and biogeochemical characteristics, have been shown to be a promising approach to understanding the mechanisms involved in structuring the community as a whole along environmental gradients (McGill et al. 2006). However, this field faces numerous challenges when applied to microscopic organisms, due to the difficulty of quantifying multiple traits at the level of the individual within a diverse community. Intraspecific trait variability (e.g. changes in cell organelles, cell size or colony formation) are also common in marine plankton communities, since their eco-physiological functions may differ depending on environmental variables, such as light levels, nutrient concentrations and grazing pressures (Litchman et al. 2007, 2010). Automated techniques may fill some of these knowledge gaps by assuring that measurements are observer-independent, in addition to providing data at the individual (or cellular) level that demonstrate links between community traits and environmental gradients (Pomati et al. 2013).

The automated quantification of multiple traits in a plankton community (e.g. cell size, shape, organelles, cell wall structure, texture, etc.) has been previously performed using several approaches. Examples include imaging flow cytometers, such as the FlowCam imaging cytometer (Sieracki et al. 1998) and the Imaging FlowCytobot (Sosik and Olson 2007). Both instruments use analysis of plankton images, as well as fluorescence properties, to capture plankton variability related to size, morphology, cell-to-cell interactions and other characteristics (Dashkova et al. 2017). More recently, the combination of fluorescence probes with automated, 3D microscopic imaging (known as environmental High Content Fluorescence Microscopy) allowed not only plankton identification but also computerised quantification of internal cell structures (Colin et al. 2017). These characteristics include DNA content, intracellular membranes, organelles (e.g. chloroplasts, food vacuoles) and cell wall structures (polysaccharides, biogenic silica, calcium carbonate), in addition to biological interactions (e.g. mixotrophy, symbiosis and parasitism) (Colin et al. 2017). These techniques generate a large amount of data that, if aligned with supervised machine learning algorithms, could tackle the diversity of traits in biological communities across a broad spectrum of spatiotemporal scales (Pomati et al. 2013; Breton et al. 2017; Colin et al. 2017).

The CytoSense instrument (CytoBuoy, b.v., NL), similar to other analytical flow cytometers, provides a non-taxonomical analysis that discriminates particles in aquatic samples, allowing classification of phytoplankton groups based on their individual optical fingerprints (fluorescence emission and light scattering properties) (Malkassian et al. 2011). A further advantage of this instrument is its capacity to record the ‘pulse shape’ (e.g., Fig. 1), which are optical fingerprint scans across each particle that provide information about the particle structure (including cellular or sub-cellular organisation) and allow microbial classification (Dubelaar et al. 2004). Moreover, the CytoSense can identify a broader range of particle sizes (from < 1 *µ*m up to 1.5 mm in diameter and up to 4 mm in length) that would not be possible with a conventional flow cytometer. The CytoSense also provides images of a selection of those particles (> 10 *µ*m, Fontana et al. 2014), which is useful for species identification of specific plankton groups. The instrument generates a large and complex dataset, where many descriptors can provide meaningful eco-physiological information to be used in trait-based analyses, such as cell size, shape, multicellular organisation (chain, colony of single cells) and pigment variability (chlorophyll-*a*, phycobiliproteins and degraded chlorophyll compounds) (McFarland et al. 2015).

Here, we apply an automated technique using the CytoSense to analyse trait variability along a longitudinal transect (55-10oW) in the subpolar North Atlantic (Fig. 2). The goals of this paper are to: 1) characterise plankton communities (phytoplankton and plastidic ciliates, herein referred to as those able to emit autofluorescence) along gradients of contrasting hydrography comprising Arctic and Atlantic water masses in the sub-Arctic North Atlantic; 2) use the CytoSense descriptors as examples of plankton functional traits; and 3) investigate the relationship between functional traits and environmental variables among these communities.

**Methods**

***Sample location and collection***

Data for this study were collected on board the *RRS James Clark Ross* during the JR302 research cruise, starting on 6th June 2014 on the western side of the Labrador Sea (Canada) and finishing on 21st July 2014, off the west coast of Scotland. Stations were sampled on a west-east transect crossing the shelves and deep ocean basin of the subpolar North Atlantic Ocean, including the Labrador, Greenland and Irminger seas (Fig. 2).

Vertical continuous profiles of temperature and salinity were measured using a Seabird 911+ Conductivity-Temperature-Density (CTD) system equipped with a 24 × 10 L Niskin bottle rosette sampler. Water samples were collected on the upward CTD casts. A stratification index (SI) was calculated as the difference in potential density (*σθ*) values between 60 m and 10 m, divided by the respective difference in depth (50 m), as reported in Fragoso et al. (2016).

***Nutrient concentrations and chlorophyll-a***

Discrete water samples were collected for chlorophyll *a* (Chl*a*) analysis from the surface (< 10 m). Samples for nutrient analysis (silicate, phosphate and nitrate) were also collected from the surface (< 10 m), and at every 30 to 40 m from 10 to 200 m, and every 100 m at depths > 200 m. Samples were measured onboard using a 7-channel SEAL AA3 AutoAnalyser (SEAL Analytical, Ltd., UK) for dissolved inorganic nutrients. Chl*a* was extracted in 90% acetone for approximately 24 hours at -20°C and fluorometrically determined using a Trilogy® Laboratory Fluorometer (Turner Designs Inc., CA, USA) equipped with Welschmeyer (1994) filters and calibrated against a Chl*a* standard (Sigma, UK) as in Poulton et al. (2016).

Nitrate to phosphate (ΔNO3/ΔPO4) and silicate to nitrate (ΔSi(OH4)/ΔNO3) utilization ratios represent the nutrient reduction in the upper water column (upper 200 m) due to phytoplankton consumption from spring to mid-summer. These reductions were calculated as the difference of the integrated surface (< 10 m) to 200 m nutrient concentration (nitrate, phosphate and silicate) from the time of sampling (spring to mid-summer) and ‘winter values’ prior to the growth (bloom) season (e.g. Fragoso and Smith 2012), which we consider as the highest concentration between 200 to 500 m at each station.

***CytoSense analysis***

Water samples (0.2 L) from the surface (< 10 m) were collected and fixed with pre-filtered 50 % glutaraldehyde (Fisher Scientific U.K., Ltd., UK) at a final concentration of 0.25 %. After 15-30 minutes of fixation, samples were stored at -80 °C prior to analysis. Although this method has been reported to cause cell losses (20-40 %) for some diatom and dinoflagellate species (Vaulot et al. 1989; Lepesteur et al. 1993), the impact of preservatives is species-specific, given that some species are more robust than others (Menden-Deuer et al. 2001). Thus, it is difficult to make a general prediction of potential cell losses for a mixed population. However, such preservation is still recommended for natural samples preserved for a long period (months to few years) (Dubelaar and Gerritzen 2000; Marie et al. 2005). Samples were analysed within 24 months of collection using a CytoSense benchtop flow cytometer (CytoBuoy, b.v., NL), which allowed particle examination within a size diameter range of < 1 to 1500 *µ*m.

Similar to other flow cytometers, the suspended particles (sample) are injected into a particle-free carrying fluid (sheath). The laminar flow of the moving sheath fluid aligns the cells in single file sample stream that intersects a laser (488 nm) (Haraguchi et al. 2017). To match the density and refractive index of the sheath fluid as closely as possible to the samples, prior to analysis, the sheath fluid was replaced with a 3% NaCl solution (w/v) made with Milli-Q water and filtered through a 0.2 *µ*m filter. To avoid bacterial growth, which could interfere with our analysis, the biocide ProClin 950 (Sigma-Aldrich) was added to the new sheath fluid at a final concentration of 0.1% (v/v) before being pumped into the CytoSense.

Triplicate samples (pseudo-replicates) were injected into the CytoSense via a volume calibrated sample pump, which enables the user to collect direct particle concentration data without the need for calibration beads. Samples were transferred to a glass beaker and kept in suspension using a magnetic stirrer. Each sample was run until ~10,000 particle events were recorded. To read 10,000 particles, the volumes analysed ranged from 200 *µ*L, where picophytoplankton were abundant, to 5 mL, for samples with low phytoplankton abundances. Particles are triggered when they intersect a flat 488 nm laser excitation beam of 5 *µ*m high and 300 *µ*m wide as they pass through the flow cell (flow rate of 10.26 **L s-1). For each particle detected, the CytoSense acquires data for the following parameters: forward light scatter (FWS, indicating cell size) and sideward light scatter (SWS), with the latter providing information about cellular granularity and surface complexity. In addition to light scattering properties, the fluorescence signatures resulting from excitation by the blue light (488 nm) was detected as emitted light at several wavelengths; red Chl*a* fluorescence (FR; 650-830 nm, about 95 % of Chl*a* fluorescence arises from Photosystem II, Johnsen & Sakshaug 2007), orange fluorescence (FO, emission from phycobiliproteins; 562-650 nm) and yellow/green fluorescence (FY/G, decaying pigments; 515-562 nm, Fontana et al. 2014). A ‘curvature’ channel adds an extra two-dimensional component by capturing the ‘split’ forward scatter signal from a double laser beam with +45° (left) and -45° (right) polarization angles (Thomas et al. 2018). If a particle has a curved or spiral shape (often observed in diatoms; e.g. *Chaetoceros curvisetus*), the forward scatter polarization ratio is high.

The trigger channel was set to only measure particles for which the emitted total red fluorescence (FR) was greater than 20 mV from each particle. The reason for this protocol is to target photosynthetic phytoplankton with a strong red fluorescence signal derived from Chl*a* (Marrec et al. 2017). This allows the acquisition of some pico-phytoplankton FR emission, such as cyanobacteria *Synechococcus* cells, but excludes the cyanobacteria *Prochlorococcus* as ~80% of their cellular Chl*a* is bonded to the non-fluorescent Photosystem I, giving a low Chl*a* emission (around 5%, Johnsen and Sakshaug 2007) and is not detected at a trigger level of total FR of 20 mV (Marrec et al. 2017). Fluorescence trigger level and photomultiplier detector sensitivities were optimised prior to running the samples in order to capture the full size range of the samples in a single acquisition, whilst minimizing background scatter detection (see the CytoSense Manual at www.cytobuoy.com).

During data acquisition, the CytoSense constructs a ‘pulse shape’ for each individual particle, based on the distribution of the fluorescence and light scatter signal along its length. This provides a visual representation of the cross section of a planktonic organism, and an estimate of cell length (*µ*m) on the longitudinal axis (according to the known laminar flow rate). It also facilitates the identification of phytoplankton cells with additional signatures from intracellular organelles (e.g. chloroplasts) and plastidic ciliates (aloricate and loricate-bearing) with photosynthetic endosymbionts (Fig. 1).

The pulse shapes of light scatter and fluorescence properties were coupled with a built-in miniature image-in-flow camera (PixeLINK PL-B741 1.3MP, magnification = 16x, pixel size = 4.8 *µ*m) (PixeLINK, Ottawa, Canada) mounted upward in the capillary tube of the CytoSense instrument (resolution in the size range > 5 *µ*m) which facilitated identification and enumeration of plankton cells based on functional traits. Further information about the functionality of the CytoSense can be found in Malkassian et al. (2011).

***CytoSense descriptors and functional traits***

For each of the six channels (FWS, SWS, FR, FY/G, FO and Curvature), the CytoClus 4 software (CytoBuoy, Nieuwerbrug, Netherlands; Dubelaar and Gerritzen 2000) calculates several parameters for each particle, including length (*µ*m), integrated total signal (area under the curve), maximum signal amplitude, average signal strength, fill factor (area of each signal compared to background), signal asymmetry and number of cells (number of peaks in pulse shape). The representation of each parameter and more detailed information can be found in the supplementary material (Fig. S1) and in Fontana et al. (2014).

The parameters used in this paper to derive functional traits are listed in Table 1. Cell size is considered a major functional trait in plankton, given that it is correlated with many physiological, ecological and life history traits (Marañón 2015). In this study, FWS length is used to represent cell size. Curvature length and number of signal peaks per particle represent multicellular organization (chains and/or colonies of single cells). The bio-optical signal peaks represent, approximately, the number of cells in a particle (chain), whilst the curvature represents how the particles deviate from the centre of the flow. Chains of cells with a high curvature value (i.e. with a spiral shape) will by their nature be longer than their FWS length suggests. Therefore a combination of increased curvature and cell number will indicate the presence of long chains (e.g. diatoms). FWS asymmetry provides information about the shape of the particles, whereas the FWS fill factor represents particle shape diversity, where a high value (near 1) represents a particle with a square shape. We, therefore, assumed the opposite (i.e. 1-Fill Factor) to represent a particle with a shape resembling a sphere.

Maximum SWS signal is correlated with the refraction index of the particle and represents high internal/external structure complexity, such as cell wall granularity (e.g. coccoliths in coccolithophores), ornaments (processes, setae and heavily silicified cell walls in diatoms) and internal vacuoles (also commonly found in large diatoms; Woods and Villareal 2008). The FWS total signal has been suggested as a suitable descriptor for particle volume (Haraguchi et al. 2017), thus, photo-physiological information for the community, such as the amount of chlorophyll-*a* per cell volume (Chl*a*/Vol), either as a single cell or chain, was analysed by FR total/FWS total. Other fluorescence signatures, such as FO total/FR total related to their size (FWS total), provide information about the pigment composition in the phytoplankton community, allowing the differentiation of cyanobacteria from other picoplankton groups, given that they contain high concentrations of phycobiliproteins (e.g. phycoerythrin, PE), which fluoresce in the orange part of the spectrum, per cell size (FWS total) (Table 1).

***Plankton group discrimination***

To allow a proper interpretation of trait variability among functional groups, photosynthetic plankton were classified and counted using the CytoClus 4 software. The groups were classified *a posteriori*. A manual clustering of the data was primarily selected based on FWS length (*µ*m) vs FO total, since size and pigments (Chl*a* and PE) were the main notable traits observed within groups (see manual gating information in the supplementary material, Fig. S2). However, cytograms of a variety of other parameters (e.g. FWS length (*µ*m) vs FR total; see previous section) were performed to confirm the identity of the phytoplankton groups (Fig. S3, supplementary material). The manual clustering consists of selecting points in the scatterplot, which represents the populations of cells with common characteristics (termed “gating” in flow cytometry) that were assigned to specific plankton groups based on their respective properties of light scatter and auto-fluorescence. Example of cytograms showing the assignment of different plankton groups as a function of their bio-optical properties are shown in Figure 3.

A total of nine groups were manually classified based on size (micro-, nano- and picoplankton and FO signal (Fig. 3, see also manual gating on Fig. S2). The gating structure was kept the same for all samples analysed to consistently categorise the distinct groups. Microplankton were classified as: (1) Micro-HighFO, including some plastidic (autofluorescent) ciliates as confirmed by images and pulse-shape; (2) Micro-HighFWS, including large, chain-forming diatoms, also confirmed by images and pulse-shapes; (3) Micro-MediumFWS, consisting of diatoms (confirmed by images) with medium FWS emission (see below); and (4) Micro-LowFO, including some diatoms (as confirmed images as well) with low FO emission (see below). Similar to microplankton, nanophytoplankton were classified regarding size (4 – 20 *µ*m) and FO signal as: (5) Nano-HighFO, which consisted possibly of PE-containing cells, including cryptophytes and some dinoflagellates; (6) Nano-MediumFO; and (7) Nano-LowFO, likely non-PE containing cells. Picophytoplankton were classified as: (8) Pico-HighFO, possibly consisting of phycoerythrin (PE)-containing prokaryotes, such as *Synechococcus*-like cells; and (9) Pico-LowFO, such as non PE-containing pico-eukaryotes.

Micro-HighFO, which included plastidic ciliates, were identified based on their large size (> 20 *µ*m), presence of FR emission (see Fig. S3a), and high FO emission characteristics (Fig. 3a). The term ‘plastidic’ for ciliates in this study is for simplification since autofluorescence is possibly due to endosymbiosis of a whole photosynthetic cell (Qiu et al. 2016) or acquisition of chloroplasts containing Chl*a* and orange fluorescent PE from cryptophytes, such as observed in the ciliate *Mesodinium rubrum* (Gustafson et al. 2000) and in the marine oligotrich *Strombidium rassoulzadegani* (Schoener and McManus 2012). High FY/G signal in plastidic ciliates has also been observed in this (Fig. 1; see Fig. S3m,o) and other studies (Dubelaar et al. 2004).

Micro-HighFWS, which included diatoms chains, were classified based on high FWS signal and FO signal (Fig. 3a-c) as well as FR signal (Bonato et al. 2015; Fig. S3a). In general, FWS total and FR total correlated positively (average signal of all sites: FR total=1.15 FWS total0.91, *r2*= 0.83), meaning that the larger the particle, the stronger the FR autofluorescence (from Chl*a*) observed. High FR total signal also correlated positively with FO total signal (FR total = 27.16 FO total0.91, *r2*= 0.85), since the higher the FR signal from chloroplasts, the higher the spillover of FO signal, due to the overlapping across these two channels. Thus, to determine whether FO originated from PE or was an artifact of strong FR signal, the relationship of FO and FWS was observed, where PE-containing cells have a higher FO/FWS than non-PE containing ones (Fig. 3a-c). Diatoms were separated into different sizes ranges (FWS length) and consequently FO (Fig 3a-c) as well as FR signal ranges (Fig S3a-c): Micro-HighFWS, often found as large diatom chains (FWS length; mean (*µ*) = 145 *µ*m; standard deviation (*σ*) = 61 *µ*m) (see example in Fig. 1b), Micro-MediumFWS, diatom found as single cell or medium-sized chains (*µ* = 37 *µ*m, *σ* = 9 *µ*m) with moderate FR signals (see Fig. S3a-c)), which also reflects a moderate FO (Fig. 3a-c), possibly due to Chl*a*), or Micro-LowFO, small diatoms (also single cells or small chains (*µ* = 25 *µ*m, *σ* = 3 *µ*m) and lower Chl*a*) (Fig. 3a-c). As a photograph of some large particles (> 10 *µ*m; Fig. 1) is taken simultaneously with each pulse shape, photographs of plastidic ciliates and diatoms were used to confirm the manual gating of these assigned groupings.

Other groups, which were too small to have their identification visually confirmed from the photographs (with the exception of some dinoflagellates (see Fig. 1d)), were classified based on their optical signature. Pico-HighFO, considered in this study as *Synechococcus*-like cells, were discriminated through their small size (FWS length < 4 *µ*m) and a significant ratio of FO/FR (mostly > 1; Fig. S3r, supplementary material) due to their high PE content compared to Chl*a* (Thyssen et al. 2014, 2015). PE-containing nanophytoplankton (Nano-HighFO), which includes cryptophytes and some dinoflagellates, were classified and grouped together based on high FO signals, but distinguished from the former group (*Synechococcus*-like) by their larger cell size (FWS from 4 to 20 *µ*m) (Fig. 3). High FO in PE-containing nanophytoplankton occurs either due to presence of PE (in the case of cryptophytes) (Thoisen et al. 2017) or due to the ingestion of small photosynthetic plankton (in the case of dinoflagellates).

Another two groups consisted predominantly of non PE-containing nanophytoplankton (Nano-MediumFO and Nano-LowFO), which likely includes mixed and un-resolved nanoplankton groups such as coccolithophores, non-calcifying haptophytes, dinoflagellates and nano-sized diatoms (< 20 *µ*m). This group was discriminated based on cell size (FWS length signal from 4 to 20 *µ*m), moderate FO signal and high SWS maximum caused by the sideward light scatter of inorganic cell components, such as coccoliths, cellulose plates and opal frustules. Coccolithophores have been resolved previously using the CytoSense (e.g. Bonato et al. 2015, 2016), however, in our study we could not confidently separate coccolithophores from other small taxa due to similarity in the CytoSense signal from cells with calcium carbonate coccoliths and cellulose plates. Other non PE-containing flagellates were classified based on their cell size (Nano-LowFO, FWS length signal from 4 to 12 *µ*m and Pico-LowFO, FWS < 4 *µ*m) and low FO and FR signals (Fig. 3a; S3a-c, supplementary material). Data that did not represent plankton cells (referred to as noise) was identified observing the pulse shape feature of the particle (see Fig. S4, supplementary material). Data classified as noise was manually grouped and removed from further analyses.

The FWS total signal has been suggested as a better descriptor for particle (cells and colonies/chains) volume than SWS (Haraguchi et al. 2017), so in this study volume (Vol) is taken as FWS total. To estimate plankton group biovolume, Vol was converted to *µ*m3 following Haraguchi et al. (2017), where biovolume (*µ*m3) = 4.24.10-6 Vol1.88. Haraguchi et al. (2017) derived their algorithm from the log-log relationship between the average integrated FWS total signal of a taxon (18 species in total) and the cellular volumes (*µ*m3, 20 to 50 cells per taxon) calculated from separate microscope measurements. Phytoplankton and ciliate biovolumes were standardized among samples to demonstrate the relative biovolume at each site.

Relative biovolume of size-based functional groups (micro- (including plastidic ciliates), nano- and pico-plankton) were defined as the fractions of the sum of the following groups divided by the total:

Micro-plankton (approx. > 20 *µ*m, Mf) = Micro-HighFO + Micro-MediumFWS + Micro-LowFO + Micro-HighFWS;

Nano-plankton (approx. 4 to 20 *µ*m, Nf) = Nano-HighFO + Nano-MediumFO + Nano-LowFO;

Pico-plankton (approx. < 4 *µ*m, Pf) = Pico-HighFO + Pico-LowFO.

***Statistical analyses***

Using the CytoClus 4 software, the mean value of each parameter (see section *CytoSense descriptors and functional traits*) was calculated from the pooled plankton groups (“all data but noise”, see manual clustering in Fig. S2, supplementary material) in each sample and exported as .csv files. Each trait derived from the CytoClus 4 (calculations are explained in Table 1) were normalised among samples, to determine the stations with lowest (value = 0 %) or the highest (100 %) trait value.

Multivariate analyses were performed on the normalised trait data using PRIMER-E (version 7) software (Clarke and Warwick 2001). Relative biovolume (percentages) of each plankton group among the different stations and among the groups themselves were displayed along the contrasting hydrography using ‘Shade Plot task’ in the PRIMER-E software.

To analyse the overall variance of plankton traits from stations of distinct hydrographical regions, principal component analyses (PCA) were applied to the normalised trait values (after square-root transformation) for each sample using the PRIMER-E software. Pie charts were constructed in the PCA plots using the PRIMER-E software to examine the associations between plankton size groups (pico-, nano- and micro-plankton) and community trait parameters.

Analysis of variance (ANOVA) and post-hoc Tukey-Kramer tests were used to determine the overall significance difference (set at *p* < 0.05) of environmental factors and traits among samples from distinct oceanographic regions using Minitab Software (Version 18, Minitab, University Park, PA, USA). Pair-wise Pearson product-moment correlations and statistical significance (at *p* < 0.05) were calculated among traits themselves and between traits and environmental values using the statistical software R (Version 3.3.3, R Development Core Team, 2017) and the *corrplot* package.

**Results**

***Hydrography and nutrient distributions***

The subpolar North Atlantic was divided into three distinct zones based on hydrography (Arctic, Northwest Atlantic and Northeast Atlantic), with temperature, salinity and nutrient utilization ratios varying in upper (0-200 m) waters (Fig. 2). In general, cold (< 3°C), fresh (salinity < 34.5) and low-density waters (*σθ* < 27.5 kg m-3) were usually found on the shelves, near Canada (the Labrador Shelf) or on the southern tip of Greenland (Greenland Shelf), indicating the influence of waters originating from the Arctic outflow, herein defined as the ARC region. Waters from ARC were the most strongly stratified in this study (see also Fig. S5a, supplementary material).

The Northwest Atlantic (NWA) region included the central portion of the Labrador Basin (between Canada and Greenland) and the Irminger Basin (Fig. 2a). The upper 200 m of the NWA had the densest (27 - 28 kg m-3) water mass observed in this study and, compared to Arctic-related waters, was warmer (2° - 9 °C), more saline (34.3 - 35.2) and had features of modified Atlantic waters (Fig. 2b). Waters from the NWA were the least stratified in this study (Fig. S5a, supplementary material).

The third water mass belonged to the Northeast Atlantic (NEA) region, which comprised the waters from the Iceland Basin and Rockall Trough near Scotland (Fig. 2a). The water mass from NEA was characterised as the most saline (> 34.8) and warmest (> 8 °C), with moderate stratification and density values (27.5 - 26.5 kg m-3) compared to the other water masses (Fig. 2b; Fig. S2a, supplementary material). The temperature and salinity (T-S) properties from the upper 200 m were not only related to the spatial distribution of these stations (Fig. 2), but also to the temporal time-frame at the interval of sampling, as waters from the Labrador Shelf (near Canada) were sampled earlier in the season (mid-June) whilst samples from the Northeast Atlantic were collected in mid-July.

***Plankton distribution***

Microplankton, including the groups Micro-LowFO (consisted of small single-celled diatoms), Micro-MediumFWS (*Ephemera* spp. (see Fig. 1c) and short diatom chains), Micro-HighFWS(large diatom chains; Fig. 1b) and Micro-HighFO (plastidic ciliates, aloricate and loricate-bearing; Fig. 1a), were more abundant in terms of both concentration (counts L-1) and relative biovolume in the ARC region, being rarely observed in the NEA (Fig. 4). In contrast, Pico-LowFO (likely pico-eukaryote cells) had the highest concentration (counts L-1) and relative biovolume in Atlantic waters (NWA and NEA). Nano-MediumFO (non PE-containing nanophytoplankton), such as coccolithophores, small dinoflagellates or diatoms (< 20 *µ*m), had higher concentrations (counts L-1) and relative biovolume in ARC and NEA waters. Nano-HighFO (PE-containing nanophytoplankton), including cryptophytes and some dinoflagellates, in addition to Nano-LowFO (non PE-containing nanophytoplankton), were all found in similar concentrations (counts L-1 and relative biovolume) throughout the regions of the subarctic North Atlantic (Fig 4). Pico-HighFO (*Synechococcus*-like cells) were observed in higher concentrations in Atlantic waters, particularly in the NEA (Fig. 4).

***Traits, plankton groups and size variability***

Plankton groups from distinct hydrographical regions of the subarctic North Atlantic showed distinct functional traits, which explained 64.1 % of the compositional variability among regions on the first PCA axis (PC1) and a cumulative proportion of 97.7 % of variability across four PCA axis (Table 2, Fig. 5). Compared to the other hydrographic regions, plankton groups from Arctic waters (ARC) were larger, more asymmetric and had higher external (setae, cell processes) or internal structural complexity (vacuoles), as well being colonial (or chain) with a greater number of cells per chain (Fig. 5a). Plankton groups in the ARC also consisted of a greater proportion of micro-plankton (plastidic ciliates and diatoms) (Fig 5b).

Conversely, functional traits of plankton in Atlantic waters were significantly different from the Arctic (ARC) (one-way ANOVA, *p* < 0.05; see also Fig. S6). Traits of plankton from the Atlantic were: small cell size (most cells were < 4 *µ*m), spherical shape, solitary form, with high PE/Chl*a* and Chl*a*/Vol (Fig. 5a). Between regions of the Atlantic (NWA and NEA), most functional traits were not significantly different (one-way ANOVA, *p* < 0.05), except for shape, which was dominated by more spherical forms in the NEA compared with the NWA region (one-way ANOVA and post-hoc Tukey-Kramer tests, *p* < 0.05; see also Fig. S6d). Atlantic waters had higher contributions of pico-phytoplankton (*Synechococcus*-like and pico-eukaryotes) and lower contributions of micro-plankton (diatoms and plastidic ciliates) than Arctic waters, whereas nano-phytoplankton contribution (PE and non PE-containing nanophytoplankton) varied in Atlantic waters (Fig. 5b).

***Traits and environmental relationships***

Pairwise comparisons showed that some traits presented positive or negative correlations among themselves, or when related to environmental variables (Pearson product-moment correlations; Fig. 6). Large, colonial and asymmetric phytoplankton, with greater extracellular (setae or processes) and/or internal cellular complexity (internal plastids and vacuoles) were positively correlated with colder, fresher and strongly stratified waters (*p* < 0.05), with higher ΔSi(OH)4/ΔNO3 utilization ratios. Conversely, small, spherical, single cells with high PE/Chl*a* were positively correlated with warm and more saline waters, with higher ΔNO3/ΔPO4 utilization ratios, whereas Chl*a*/Vol correlated negatively with temperature (*p* < 0.05, Fig. 6).

**Discussion**

***Patterns of plankton community structure***

The subpolar North Atlantic presents a complex hydrographic environment, where waters of Arctic and Atlantic origin divide the region into different zones with defined biogeographical provinces (Longhurst et al. 1995; Head et al. 2003; Fragoso et al. 2016). In this study, the distinct water masses of the sub-Arctic North Atlantic showed dissimilar phytoplankton functional groups. In waters of Arctic origin (ARC), a great proportion of large (> 20 *µ*m) micro-plankton were observed, including diatoms as both single cells and chains, as well as plastidic ciliates, when compared to Atlantic waters (NWA and NEA). Conversely, Atlantic waters, particularly the Northeast Atlantic (NEA), had a higher proportion of small-sized plankton groups, such as pico-eukaryotes and *Synechococcus*-like cells. In the Northwest Atlantic (NWA), the plankton composition gradually changed between the ARC (45 ºW to 53ºW) and NEA (10 ºW to 30 ºW) (Fig. 2a and 4b).

The surface waters of the NWA, which includes the central region of the Labrador Sea and the Irminger Basin, had higher contributions of Arctic waters than the NEA (Yashayaev and Clarke 2008). This explains the gradual increase from west to east in temperature and winter (> 200 m depth) NO3 concentrations, typically attributed to Atlantic-related waters (Harrison et al. 2013) and the transitional shift in phytoplankton functional groups observed in this study. Moreover, the central deep basin of the Labrador Sea is known to possess highly dynamic hydrography from spring to summer due to the transition from un-stratified to thermally-stratified waters, which drive variability in species composition (Fragoso et al. 2016, 2017).

The taxonomical composition and relative biovolume of some plankton groups, as derived from the CytoSense analyses (recognised from the images provided), were similar to results from separate microscope (Fragoso et al. 2016, 2018), flow-cytometry (Li and Harrison 2001) and pigment-based studies (Stuart et al. 2000; Fragoso et al. 2017) in the Labrador Sea. For instance, polar or ice-related diatoms have been found to dominate shelf waters of Arctic influence, whereas Atlantic diatoms, such *Ephemera planamembranacea* (Fig. 1c), dominate the central Labrador Sea (Fragoso et al. 2016). The high relative biovolume of diatoms in Arctic waters, decreasing eastwards in Atlantic waters, explains the strong drawdown of Si(OH)4 observed (see Fig. S5b, supplementary material). The higher concentration of Si(OH)4 relative to NO3 found in Arctic waters compared to Atlantic waters (Harrison et al. 2013) has also been suggested to shape diatom species composition and the degree of cell wall silicification, with Arctic species having more heavily silicified cells than Atlantic species (Fragoso et al. 2018).

Pulse-shape recording flow cytometry, when compared to other methods, shows good biogeographical agreement for large-sized plankton, such as diatoms (Fragoso et al. 2016, 2017). As expected, taxonomical resolution is limited in terms of nanoflagellates as observed in other studies (Haraguchi et al. 2017). For example, pigment-based community analysis is able to identify chlorophytes as co-dominating Arctic waters using chlorophyll *b* as a biomarker for this group (Fragoso et al. 2017), whereas this group is not distinguishable using the CytoSense parameters. The colonial prymnesiophyte *Phaeocytis pouchetii*, which constitutes an important component of phytoplankton spring blooms in the West Greenland Current (eastern Labrador Sea) (Frajka-Williams and Rhines 2010; Fragoso et al. 2016, 2017) has been previously detected using the CytoSense (Bonato et al. 2015, 2016). In this study, however, *Phaeocystis* colonies were not observed, potentially due to their low abundance in the Labrador Sea during the summer of June 2014 (Fragoso et al. 2016, 2017).

Plastidic aloricate and loricate-bearing (tintinnids) ciliates are abundant in Arctic waters (Onda et al. 2017; Kauko et al. 2018; this study) and act as early grazers of the spring plankton community, regulating the size structure by ingesting small flagellates (Pomati et al. 2013) in highly stratified waters (McManus and Fuhrman 1986). In this study, plastidic ciliates graze on small plankton, which could favour the growth of large diatoms and explain the high percentage of micro-plankton in Arctic compared to Atlantic waters. Moreover, the plastidic ciliates detected in this study presented internal orange and yellow/green fluorescence, which may relate to their grazing on cryptophytes and/or cyanobacteria (Schoener and McManus 2012), although internal red fluorescence was also observed. The ciliate *Mesodinium rubrum* is known to preferentially prey on cryptophytes, ingesting and retaining their chloroplasts for their own use (Gustafson et al. 2000; Myung et al. 2013). Although the plastidic ciliates observed in this study were not *M. rubrum* but loricate-bearing tintinnids (Fig. 1a) and other aloricate ciliates (e.g. *Strombidium* spp.), other studies have suggested that ingestion and retention of cryptophyte chloroplasts is a common feature in many ciliates and certain dinoflagellate taxa (Sjoqvist and Lindholm 2011; Schoener and McManus 2012; Pereira et al. 2017).

In this study, pico-eukaryotes were the most abundant plankton group found in Atlantic waters, although they were also present in the other water masses. This group, which includes the prasinophyte *Micromonas pusilla*, has been shown to numerically dominate the entire sub-Arctic North Atlantic (Li et al. 2009; Pettersen et al. 2011; Harrison et al. 2013) and has been assumed as a baseline component that persists throughout the seasons in subpolar waters (Not et al. 2005; Lovejoy et al. 2007). Prokaryotic picoplankton includes *Synechococcus*-like cells, being predominantly observed in the NEA. *Synechococcus* abundance has been shown to have a strong link with temperature, having an optimum temperature of 10°C and being rarely detected in sub-zero waters (Flombaum et al. 2013). Low abundance of cyanobacteria, presumably *Synechococcus*, observed through pigment-based approach has also been observed in cold (< 0°C) Arctic waters of the Labrador Shelf in previous studies in the Labrador Sea (Fragoso et al. 2017). A relationship between *Synechococcus* and warm, saline and NO3-rich waters has already been noted in the subpolar Northwest Atlantic, indicating its strong affinity to waters of Atlantic origin (Harrison et al. 2013).

***Trait analyses derived from CytoSense***

Trait-based approaches have been considered a successful method in ecology because they offer a “common currency” that numerically compares taxa based on their ecological significance (Pomati et al. 2013). To date, the influence of multiple traits in a plankton community has been investigated either using mean community weights from continuous and/or categorical traits (e.g. fuzzy code) (Rosati et al. 2017; Klais et al. 2017; Fragoso et al. 2018) or modelling approaches (Edwards et al. 2013, 2016; Breton et al. 2017). The use of optically-derived descriptors adds to the state-of-the-art in trait-based analyses as multiple traits are quantified based on direct observations at the individual cell level, which accounts for variability within plankton groups (Fontana et al. 2016). Trait plasticity within species or taxa are often neglected in trait-based studies, although it has been established as strongly influencing community structure and ecosystem function as much as interspecific variability (Albert et al. 2010; Fontana et al. 2016; Des Roches et al. 2018).

The application of bio-optical descriptors derived from pulse-shape recording automated flow cytometers, including the CytoSense and CytoBuoy, as a way of quantifying plankton traits has been previously applied in natural freshwater communities (Pomati et al. 2013; Fontana et al. 2014, 2016), mesocosms (Pomati and Nizzetto 2013) and laboratory experiments (Takabayashi et al. 2006). These previous studies used the trait descriptors as is, whereas, in this study we used the combination of these descriptors to translate into functional traits typically observed in ecological studies (Table 1). Similar to studies in freshwater systems, functional traits were intrinsically related to their taxonomic grouping in our study (e.g. large diatoms are either large single cells and/or cells living as chains, whereas *Synechoccocus* are small and have high ratios of PE/Chl*a*), where cell size is the master trait (Pomati et al. 2013; Fontana et al. 2014, 2016). The positive correlation of cell size with other morphological characteristics in this study highlights that a number of these traits are evolutionarily interrelated (Finkel et al. 2009a; Litchman et al. 2010). Functional redundancy of cell size in relation to other morphological traits (asymmetry, coloniality, structural complexity) can provide further ecological information on a biological community, and may influence ecosystem stability (Peterson et al. 1998) and biodiversity (Nock et al. 2016).

***Trait patterns along environmental gradients***

While plankton community structure differed between hydrographic regions (ARC, NWA, NEA), traits defined in this study were only statistically different between Arctic and Atlantic waters and not between sub-regions of the Atlantic (NEA, NWA). A major factor determining these regional difference is due to the traits between water masses being cell size-related, where large and more complex plankton in terms of morphological structure (e.g. diatoms) occur in Arctic waters whilst small picoplankton dominate in Atlantic waters.

In this study, asymmetry, coloniality (e.g. cells per chain), structural complexity and cell size are common traits found in species assemblages (i.e. diatoms) which correlated negatively with salinity and temperature, meaning that these traits were found in cold, fresh and more strongly stratified Arctic waters (Fig. 6). Compared to other plankton groups, diatoms are recognised for being large (Le Quéré et al. 2005), often colonial, and exhibiting high structural complexity (i.e. possessing cellular processes, setae and internal vacuoles) (Tréguer et al. 2018; Fragoso et al. 2018). Diatoms are also known for their high package effect or potential ‘self-shading’ of chloroplasts within the cell (Agustí 1991; Stuart et al. 2000) compared to other (smaller) plankton groups. Strongly illuminated, stratified, ice-melt influenced Arctic waters may select for large cell-sized and colonial diatoms, given that their low optical absorption cross-section protects them from light (including ultraviolet) damage at high intensities (Key et al. 2010).

Highly stratified ice-melt waters, however, may be an unfavourable environment for large diatoms chains and colonies, which can sink more readily assuming that they are dense (Margalef 1978). The trade-off for this group may be their highly non-spherical shape (large asymmetry, particularly chains, in addition to their external cellular processes), as observed in this study, which increases drag in the water column, slowing their sinking and maintaining their positive buoyancy (Smetacek 1985; Nguyen et al. 2011). Large diatom cells also possess internal cell vacuoles, often filled with low density ions (lower than the surrounding seawater), which also allows the cells to maintain a positive or neutral buoyancy (Moore and Villareal 1996; Woods and Villareal 2008; Miklasz and Denny 2010). Large diatom cell-size and colony formation are common features of phytoplankton from ice-related waters (Arrigo et al. 2010, 2014; Johnsen et al. 2018; Fragoso et al. 2018).

In Atlantic waters (NEA), traits including spherical shape, high PE/Chl*a* ratios and high intracellular Chl*a*/Vol ratios may all be attributed to dominance by small phytoplankton, such as pico-eukaryotes and *Synechococcus*-like cells. Likewise, the drawdown of NO3 relative to PO4 was higher in Atlantic compared to Arctic waters. The sub-Arctic North Atlantic presents a gradual increase in east-west winter NO3 (> 200 m) (Harrison et al. 2013), suggesting that phytoplankton in surface waters of the NEA were exposed to low NO3 availability at the time of the study. Small cell size, in addition to high spherical cell shape, is a favourable trait when it comes to low nutrient concentrations because of their inherent high surface area to volume ratio, which promotes rapid nutrient assimilation compared to large cells (Li 2002; Finkel et al. 2009; Marañón 2014). Small cells also have less of a pigment packaging effect than larger cells (i.e. less intracellular shading of the chloroplasts and higher chlorophyll *a* specific absorption; Stuart et al. 2000; Fujiki and Satotu 2002; Johnsen and Sakshaug 2007) so that they are better able to thrive under low light intensities. In this and previous studies (Frajka-Williams et al. 2009; Frajka-Williams and Rhines 2010; Lacour et al. 2015), Atlantic waters were in general less stratified and potentially less well illuminated than Arctic waters, which favours phytoplankton groups that have higher internal concentrations of Chl*a* and accessory pigments per cell (Fragoso et al. 2017).

Photo-physiological traits, such as high PE to Chl*a* ratios observed in *Synechoccocus* in Atlantic waters, can also be an advantageous trait given that this accessory pigment provides a chromatic adaptation to clear, open and highly dynamic (variable in mixing conditions) waters of the Atlantic Ocean. The ubiquitous distribution of *Synechococcus* partly relates to the pigment diversity of its light harvesting antennae and phycobiliproteins (Shukla et al. 2012). Compared to Chl*a*, which absorbs in a narrow band of blue and red light, PE absorbs in a wide part of the spectrum, from blue (460 nm) to yellow (580 nm) (Johnsen and Sakshaug 2007). Thus, the presence of PE in *Synechococcus* is a useful trait as it allows them to adjust for changes in the ambient light colour and ultimately maximize photon capture for photosynthesis (Shukla et al. 2012), given that Atlantic waters, in this study, were less stratified (and possibly more dynamic) than Arctic waters.

**Conclusions**

Here we have shown that the CytoSense flow cytometer has the capacity to quantify morphological and pigment functional traits based on the optical fingerprints associated with light scattering (forward and sideward) and fluorescence (red, yellow and orange for Chl*a*, degraded pigments and phycobiliproteins, respectively) of plankton cells. We used simple output descriptors and translated them into functional traits to demonstrate their variability along an environmental gradient. Functional traits derived from the CytoSense are demonstrated to be a good proxy to explain the segregation of plankton communities, including size spectrum, in contrasting water masses of distinct origin (Arctic versus Atlantic) in the subarctic North Atlantic Ocean.

Functional traits have previously been used as a common currency to explain the success of certain species in biological communities. This field promises to simplify our interpretation of functionality in biological communities and reduce the information complexity of ecological roles, processes and interactions, which are fundamental in modelling approaches (Merico et al. 2009). The use of the CytoSense data as a way to quantify traits holds further promise when dealing with plasticity, variability and dynamics within plankton groups, such as quantifying variability in cell or colony size, which are difficult to quantify via conventional microscopic approaches. Current advances in automated platforms that record in vivo continuous optical measurements, such as the FlowCytoBot (Lambert et al. 2017), CytoSub (Thyssen et al. 2008) and CytoBuoy (Pomati et al. 2011), combined with the trait-based approach used in this study can offer an unique opportunity to study phytoplankton functional trait dynamics at high spatial and temporal scales. Moreover, the use of artificial fluorescence probes to label plankton cellular ultrastructure (e.g. diatom silicification; Leblanc and Hutchins 2005; McNair et al. 2015), as well as gene expression (e.g. fluorescence in situ hybridization (FISH); Colin et al. 2017) could be quantified using automated flow cytometers, such as the CytoSense. Such unique approaches can bring forward new opportunities that unravel the linkages between cell biology, evolution, ecosystem structure and the biogeochemical function of plankton communities.

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**Fig. 1.** Example showing the pulse shape and photographic image of: (a) plastidic loricate-bearing ciliate (tintinnid), (b) *Thalassiosira* sp. chain (diatom), (c) *Ephemera* sp. (diatom), and (d) thecate dinoflagellate. Colors in the pulse shape refer to the following signals: Forward (FWS, black) and sideward light scatter (SWS, blue), yellow/green (FY/G, green), orange (FO, orange) and red (FR, red) fluorescence. Scale bars are 80 *µ*m in a-c and 60 *µ*m in d.

**Fig. 2.** Biogeographical zones of the North Atlantic showing (a) spatial distribution of stations and (b) their respective potential temperature and salinity vertical profiles (upper 200 m) with isopycnal contours. Colours are categorised as belonging to the following regions: Arctic (ARC, blue), Northwest Atlantic (NWA, red) and Northeast Atlantic (NEA, green).

**Fig. 3.** Cytograms for samples from stations represented in Figure 2 that belongs to the distinct hydrographical regions of the subpolar North Atlantic: Arctic (ARC, station 5, left), Northwest Atlantic (NWA, station 22, middle) and Northeast Atlantic (NEA, station 148, right) based on: (a-c) orange fluorescence (FO) total and forward scatter (FWS) length and (d-f) FO total and sideward scatter (SWS) maximum. Plankton groups are represented in different colours: Pico-LowFO (likely pico-eukaryote, light green), phycoerythrin (PE)-containing nanophytoplankton (defined as Nano-HighFO, pink) and non-PE containing nanophytoplankton (defined as Nano-MediumFO, yellow, and Nano-LowFO, cyan), Pico-HighFO (*Synechococcus*-like cells, red), Micro-HighFO (identified as plastidic ciliates in some images, dark green), Micro-LowFO (which includes small diatoms, brown), Micro-MediumFWS (including medium diatoms, grey) and Micro-HighFWS (mostly large, chain-forming diatoms, dark blue) and noise (black). Additional cytograms are shown in Fig. S3, supplementary material).

**Fig. 4.** Shade plot showing the a) relative biomass (percentage) and b) concentration (counts.L-1) of plankton groups at each site belonging to the distinct hydrographical regions of the subpolar North Atlantic: Arctic (ARC, blue), Northwest Atlantic (NWA, red) and Northeast Atlantic (NEA, green). Arrows refer to station name, where cytograms were plotted as in Fig. 3.

**Fig. 5.** Principal components (PC) analysis of trait variables as a function of (a) stations from different regions (Arctic: ARC, blue; Northwest: NWA, red; Northeast Atlantic: NEA, green) and (b) relative biovolume of size classes: pico- (Pf, white), nano- (Nf, grey), and micro-plankton and plastidic ciliates (Mf, black). Abbreviations refer to phycoerythrin (PE) to chlorophyll *a* (PE/Chl*a*) and Chl*a* to volume (Chl*a*/Vol) ratios.

**Fig. 6.** Correlation plot of environmental factors and the different traits showing statistically significant (*p* < 0.05) positive (red) and negative (blue) correlations. White squares refer to non-statistically significant relationships. Environmental abbreviations refer to the NO3 to PO4 (ΔNO3/ΔPO4) and Si(OH)4 to NO3 utilization ratios (ΔSi(OH)4/ΔNO3), stratification index (SI) and phycoerythrin (PE) to chlorophyll *a* (PE/Chl*a*) and Chl*a* to volume (Chl*a*/Vol) ratios).