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Interoperable vocabulary for marine microbial flow cytometry

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The recent development of biological sensors has extended marine plankton studies from conducting laboratory bench work to *in vivo* and real-time observations. Flow cytometry (FCM) has shed new light on marine microorganisms since the 1980s through its single-cell approach and robust detection of the smallest cells. FCM records valuable optical properties of light

scattering and fluorescence from cells passing in a single file in front of a narrow-collimated light source, recording tens of thousands of cells within a few minutes. Depending on the instrument settings, the sampling strategy, and the automation level, it resolves the spatial and temporal distribution of microbial marine prokaryotes and eukaryotes. Cells are usually classified and grouped on cytograms by experts and are still lacking standards, reducing data sharing capacities. Therefore, the need to make FCM data sets FAIR (Findability, Accessibility, Interoperability, and Reusability of digital assets) is becoming critical. In this paper, we present a consensus vocabulary for the 13 most common marine microbial groups observed with FCM using blue and red-light excitation. The authors designed a common layout on two-dimensional log-transformed cytograms reinforced by a decision tree that facilitates the characterization of groups. The proposed vocabulary aims at standardising data analysis and definitions, to promote harmonisation and comparison of data between users and instruments. This represents a much-needed step towards FAIRification of flow cytometric data collected in various marine environments.

KEYWORDS

flow cytometry, marine microorganisms, standardization, vocabulary, FAIR principle interoperable vocabulary for marine flow cytometry

Introduction

The biological understanding of the marine environment starts with the unicellular world of microorganisms. Unicellular marine microorganisms, including autotrophic, heterotrophic, and mixotrophic prokaryotes and eukaryotes, as well as viruses, are the principal biogeochemical actors of the oceans (Pomeroy, 1974; Worden et al., 2012). They play a crucial role in the marine environment, and by extension, the Earth climate, due to their physiological capacity to oxidize and reduce elements (essentially carbon) through photosynthesis, respiration and exudation, setting the ecological and trophic status of marine ecosystems. Unicellular autotrophic prokaryotes and eukaryotes, hereafter called phytoplankton, produce up to half of the oxygen and organic matter on Earth (Field et al., 1998), and form the base of the marine food webs. Unicellular heterotrophic prokaryotes are ubiquitous and play a key role in biogeochemical cycles (Fenchel et al., 2012) including the recycling of organic matter and nutrient regeneration. Unicellular heterotrophic and mixotrophic eukaryotes consume phytoplankton and heterotrophic prokaryotes and, depending on their cell size, facilitate the transfer of the produced organic matter to higher trophic levels (Azam et al., 1983) or its recycling by marine heterotrophic prokaryotes (Ducklow et al., 1986). Marine viruses as well are indirectly responsible for facilitating nitrogen cycling and therefore phytoplankton growth and for reducing carbon dioxide release

into the atmosphere. They are also a major driver of evolution by increasing genetic diversity (Suttle, 2005). In addition to this high biological and functional diversity, their dynamics and distribution are characterised by high spatio-temporal heterogeneity, making studies of their activity challenging.

Observations and modelling of plankton dynamics and functionality traits (Dubelaar et al., 1999; Quéré et al., 2005; Litchman et al., 2007; Fontana et al., 2018; Lévy et al., 2018) highlight the importance of fine spatio-temporal and functional resolutions when assessing the role of microorganisms in marine ecosystems. The most accurate way to study the distribution, composition, dynamics, and functions of microorganisms is to resolve them at their unit scale, i.e., the single cell. This is possible with FCM, widely used in oceanography since the 1980s (Yentsch & Yentsch, 1979; Trask et al., 1982; Yentsch et al., 1983; Olson et al., 1985). FCM has revolutionised marine microbiology by accessing the abundance distribution of plankton size classes (PSCs) and several existing phytoplankton functional types (PFTs) or functional groups (PFGs) (Salmaso et al., 2015). FCM also helped in the discovery of unexpectedly small organisms (< 1 µm) playing an important role in the trophic web and the marine carbon cycle such as *Prochlorococcus* (Chisholm et al., 1988) and *Ostreococcus* (Courties et al., 1994). The power of conventional FCM relies on the hydrodynamic focusing of a laminar flow where particles are lined up and separated. Their intersection with a laser beam generates light scattering which can be used as

a proxy of cell diameter or cell structure, and fluorescence from pigments or user-applied dyes. The identification of photosynthetic microorganisms does not require any staining due to the auto-fluorescence of chlorophylls and accessory pigments. Those auto-fluorescence properties, combined with size and structure, make it possible to separate photosynthetic cell populations from other marine particles. Heterotrophic microorganisms and viruses require specific staining (usually of Nucleic Acids) to distinguish them from the detrital organic matter pool (Grégori et al., 2001; Zubkov et al., 2001; Christaki et al., 2011). Automated staining systems dedicated to heterotrophic micro-organisms high-frequency observation were set up and deployed, either for drinking waters (Hammes et al., 2012), or marine environments (Brownlee et al., 2016; Silovic et al., 2017) monitoring. Flow cytometers can be equipped with lasers emitting light at different wavelengths to measure signals from various chromophores.

Over the last 20 years, automated systems (i.e. systems that can be operated autonomously for months or years and remotely controlled) such as CytoSense/Sub/Buoy (Dubelaar et al., 1999), FlowCytobot and Imaging FlowCytobot (Olson et al., 2003; Olson & Sosik, 2007), SeaFlow (Swalwell et al., 2011), have produced highly resolved spatial and temporal datasets of numerous phytoplankton groups, including relatively large and rare groups thanks to increasing sample volume analysed. These instruments can be deployed *in situ* and underwater (Sosik et al., 2003; Thyssen et al., 2008b; Peacock et al., 2014; Hunter-Cevera et al., 2016), on a ship (Li et al., 1995; Thyssen et al., 2009; Bonato et al., 2015; Thyssen et al., 2015; Marrec et al., 2018; Aardema et al., 2019; Ribalet et al., 2019; Fowler et al., 2020; Louchart et al., 2020) or a buoy (Thyssen et al., 2014). Imaging in flow systems can be combined with flow cytometry acquisition, affording additional taxonomic identification for microplankton size range. The investigation of machine learning approaches for generating automatic recognition of microorganisms at individual or cluster levels in marine samples has been explored (Malkasian et al., 2011; Ribalet et al., 2011; Hyrkas et al., 2016; Olusoji et al., 2021; Fuchs et al., 2022), and will benefit from medical field research for additional computational processes and data analysis workflow (Aghaeepour et al., 2013), promising to ensure near real-time data availability.

The worldwide increase in marine FCM datasets provides evidence of the technique's utility and reliability in marine research. Indeed, FCM datasets are now widely accepted as a core parameter and a common reference in oceanographic cruises (Manti et al., 2012). Unfortunately, the datasets collected are, in most cases, maintained in local data stores and are not routinely set up for open access through international data portals, despite the need for sharing and disseminating valuable information on ocean plankton functional-group distribution. Consequently, scientists are facing the difficult challenge of handling a large amount of data which needs to be processed rapidly and harmonised

before being stored in databases to be accessible to scientific and environmental management communities. Best practices in data management (Boss et al., 2018) have received relatively little attention despite the need for a data flow framework to follow the Findability, Accessibility, Interoperability, and Reusability (FAIR) principles (Wilkinson et al., 2016). In 2016, the SeaDataCloud project (H2020 EU, ID: 730960) supported a first initiative to harmonise FCM datasets *via* an interoperable vocabulary for the most common phytoplankton and heterotrophic prokaryotic groups. This first step was a valuable attempt to gather input from a large and international panel of FCM experts, but needed maturation as the names of groups first suggested as standards referred to some vague taxonomic descriptions. In addition, the definitions of those groups lacked precision. This paper stems from this common initiative, aiming to promote accessibility to the marine FCM datasets, proposing a consensus vocabulary and its definition for microbial groups discriminated by FCM in marine environments. The virus and microzooplankton groups were excluded from this exercise as they are not yet routinely analysed by these instruments.

Standardized vocabulary and definition

Authors of this manuscript provide a commonly agreed standardized vocabulary with definitions of marine microbial groups observed by FCM, resulting from the consultation among FCM users and experts (co-authors of this manuscript) and a literature review spanning the period 1983-2020 (Annex 1 in Supplementary material). Datasets in which FCM groups are identified by the experts are highly informative and are compatible with a consensus nomenclature, unlike raw data which are saved in various binary formats (e.g. *cyz* for the CytoSense, *adc* for the Imaging FlowCytobot, *evt* for the SeaFlow, *fcs* for more conventional flow cytometers). The interoperable vocabulary needs to apply to a variety of groups present in many marine environments while being specific enough to reveal the functional diversity observed with any flow cytometer.

From this consultation, a total of 13 groups are identified and defined in the look-up Table 1. The last column presents some examples collected in the literature and extracted from Annex 1, revealing the diversity of names given for a specific group. In the literature, they are either named using the known and referenced, or supposed, taxonomic nomenclature, such as bacteria, *Synechococcus*, *Prochlorococcus*, prochlorophytes, cryptophytes, *Crocospaera*, coccolithophorids, or by using a classification based on their size-class or cellular structure, such as picophytoplankton, picoeukaryotes, picocyanobacteria, ultraphytoplankton, nanophytoplankton, nanoeukaryotes, microphytoplankton, among others. Several identified groups share the same descriptions as well as similar optical properties or position on a cytogram, yet are

TABLE 1 Look-up table between terms identifying the main FCM groups that appear in the literature (extracted from Annex 1), and the common nomenclature findable in the <https://vocab.nerc.ac.uk/collection/F02/current/>.

Identifier	Common vocabulary (Alternative label)	Short description (Preferred label)	Example of terms that appear in the literature (Annex 1 extract)
SDN:F02::F0200002	RedPicoProk	Red only fluorescing prokaryote picophytoplankton	Prochlorophytes, Prochlorococcus, very small red fluorescing bodies, PRO, Prochl
SDN:F02::F0200013	RedRedPico	Red and Red only fluorescing prokaryote picophytoplankton, rich in the pigment phycocyanin (PC)	PC-rich <i>Synechococcus</i> , PC-CYAN, PC SYN
SDN:F02::F0200003	OraPicoProk	Orange fluorescing prokaryote picophytoplankton, rich in the pigment phycoerythrin (PE)	Syn, <i>Synechococcus</i> , cyanobacteria, PE-type <i>Synechococcus</i> , <i>Crocospaera</i> , PE-CYAN
SDN:F02::F0200004	RedPico	Red only fluorescing picophytoplankton	Picoeuk, redpico, Peuk, picophytoplankton, PicoEuk1
SDN:F02::F0200005	RedNano	Red only fluorescing nanophytoplankton	Nanoeuk, rednano, Neuk, nanophytoplankton
SDN:F02::F0200014	OraPico	Orange and red fluorescing picophytoplankton, rich in the pigment phycoerythrin (PE)	Pico cryptophytes
SDN:F02::F0200006	OraNano	Orange and red fluorescing nanophytoplankton, rich in the pigment phycoerythrin (PE)	<i>Cryptomonas</i> , Cryptophytes, HighOrgnano, Nanocyanobacteria, CRYPTO
SDN:F02::F0200015	RedRedNano	Red and red only fluorescing nanophytoplankton, rich in the pigment phycocyanin (PC)	Nanocyanobacteria
SDN:F02::F0200007	HsNano	Red fluorescing nanophytoplankton with relatively high sideward light scattering properties	Coccolithophorids, HighSWSnano, COCCO
SDN:F02::F0200008	RedMicro	Red only fluorescing microphytoplankton	Microphytoplankton
SDN:F02::F0200016	OraMicro	Orange fluorescing microphytoplankton, rich in the pigment phycoerythrin (PE)	Microphytoplankton
SDN:F02::F0200010	HetHNA	Heterotrophic prokaryotes with relatively high nucleic acid	HNA
SDN:F02::F0200011	HetLNA	Heterotrophic prokaryotes with relatively low nucleic acid	LNA

Short description is identified as the preferred label and common vocabulary as the alternative label. Descriptions of the groups are detailed in the text.

assigned a different nomenclature (photosynthetic picoeukaryotes or PPE, picoEuk, picophytoplankton) or can be spread into sub-groups (like picocyanobacteria and picophytoplankton, that both include *Synechococcus* and *Prochlorococcus*). Additionally, some groups were identified by sorting (*Ostreococcus* sp., Courties et al., 1994; *Richiella* sp., Zhao et al., 2012) or image acquisition (*Phaeocystis* sp., Rutten et al., 2005) as their light scattering and fluorescence signatures are not specific enough to be resolved at a taxonomical level from cytograms only. As said before, these groups are named on a basis of post-treatment procedures by expert's, and should appear in any interoperable database in line with a consensual and interoperable vocabulary. As a best practice recommendation, any manuscript dealing with marine FCM results should use the standardised common vocabulary or include a correspondence table that lists the groups or subgroups identified by a flow cytometry user with the standards, as in Table 1, and referenced to their sources (Uniform Resource Identifier (URI)). The URIs (e.g. <http://vocab.nerc.ac.uk/collection/F02/current/F0200002/>) are the only reliable, machine-readable and persistent identifiers of the concepts defined in the F02 vocabulary.

The proposed interoperable vocabulary combines all the existing FCM groups found in the literature so far into a unique framework defined by arbitrary optical parameters and size classes. Defining the FCM groups relies on their position on cytograms using relative proxies. Indeed, optical signatures of the recorded particles are modulated by the instrument settings, the instrument

types, as well as the environmental conditions affecting cell physiology. As an example, daily cell cycle and depth affect cell size and fluorescence signals, by increasing cellular biovolume before division (Jacquet et al., 1998; Sosik et al., 2003; Thyssen et al., 2008b; Ribalet et al., 2015), by adaptation to light availability with depth (Vaulot et al., 1995; Partensky et al., 1999), or by variable incident irradiance favouring non-photochemical quenching (Brunet et al., 2008). These effects might impact the group position within cytograms over time. This is observed when sampling at short time intervals (e.g. hourly) to cover diel cycles, or when sampling in the water column, or in different ecosystems. Furthermore, the inference of cell diameter from light scattering measurements is challenging due to the difficulties in estimating the refractive index of micro-organisms. For example, the standard pico-nano size cut-off, originally defined using 2 µm filters, is not accurately estimated using calibrating standards (e.g. Polystyrene (PS) beads, cultures of known sizes) light scattering, and may separate otherwise well-defined pico-size clusters whose size fluctuate around 2 µm due to their cell cycle (Jacquet et al., 2001) or specific composition. This issue is a source of ambiguity and may lead to large uncertainties in the enumeration of both pico- and nano planktonic groups, and their size (Green et al., 2003; Duforêt-Gaurier et al., 2015; Ribalet et al., 2019), or even their carbon quota, using known conversion factors (Buitenhuis et al., 2012). In our document, we have used the forward scatter of 3 µm silica beads to separate pico from nano groups (Vaulot et al., 2008).

We do not provide a standard vocabulary for the optical parameters collected by flow cytometry, such as fluorescence or scatter, as these values are arbitrary. The relative units and the different configurations and settings of the instruments make the recorded optical values per cell not comparable. The conversion into quantitative values such as the conversion between FSC and SSC and size, or the conversion of the FLR into chlorophyll a concentration requires some comparison with *in situ* collected data from conventional methods (Haraguchi et al., 2017) or additional sorting (Bock et al., 2022).

Definition section = detailed description of the groups

Red only fluorescing prokaryotic picophytoplankton: RedPicoProk

The RedPicoProk are commonly identified as the unicellular photosynthetic cyanobacteria *Prochlorococcus* spp., sized on average 0.6 μm (Partensky et al., 1999). Due to their auto-fluorescence, staining is not required to detect them by FCM. When excited by a blue laser, their forward scatter (FSC or FWS) and red auto-fluorescence (FLR) emitted by chlorophylls (divinyl chlorophyll *a* for the prochlorophytes) allow their discrimination from the background noise. These are the smallest known photosynthetic marine microorganisms (Chisholm et al., 1988) and their detection requires sensitive photodetectors or high-power lasers (optimally at 445 nm wavelength). Due to their strong photo-acclimation properties, their red autofluorescence is at times too dim to be detected by FCM, especially on the surface in the summer, hence some cells may be lost in the background noise depending on the instrument's sensitivity. At depth, RedPicoProk often shows a bimodal distribution of the FLR, due to the co-occurrence of two different ecotypes with different photo physiological properties (Campbell and Vault, 1993; van den Engh et al., 2017).

In samples incubated with a DNA-staining fluorescent dye and excited with a blue laser to enumerate heterotrophic prokaryote analyses, *Prochlorococcus* group(s) can sometimes be distinguished using sideward scattering (SSC or SWS) vs FLR or FLR vs green fluorescence emitted by DNA-staining fluorescent dye.

Orange fluorescing prokaryote picophytoplankton, rich in the pigment phycoerythrin: OraPicoProk

The OraPicoProk, usually identified as *Synechococcus* spp. cells are unicellular photosynthetic cyanobacteria with forward scatter (FSC) and sideward scatter (SSC) signatures larger than

those of most marine heterotrophic bacteria and of RedPicoProk. Depending on the instrument settings, their optical signatures can span nearly two decades of signal sensitivity range (Vault et al., 1995). Staining is not required to distinguish them by FCM due to the presence of photosynthetic pigments. When excited by a blue laser, the OraPicoProk cluster displays higher FSC and FLR signatures than RedPicoProk at any given depth. They contain phycoerythrin, and have a distinct FLO signature, which can be excited by either 488 or 561 nm wavelength lasers. Depending on the optical band pass filters for fluorescence detection, the OraPicoProk cluster is well resolved in red vs green (FLR/FLG) and/or in FLR/FLO cytograms.

Red and Red only fluorescing prokaryote picophytoplankton, rich in the pigment phycocyanin: RedRedPico

The RedRedPico is a group mainly identified as the phycocyanin (PC)-containing picocyanobacteria (i.e. *Synechococcus* or *Crocospaera*). This group can only be resolved using a red laser (615-640 nm). These PC-rich species are expected to be more abundant in turbid estuaries, as PC is optimized for orange-red light, whereas phycoerythrin (PE) pigments present in some other *Synechococcus* are optimally tuned to the blue-green light present in clear waters (Stomp et al., 2007). These RedRedPico overlap with RedPicoProk or RedPico on a FLO vs FLR cytogram when excited by a blue laser (Liu et al., 2014).

Red-only fluorescing picophytoplankton: RedPico

The RedPico is a polyphyletic group. Based on the literature, this group is defined by chlorophyll-containing cells with a diameter <3 μm (Buitenhuis et al., 2012 and references therein) with higher scatter than RedPicoProk and can include RedRedPico when only a blue laser is used. Staining is not required to distinguish them by FCM due to the presence of chlorophyll. RedPico exhibit a well-defined FCM signature when excited with a blue laser, with scatter signals (FSC or SSC) and FLR signals larger than those of RedPicoProk, but smaller than those of the RedNano (below). The FSC signal of either 2 or 3 μm fluorescent beads is widely used as an optical standard to localize this group, even though polystyrene beads are not a reliable indicator of cell size due to their refractive index higher than the one of cells. When possible, size-fractionated by sequential gravity filtration or cultures should be used to define this group (Green et al., 2003; Mojica et al., 2016; Ribalet et al., 2019).

Red only fluorescing nanophytoplankton: RedNano

The RedNano group is also a polyphyletic group, including phytoplankton cells with a size between approximately 3 and 20 μm . They auto-fluoresce red when excited by a blue laser due to the presence of chlorophyll. They differ from RedPico due to their higher FLR, SSC, and FSC. RedNano cells are distinguished from PE-containing cells as they lack orange fluorescence. This group may include the RedRedNano if the instrument used does not have a red laser to separate those phycocyanin-containing cells.

Orange and red fluorescing picophytoplankton, rich in the pigment phycoerythrin: OraPico

The OraPico group consists of $\sim 3 \mu\text{m}$ auto-fluorescing prokaryotes or eukaryotes that contain the pigment PE and fluorescing in the orange and in the red. When excited by a blue laser, their SSC and FSC signatures are larger than those of OraPicoProk but with a FLR signature similar to the RedPico. This group includes cyanobacteria such as *Crocospaera* spp. (Wilson et al., 2017) and cryptophytes.

Orange and red fluorescing nanophytoplankton, rich in the pigment phycoerythrin: OraNano

The OraNano group belongs to the size class of nanoplankton (3–20 μm). They are auto-fluorescing in the FLR due to the presence of chlorophyll. They contain PE and may also contain PC that can be excited by a red laser beam (615–640 nm). When excited by a blue laser, they exhibit FSC signatures similar to the RedNano but with a high FLO, often observed above the OraPico group in FLO vs FLR cytograms. If the OraNano cells contain both PE and PC and are excited by a blue laser beam they will emit a higher FLR signal compared to RedNano (due to the energy transfer to FLR, (Bazzani et al., 1992)). This group includes larger cyanobacteria (e.g. large-size *Crocospaera*, *Richelia*), red algae and cryptophytes.

Red and red only fluorescing nanophytoplankton, rich in the pigment phycocyanin: RedRedNano

The RedRedNano group belongs to the nanoplankton size class (3–20 μm) and its cells emit red light when excited by a red laser (615–640 nm), due to PC (Read et al., 2014), but no or little in the orange when excited by a blue laser, due to lack of PE. Their FSC and FLR signatures are similar to RedNano. They

include cryptophytes and some cyanobacteria, especially when they form small colonies (Wiśniewska et al., 2021).

Red fluorescing nanophytoplankton with relatively high sideward light scattering properties: HsNano

The HsNano group consists of photosynthetic, mostly eukaryotic cells, that have a high SSC signal when excited by a blue laser, compared to the RedNano and OraNano groups, but similar FSC and FLR signatures. These cells also display a high depolarization ratio (parallel and vertical polarized forward light scatter). This optical property is very useful to identify coccolithophores that are covered with coccoliths made of CaCO_3 (Green et al., 1996; Von Dassow et al., 2012), as well as some autotrophs and armoured dinoflagellates.

Red only fluorescing microphytoplankton: RedMicro

The RedMicro group is a polyphyletic group defined by cells or chains $> 20 \mu\text{m}$ in size. When excited by a blue laser, this auto-fluorescing group is discriminated by its FSC and FLR signals, which are the largest observed by FCM. Due to the small sample volumes usually analysed by traditional FCM (less than 1 cm^3 in most cases), counts for this group can have high uncertainties (due to their typically low concentrations). When FSC is calibrated with appropriate standards or phytoplankton cultures, it enables microphytoplankton to be distinguished from nanophytoplankton with a size of about 20 μm . Chains or colony-forming cells may be too large for FCM analysis depending on instrument characteristics (tubing size, flow cell dimension, pulse shape recording or not).

Orange fluorescing microphytoplankton: OraMicro

The OraMicro group consist of cells or chains $> 20 \mu\text{m}$. They display higher FSC and FLO signals than OraNano and a high FLO/FLR compared to the RedMicro group. Cells have a high PE content and may also contain PC that can be excited by a red laser. In this case, when a laser beam with a wavelength of approximately 533 nm (green) excites cells, they will emit a higher FLR signal compared to chlorophyll *a* only containing-cells due to the energy transfer to the pigment PC that will also emit fluorescence in the FLR channel. This group can include cryptophytes of $> 20 \mu\text{m}$, colonial cyanobacterial filaments, but also mixotrophic dinoflagellates and ciliates, according to their pigmentation. Due to the small volumes usually analysed by FCM (typically less than 1 cm^3), counts for this group can have

high uncertainties when cells are not abundant enough. When FSC is calibrated with appropriate standards or phytoplankton cell cultures, it is possible to distinguish microphytoplankton from nanophytoplankton cells with a size close to 20 μm . Chains or colonies may be too large for FCM analysis, depending on instrument characteristics (tubing inner diameter, flow cell dimensions, pulse shape recording or not).

Heterotrophic prokaryotes (Heterotrophic prokaryotes with relatively high nucleic acid= HetHNA and Heterotrophic prokaryotes with relatively low nucleic acid= HetLNA)

Heterotrophic prokaryotes include both *Bacteria* and *Archaea*. To be detected by FCM, staining is necessary. While initial studies have used nucleic acid-stained dyes like DAPI and Hoechst 33342 (Monger and Landry, 1993), nowadays a visible-light green-emitting dye is the most common choice. Blue laser excitation enables heterotrophic prokaryotes to be distinguished due to their SSC (or FSC) and green fluorescence (FLG) signatures. Cells with a lower FLG correspond to heterotrophic prokaryotes with a relatively lower nucleic acid content (HetLNA) and cells with a higher FLG correspond to heterotrophic prokaryotes with a relatively high nucleic acid content (HetHNA). Their scatter signals (FSC, SSC) are equal to or lower than those of OraPicoProk and RedPico and may overlap those of RedPicoProk. The LNA and HNA are apparent nucleic acid contents. By creating a cytogram of FLO vs FLG of the cells, it is possible to discriminate small size autotrophs (such as RedPicoProk) from heterotrophic prokaryotes in the same sample to be subtracted to get accurate heterotrophic prokaryote abundances.

Discrimination on cytograms: A conceptual view

The classification of phytoplankton and heterotrophic prokaryotes from FCM raw data is based on several two-parameter cytograms presenting scatter and fluorescence intensities (arbitrary units) on a log scale. On such cytograms, the selection of FCM groups relies on manually gating density distribution following higher densities in the centre and well separated from other groups on at least two optical parameters. When a FCM group is not abundant enough to estimate its distribution, or when a group has a multimodal distribution, the manual separation into individual groups relies on a-priori knowledge by experts. Output intercomparing between instruments is complex but can be facilitated by standard beads as a reference for approximating relative positions of groups and signal intensities. Despite the differences between

flow cytometers and the physiological influences affecting optical properties, the relative positions of the 13 marine microbial groups defined in this paper follow a common pattern, leading to an agreement between FCM experts. Abundance comparison between instruments is then possible with high accuracy (Thyssen et al., 2014; Marrec et al., 2018). Conceptual cytograms are proposed in Figure 1 to help discriminate the most commonly observed plankton groups in seawater and a decision tree (Figure 2) inspired by Olson et al. (1989) illustrates the steps in identifying the red and orange fluorescing particles when using a blue laser. The yellow/green fluorescence has not been illustrated in the conceptual cytograms for the common phytoplankton identification, as it is not used to identify them.

In summary, RedPicoProk (Figures 1, 2) which includes *Prochlorococcus* spp. (Table 1) is usually very close to the instrument noise and the non-fluorescing particles when they come from well-lit waters (Gérikas Ribeiro et al., 2016). RedPico and RedNano (Figures 1, 2) belong to pico and nanophytoplankton size classes, 3 μm sized beads used as an internal standard may help in separating one from the other (Figure 2). OraPicoProk has a specific signature in the FLO (Figure 1B) due to phycoerythrin. The group typically presents an elongated ellipse signature in the red vs orange fluorescence, belonging in most cases to the *Synechococcus* genus (Olson et al., 1985, Figure 2). The OraPico (Figures 1A, B) has been found to belong to picoplanktonic cryptophytes (Metfies et al., 2010; Hamilton et al., 2017), but can also include, depending on the size and the environment, cyanobacteria such as *Crocospaera* spp. They can share similar or higher orange fluorescence intensity as OraPicoProk, but with higher scatter signatures, similar to the RedPico group (Figure 1A). HsNano cells (Figure 1C) have a FSC signal similar to RedNano cells but are separated by their SSC signals. These are identified as coccolithophorids with coccoliths that generate high SSC signatures by light scatter (Green et al., 1996; Jacquet et al., 2002). RedMicro and OraMicro are only adequately counted when sample volume is high enough (several cm^3) or by integrating several samples when collected within an appropriate time interval (Thyssen et al., 2008a) or if there is a high concentration of algal cells such as during bloom events. Their size class can be estimated from collected images when available, or after light scattering calibration using algal cultures or size fractionated filtration. RedRedPico and RedRedNano cells are only distinguished using a red laser with emission collected > 650 nm. The RedRedNano group is barely found in marine samples although it could include nanocyanobacteria or chain-forming cyanobacteria most often found in brackish and freshwaters (Read et al., 2014). The distribution of heterotrophic prokaryotes (HetLNA and HetHNA) is based on the fluorescence of the nucleic acid-stained intensity, with generally two groups observed, one over the other, in terms of intensity. HetHNA can also have a higher SSC signature (Gasol et al., 1999).

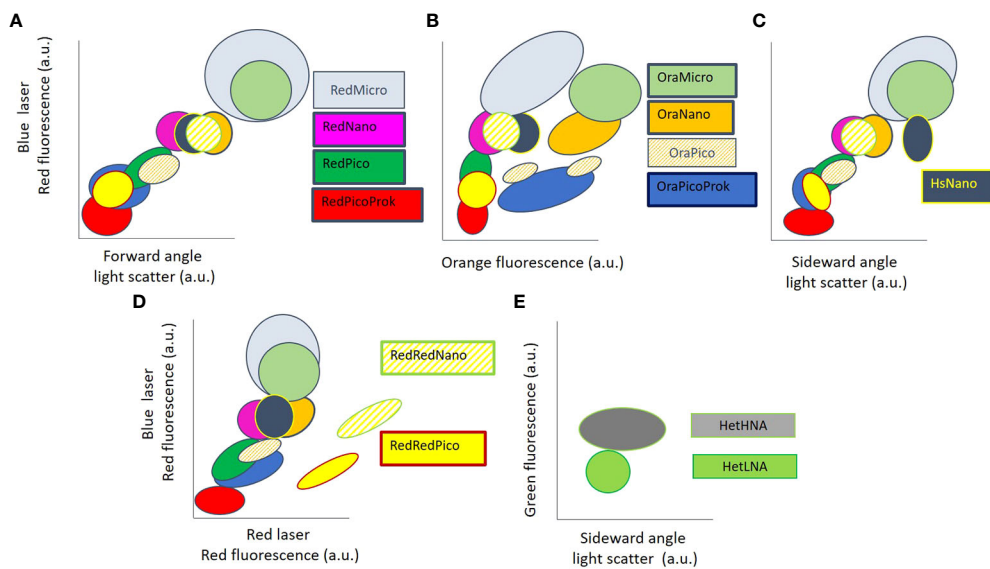


FIGURE 1

Conceptual cytograms depicting the relative position of the most commonly observed groups in marine waters. Axes are in log vs. log scale. (A) Relationship between forward light scatter (FSC), a proxy for cell size, and chlorophyll a FLR from a blue laser. (B) Phytoplankton group separation based on phycoerythrin orange fluorescence and chlorophyll a fluorescence. (C) Relationship between sideward angle light scatter (SSC), a proxy for cell surface roughness, and chlorophyll a FLR from a blue laser. (D) Relationship between chlorophyll a and phycocyanin FLR from a red laser, and chlorophyll a FLR from a blue laser. (E) HetHNA and HetLNA are evidenced after DNA staining with a green emission dye such as SybrGreen I® and are separated on a green fluorescence vs (SSC) with a blue laser. Group denomination and fluorescence terminology are further defined in the main text.

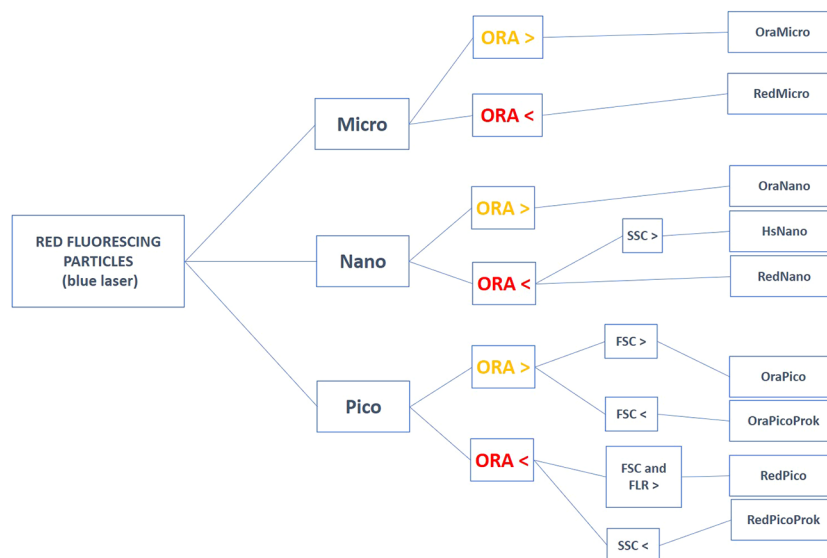


FIGURE 2

Decision tree for helping in the group's discrimination on cytograms for unstained natural red fluorescing particles excited with a blue laser. Red fluorescing particles corresponding to the phytoplankton groups are selected above a threshold that corresponds to the lowest limit of detection of the FCM. The lowest limit of detection is defined when the optical noise and the signature of the detritus in the sheath signal are well identified and do not affect the smallest resolved phytoplankton group the FCM can count (at best, the RedPicoProk). FSC= Forward scatter, FLR= Red fluorescence, FLO= Orange fluorescence, SSC= Sideward scatter. The groups RedRedPico and RedRedNano (discriminated with a red laser), as well as the HetHNA and HetLNA, are not presented here.

Vocabulary setup and governance

The FCM vocabulary is hosted on the NERC (Natural Environment Research Council) Vocabulary Server (NVS, <https://vocab.nerc.ac.uk>). The NVS is a dedicated semantic repository for terminologies used in the marine and related domains. It is managed by the British Oceanographic Data Centre (BODC) at the National Oceanography Centre in the United Kingdom. The NVS publishes terminologies like the FCM vocabulary, in standardized formats using the World Wide Web Consortium's (W3C) Simple Knowledge Organization System (SKOS, <https://www.w3.org/2004/02/skos/>) to represent knowledge in a format that is understandable by both computers and humans. As part of the SeaDataCloud project, the terms and definitions needed to represent the commonly agreed FCM groups were converted into SKOS concepts and added to a SKOS collection. For the FCM concepts, a new collection had to be created. It was given its collection identifier (F02) to form its unique Uniform Resource Identifier (URI) <https://vocab.nerc.ac.uk/collection/F02/>. Within the collection, the SKOS concepts are made of an identifier, a mandatory preferred label, an optional alternative label, and an optional description field. The SKOS identifier enables each concept to be individually and uniquely accessible through its URI.

The technical governance of the FCM vocabulary is entirely managed by the NVS team at BODC while the content governance is currently coordinated by the authors of this paper. A GitHub repository (<https://github.com/nvs-vocabs/F02>) was set up to enable any interested person to propose changes, or additions or ask questions about the new vocabulary in a publicly open forum. The management of a vocabulary within a formal semantic repository infrastructure such as the NVS means that several guarantees are given to the users. One of these guarantees is that a term will never be deleted or modified in such a way that its meaning changes. Instead, terms that are no longer considered valid are deprecated meaning that their unique URI will remain valid but the concept will be marked as "deprecated" and, unless requested specifically, they will be, by default, filtered out of the BODC' NVS search tool (https://vocab.nerc.ac.uk/search_nvs/F02/).

Discussion

Large and curated datasets of fundamental ecological traits and abundances of marine pelagic microorganisms should be made available worldwide, given their relevance in balancing Earth's climate, controlling food webs, and affecting the ecological status of the oceans. FCM is a powerful tool to provide such datasets as it allows fast and reliable access to phytoplankton and heterotrophic prokaryotes abundance with a

relatively good separation into important ecological traits such as size and pigment classes. Consequently, a wider scientific community and managers became interested in implementing FCM data more routinely in monitoring programmes (e.g., Benedetti-Cecchi et al., 2018; Delpy et al., 2018; Eriksen et al., 2019) increasing the FCM datasets in different pelagic habitats. However, homogenisation of datasets is as far as we know, not existing, preventing integration of knowledge at large scales. These datasets are undervalued and seldom integrated into global studies, due to a lack of sharing facilities and interoperability solutions. Without establishing a standardization framework also referred to as Ocean Best Practices (Pearlman et al., 2019), the FCM datasets cannot be shared worldwide, preventing their fundamental contribution to Essential Ocean Variables (EOVs) and Essential Biodiversity Variables (EBVs) for calculating indicators of biodiversity and ecosystems status and trends. During the last 10 years, steps towards best practices and quality control procedures for sample collection, calibration, standardization procedures and data management (including international ISO standards) have been started and discussed in national and international programs such as SOMLIT (<https://www.somlit.fr/en/>), Dymaphy (<http://www.dymaphy.eu/>), SeaDataCloud (H2020 EU project, ID: 730960; <https://www.seadatanet.org/About-us/SeaDataCloud>), JERICO-NEXT and JERICO-S3 (H2020 EU projects, ID: 654410/871153; <https://www.jerico-ri.eu/>) by a network of experts. Besides building best practices to make the handling of the flow cytometers similar between users, historically widely discussed and treated in the frame of immunology and medical use (Pachón et al., 2012), one of the first outcomes of these collaborative efforts is the establishment of the 13 groups of autotrophic (including mixotrophic) and heterotrophic microorganisms categorized and quantified based on a common non-taxonomic vocabulary. A second reason for building such a vocabulary is the continued development of machine learning and other techniques for interpreting the ever-growing number of studies involving multiparametric FCM analysis of marine samples, especially with the increasing use and diversity of autonomous high frequency sensors. Indeed, inter-laboratory data analysis based on manual classification of the FCM groups can be affected by strong variability, especially for the dimmest and the least concentrated groups such as the RedPicoProk, the HsNano or the RedMicro and OraMicro groups (Fuchs et al., 2022), and for data generated by different instruments (Peperzak et al., 2020). Annotation of groups based on a consensus definition will facilitate the process of building the workflow from the original dataset to the interoperable database. It will also strengthen and guide the users, by inferring and assessing the expected outcomes from marine water analyses. This step is one of several to proceed toward FAIR data, and it also paves the way for European (EMODnet; <https://www.emodnet-biology.eu/>) and global

(OBIS; <https://obis.org/>) databases to consider trait-based biological information from FCM with high spatial and temporal resolution as valuable as and complementary to taxonomical data for developing EBVs for future policy requirements. However, by applying standards and trained neural networks dedicated to the recognition of the described groups presented in this manuscript, we advise it could hide some interesting groups not specifically recognised by the training set. Indeed, rare events or sub-groups should find their place in one of the 13 groups described here, still, it will belong to the scientist dealing with his instrument to make the choice on which level of complexity its study should focus on.

A similar effort on inter-laboratory validations and guidelines to ensure optimization of the settings for an instrument in observing marine microorganisms is urgently required. This can be supported by training, using similar standards such as reference beads and fixed samples. The need for large volume analyses to count rare groups is an unresolved problem, and limitations are strongly instrument-dependent. Harmonizing sample and data acquisition procedures should be assessed at the international level by sharing knowledge between experts. Open forums for discussions are a powerful source of success, enhancing collaborations (<https://marinecytometry.slack.com>). In addition, great efforts are done to sample and analyse viruses, heterotrophic flagellates, microzooplankton and/or microphytoplankton, *via* automatic staining or large volumes combined with cameras that allow some taxonomic identification for particles > 10 μm (Sosik and Olson, 2007; Lombard et al., 2019; Fragoso et al., 2019).

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Author contributions

SL initiated the vocabulary standardization procedures with the sustain of GM. MT and GG wrote the first definition of each group. MT and VC wrote the first draft of the paper. The validation and rewriting of the definitions and the standard vocabulary were decided by all co-authors. MT lead the paper and all co-authors participated in the manuscript redaction. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author GD was employed by Cytobuoy b.v.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The Reviewer AK declared a shared affiliation with the author SE to the handling Editor at the time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.975877/full#supplementary-material>

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