A Guide For Field Studies And Environmental Monitoring Of Mixoplankton Populations

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Bringing marine ecology into 21st century

Training next generation marine ecologists in the mixotroph paradigm

MixITiN

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Work Package 5

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A guide for field studies and environmental monitoring of mixoplankton populations

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1 Executive Summary

- A guide for the sampling and analysis of mixoplankton in natural environments is provided that offers guidelines to assist students and research scientists initiating studies of mixoplankton in natural waters.

- The guide contains methods on how to sample, preserve and analyse mixoplankton abundance and diversity directly from natural environments. As mixoplankton are fully integrated constituents of the protist plankton community, many of the sampling strategies and techniques described in this guide are applicable also to phytoplankton and protozooplankton. Accordingly, methods cover traditional methods but also evolving new molecular techniques developed for applications to field and discrete studies of plankton diversity.

- The guide contains specific information on various topics including:
    - Sampling and sample preservation for optical microscopy analyses.
    - Protist microplankton and mixoplankton community sampling shipboard.
    - Size-fractionated eukaryotic protist plankton sampling for molecular purposes.

- Topics not considered in this work include continuous and autonomous methods of sampling (though these are commented upon) and identification of environmental parameters required to contextualise drivers of changes in diversity.

2 Glossary

Items in italics are described elsewhere in this glossary. Some terms included here are not strictly required within this guide but may help interpreting other published materials.

18S rRNA (gene): the most widely used marker for detection and classification of marine eukaryotic microbes.

Chl: chlorophyll and often specifically chlorophyll *a*. The core photopigment, which is usually augmented by various accessory pigments that collect energy across other parts of the *PAR* spectrum.

Chl:C: the ratio (usually as mass) of chlorophyll to C-biomass. This ratio varies between species; typically with a maximum of 0.06 gChl $(gC)^{-1}$.

Ciliates: Phylum Ciliophora. A group of protists characterized by the presence of hair-like organelles called cilia, which are identical in structure to eukaryotic flagella. Cilia are in general shorter than flagella and present in much larger numbers, with a different undulating pattern than flagella. Cilia occur in all members of the group with a few exceptions. They have various different uses such as for swimming, crawling, attachment, feeding, and sensory. CIliates are common in marine, brackish and freshwaters. Size range: 10-4000 µm. Most species are heterotrophic, but some (as non-constitutive mixoplankton; *NCM*) engage in symbiosis with ingested photo-trophic prey or sequester functional chloroplasts from ingested photosynthetic prey. Prey types of ciliates: bacteria and other protists.

CM: constitutive *mixoplankton*; a protist that has an innate, constitutive, ability to conduct photosynthesis and that is also able to phagocytose. (Cf. *NCM*)

Cryptophytes: Phylum Cryptophyta. They have also been termed cryptomonads. Most species of this group of protists have chloroplasts, but some species are known to be without chloroplasts. Cryptophytes contain, besides Chl*a*, also the accessory pigments phycocyanin, allophycocyanin and/or phycoerythrin They are common in marine, brackish and freshwater habitats. Size range (length): 6-50 µm; cells are often flattened in shape, with an anterior groove or pocket. At the edge of the pocket, there are typically two slightly unequal flagella. The groove contains extrusomes, called ejectosomes. Cryptophytes include species which are constitutive mixoplankton (*CM*). Prey: bacteria.

Diatoms: Phylum Bacillariophyta. Photosynthetic protists that (except for very few species) are characterised by a silica cell wall. Non-motile (except pennate diatoms may move slowly when in contact with a hard surface), robust, fast growing, and characteristic of early spring bloom plankton growths in turbulent water. Sizes from 5 - 500µm as pennate (canoe-shaped) or centric (cylindrical) forms. Invariably mixotrophic by virtue of osmotrophy (many grow well in complete darkness on sugars and amino acids), but they are not mixoplankton as they cannot eat.

DIC: dissolved inorganic carbon, comprising CO_2 (the substrate for *RuBisCO*, for photosynthesis), bicarbonate (HCO_3^-) and carbonate ($CO3^{2-}$).

DIN: dissolved inorganic nitrogen, comprising ammonia (NH_3), ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-). NH_4^+ and NO_3^- are the usual main forms of DIN; NH_4^+ is

the “preferred” N-source in algal physiology but it is toxic at high residual concentrations (such as in undiluted anaerobic digestate liquors). Protists isolated from natural (low nutrient) waters may be highly sensitive to even moderate concentrations of ammonium (e.g., >50 µM), even though ammonium is suspected to be the major DIN source in nature.

Dinoflagellates: Phylum Dinoflagellata. Group of protists with approximately half of known species within this group lack chloroplasts, and the other half capable of phototrophy by virtue of being a *CM*. Some are *SNCM* and *eSNCM*. They are found in marine, brackish, and freshwaters. They can form blooms, often considered as harmful. Size range: 6-2000 µm. About half of the species (thecate species) carry cellulosic plates in vesicles (alveoli) under the cell membrane. Some species produce phycotoxins, which can accumulate in the food web or cause fish kills. Some species are parasitic. Prey: other protists and in some cases metazoans. Their feeding mechanisms allow them to feed on relatively large prey, including, even prey that exceed their own size.

DIP: dissolve inorganic phosphorous, PO_4^{3-}.

DOC: dissolved organic carbon.

ESD: Equivalent Spherical Diameter, a transformation used to gain a diameter for organisms or particles from information of their volume; this thus assumes a spherical form for organisms and thus standardises comparison between particles. Very few organisms are actually spherical and care should be taken in comparing, for example, organism ESD with pore sizes in filter-fractionations.

eSNCM: endosymbiotic specialist non-constitutive *mixoplankton*; these mixoplankton harbour selected species of intact endosymbiotic microalgal communities (prokaryotic as well as eukaryotic species). The symbionts continue to perform photosynthesis, while growing inside the host protist. (Cf. *CM, GNCM, SNCM*)

FastQ: a text-based format for storing both a biological sequence (usually nucleotide sequence) and its corresponding quality scores. They are the starting point for all downstream bioinformatics data analysis.

GNCM: generalist non-constitutive *mixoplankton*; a protist that lacks an innate, constitutive, ability to perform photosynthesis and acquires its phototrophic potential from various other organisms. (Cf. *CM, GNCM, SNCM*)

HAB: Harmful Algal Bloom. Most eukaryotic HAB species are now recognised to be *CM*, or *NCM*.

Haptophytes: a clade of organisms within the phylum Heterokontophyta. Most species have chloroplasts. The cells typically have two slightly unequal flagella, both of which are smooth, together with a unique organelle called a haptonema, which is superficially similar to a flagellum but differs in the arrangement of microtubules and in its use. The mitochondria in this group have tubular cristae (taxonomic aid). Many are suspected mixoplankton, feeding on prey ranging from bacteria to ca. 1/3rd their own size.

Heterotrophy: nutrition and growth supported by organic sources of carbon. Osmotrophy and phagotrophy are subsets of heterotrophy. (Cf. *mixotrophy*, *osmotrophy*, *phagotrophy*, *phototrophy*)

LM: Light-microscopy

Lugol's iodine: a common relatively-safe fixative used to immobilise and stain microbes prior to microscopy. It comes in many varieties, but the standard recipe contains crystalline iodine (I2) and potassium Iodine (KI). To make Lugol's iodine: mix 10 g KI and 5 g I2 with 100 mL HPLC-grade water. For an acidified version, 10 mL of glacial acetic acid is added per 100 mL Lugol's solution. The solution should be stored in dark brown glass bottles. To fix a plankton sample add 1 mL Lugol's per 100 mL water sample (= 1% v/v final concentration).

Microplankton: Plankton in the size range of 20-200 µm. While the term microplankton should be used strictly for plankton within the is size range, often it is used as a general term for all microscopic plankton including those outside of this size range. (Cf. *nanoplankton*)

Mixoplankton: Planktonic protists that combine *phototrophy* and *phagotrophy*.

Mixotrophy: combining *phototrophy* and *heterotrophy.* (Cf. *heterotrophy*, *osmotrophy*, *phagotrophy*, *phototrophy*)

Nanoplankton: Plankton within the size range of 2-20 µm. (Cf. *microplankton*)

NCM: non-constitutive *mixoplankton*; a planktonic protist that lacks an innate, constitutive, ability to conduct photosynthesis and thus acquires its phototrophic potential from (an)other organism(s). (Cf. *CM, GNCM, SNCM*)

Osmotrophy: A mode of heterotrophy (i.e., osmo-hetero-trophy) involving the uptake and consumption of dissolved organic compounds.

PAR: photosynthetically active radiation; the portion of the light spectrum that is exploited by photosynthetic organisms. Coincidentally, this is the same as the visible spectrum for humans (light of wavelengths 400-700 nm).

Phagotrophy: a form of *heterotrophy* in which nutrition and growth is supported by the consumption of organic particles; usually such particles are other organisms and thus phagotrophy is *de facto* a form of predation. (Cf. *heterotrophy*, *mixotrophy*, *osmotrophy*, *phototrophy.*)

Phototrophy: nutrition and growth supported by assimilation of inorganic sources of carbon (*de facto*, CO2) through photosynthesis. (Cf. *heterotrophy*, mixotrophy, *osmotrophy*, *phagotrophy.*)

pSNCM: plastidic specialist non-constitutive mixoplankton; these acquire and exploit only the plastids originating from another organism. (Cf. *CM, GNCM, SNCM*)

SNCM: specialist non-constitutive mixoplankton; these acquire and exploit plastids and, in some instances, also other cell organelles and cytoplasm originating from a different "prey" organism. (Cf. *CM, GNCM*)

SSU: Small subunit ribosomal

3 Introduction

The accepted view in the marine research community, over decades, has been that the single-celled plankton can be divided between phytoplankton (primary producers) and protozooplankton (primary consumers) akin to the plant-animal dichotomy in terrestrial ecosystems. In between these "plant-like" phytoplankton and "animal-like" protozooplankton, are the single-celled mixoplankton.

The mixoplankton are so named because they use a mixture of photo-auto- and phago-hetero- trophic strategies for growth (Mitra *et al.* 2016; Flynn *et al.* 2019). While mixoplankton *per se* are not new to science, they have typically not been provided the same status as other marine plankton such as phytoplankton, bacterioplankton, zooplankton etc. For decades mixoplankton have been considered to be curiosities of nature with the assumption that they prosper only when the strict primary producers (phototrophic phytoplankton) and the strict primary consumers (phagotrophic protozooplankton) are disadvantaged. Alternatively, they have been labelled as phytoplankton that eat (e.g., Zubkov & Tarran 2008), or perhaps as protozooplankton that photosynthesise.

Over the last decade there has seen a radical reshaping of how scientists think that the marine food-web operates. It has been shown that in nature mixoplankton are often common components of the plankton community, and thus are closer to the norm rather than the exception. In short, the traditional dichotomy between "plant-like" phytoplankton and the "animal-like" protozooplankton used to describe the oceanic food-web is no longer tenable (Flynn *et al.* 2013; **Fig. 3.1**) and the protist plankton can now be broadly divided into six functional groups (**Fig. 3.2**; Mitra *et al.* 2016; Flynn *et al.* 2019).

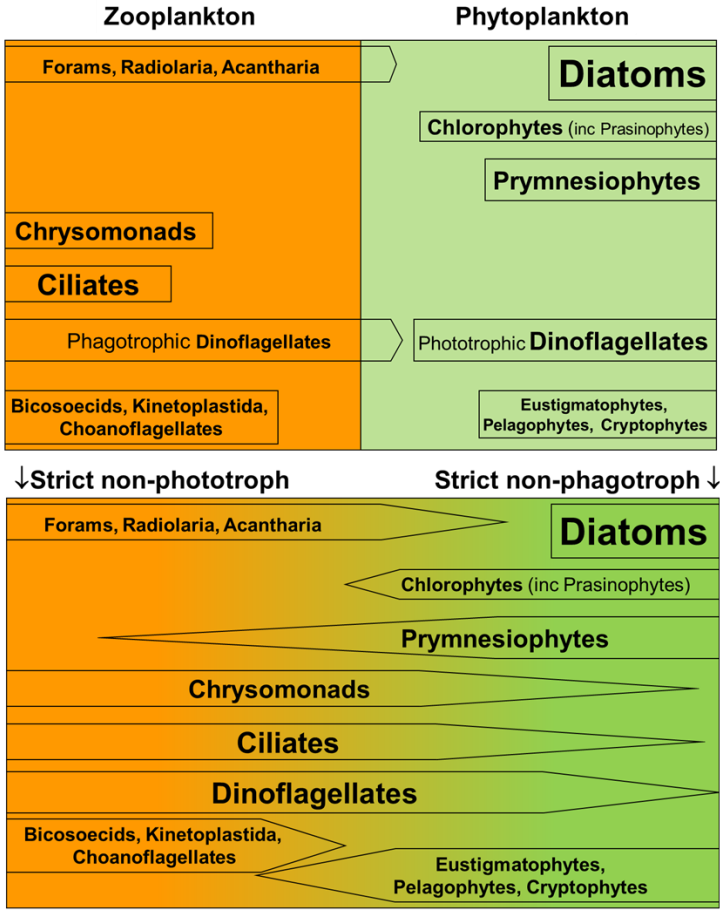


Fig. 3.1 |

The traditional dichotomy of protist plankton (top panel) is now replaced by a gradation (lower panel) from non-phototroph to strict non-phagotrophy. In contrast to the classic misrepresentation shown in the upper panel, the potential for individual organisms to contribute both to primary and secondary production is now acknowledged. Figure modified from Flynn et al. (2013).

At opposite ends of the spectrum are the non-phagotrophic phytoplankton (comprised primarily of diatoms) and the non-phototrophic protozooplankton; these two groups define the archetypical components of the traditional “plant-animal”-like paradigm (upper panel, **Fig. 3.1**). The new mixoplankton paradigm shows a continuum between these extremes, with many combining some degree of both trophic routes (lower panel, **Fig. 3.1**).

The mixoplankton themselves are divided into two major functional groups, with one of those being split into three sub-groups (**Fig. 3.2**). Constitutive mixoplankton (CM) have inherent capability to photosynthesize; these would have traditionally been labelled “phytoplankton”. Other mixoplankton do not have a constitutive capability to photosynthesize but acquire phototrophic capabilities through retention of “body” parts from their prey or entire prey cells as symbionts. These non-constitutive mixoplankton (NCM) would have traditionally been labelled as “microzooplankton”.

NCM comprise:

- Generalist Non-Constitutive Mixoplankton (GNCM) which acquire phototrophy from a range of different prey types;
- Specialist Non-Constitutive Mixoplankton (SNCM) which are “fussy” and acquire their phototrophic capabilities from very specific prey types through retention of i) parts of prey (plastidic SNCM; pSNCM) or, ii) entire prey cells as symbionts (endosymbiotic SNCM; eSNCM).

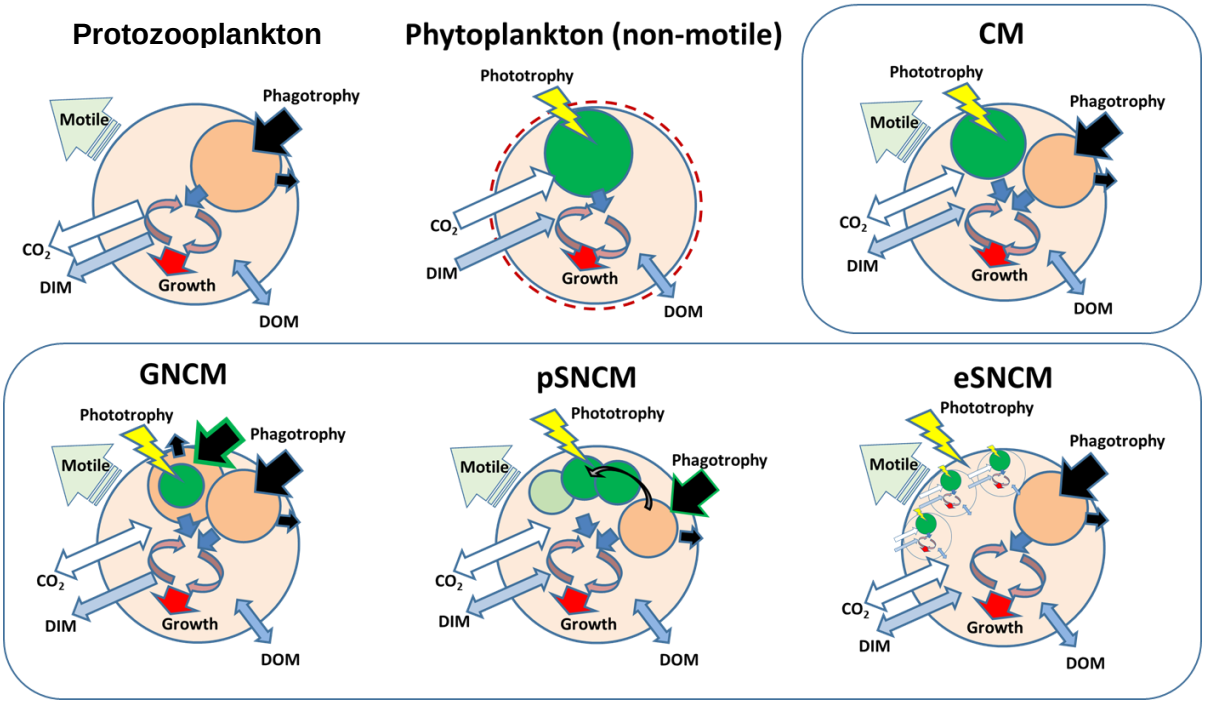


Fig. 3.2 | Schematics showing differences between protist plankton physiologies. Protozooplankton are osmo–phagotrophic; they are incapable of phototrophy. Phytoplankton are photo-osmo-mixotrophic; they are incapable of phagotrophy. The constitutive mixoplankton (CM) and non-constitutive mixoplankton (NCM) are all photo-, osmo- and phago-mixotrophic. The generalist NCM (i.e., GNCM) may acquire phototrophy from many types of phototroph prey; pSNCM are plastidic specialists acquiring phototrophy from specific prey only. eSNCM are endosymbiotic NCM, acquiring phototrophy by harbouring specific phototrophic prey cells. See Mitra *et al.* (2016) and Flynn *et al.* (2019) for further information.

This new marine paradigm thus sees a radical shift from the traditional view of a food-web dominated by phytoplankton (“microscopic plants”) and microzooplankton (“microscopic animals”) to one that contains as a major component the mixoplankton in global oceans (Leles *et al.* 2017, 2019). However, routine field sampling and monitoring techniques are very much based on the phototrophy-phagotrophy dichotomy. Within this guide we look at the different methods required for mixoplankton field sampling.

The guide contains methods on how to sample, preserve and analyse mixoplankton abundance and diversity directly from natural environments. As mixoplankton are fully integrated constituents of the protist community, many of the sampling strategies and techniques described in this guide are applicable also to phytoplankton and protozooplankton. Also, covered are the evolving new molecular techniques developed for applications to field and discrete studies of plankton diversity.

Topics not considered in this work include continuous and autonomous methods of sampling and identification of environmental parameters required to contextualise drivers of changes in diversity.

To draw explicit attention to any special precautions or steps to handle mixoplankton, in comparison with other protist plankton, each protocol carries a text box alerting the reader to any specific aspects.

Key amongst these is that, as a group, mixoplankton are relatively fragile and are easily damaged so complicating identification, counting and preventing physiological studies.

HEALTH & SAFETY

Always observe all local health and safety requirements, including conducting risk assessments, chemical dose exposure calculations, and following waste disposal protocols.

4 Sample collection, preservation and storage

4.1 Sample collection

Precautions for mixoplankton: In general, handle as per phytoplankton and/or protozooplankton; no additional steps are required other than being particularly careful not to subject the samples to hydrodynamic, light and/or temperature shock/s.

Discrete samples are taken at specific depths and locations according to the physical, chemical and biological characteristics of the water column. Water samples are taken by means of a CTD-rosette with Niskin bottles and characteristics of the water column are measured by the instruments mounted on the rosette. Once the rosette is on deck, the samples are taken from the Niskin bottles and processed to be either analyzed on board or stored for further analysis.

4.2 Sampling tools and equipment

Precautions for mixoplankton: No additional equipment required; handle as per phytoplankton and/or protozooplankton.

In situ sampling will require a research vessel equipped with all standard sampling equipment (**Fig. 4.1**) including:

- CTD-rosette with Niskin bottles or Niskin bottles alone
- Conductivity-temperature-depth (CTD) probe
- Vacuum and peristaltic pumps and filtration units
- Fridges (4°C) and freezers (-20°C and -80°C)

Fig. 4.1 | Left: Sea-bird SBE25 (on board RV Simon Stevin). Right: Sea-bird SBE911 CTD probe (on board RV Heincke).

4.3 Sample preservation

Precautions for mixoplankton: In general, the minimum amount of preservative to fix material should be used as mixoplankton are fragile and their shape and internal contents are key defining characteristics for identification. The situation is particularly acute for the smallest, and also the largest forms (e.g., eSNCM colonial Radiolaria). Identification should be performed as soon as practically possible.

Samples should be preserved immediately for further analyses.

4.3.1 Fixatives/preservatives

The preservative used must guarantee a good recognition of taxa at least during the storage period of the samples. The most frequently used preservatives in phytoplankton research are Lugol's solution (basic Lugol if intact coccolithophorids are examined, otherwise acid Lugol and adaptations of it) and formaldehyde-based solutions.

According to Stoecker *et al.* (1994), formaldehyde fixation may underestimate the total ciliate abundance by up to 65%. Acid Lugol's solution is considered the most effective fixative in terms of ciliate cell loss (Gifford 1985; Leakey *et al.* 1996) but does not allow observation of chloroplast autofluorescence.

Particularly for eSNCM Radiolaria ethanol alcohol fixation would work up to some degree but some loss is expected. Borax formaldehyde would also work for single celled but not for colonial Radiolaria.

Since both fixative methods have advantages and disadvantages, it is recommended to count duplicate samples fixed by both methods (Lugol's and formaldehyde; Karayanni *et al.* 2004; Romano *et al.* 2021).

Thus, for identification and enumeration of organisms under microscope, duplicate water samples (200-1000 mL, depending on trophic status of the environment and depth) are collected from the desired depth into different amber glass or polypropylene bottles as follows:

- one containing Lugol´s solution (1-2% v/v final concentration), and,
- the other containing borax-buffered formalin or glutaraldehyde (final borax concentration 1-2% w/v, pH 7).

Samples fixed with Lugol's are kept at 4°C in darkness until analysis, while aldehyde samples are best kept in the freezer.

4.3.2 Filtration

Precautions for mixoplankton: No additional steps required; handle as per phytoplankton and/or protozooplankton.

For species identification using molecular techniques, water samples need to be filtered.

There are two different water sampling procedures for deployment onboard for obtaining samples for molecular biological analysis as described here.

I. **Entire size-range community:** Vacuum filtration through 0.22 µm PVDF (Millipore Durapore) filters a volume of typically 300-500 mL water directly obtained from the Niskin bottle in order to obtain the entire size-range of eukaryotic planktonic community, followed by immediate storage of filters at -20°C (the volume to be filtered depends on the trophic status of the environment; see also **Fig. 4.2**).

II. **Size-fractionated community**: filtration of 15-20 L of water through three different size meshes and filters. First, water is filtered through 200 and 20 µm meshes by gravity-filtration and next, size fractions of 20-3 µm and 3-0.2 µm are obtained by means of a series of tripod-mounted (for 142 mm diameter filters) or in-line filters (for 47 mm diameter filters) filtration units using peristaltic pumps and polycarbonate filters (**Figs. 4.3, 4.4**). Filters can be cut in 4 pieces by the use of a filter cutting tray. Filters need to be stored immediately at minimally -20°C.

Fig.4.2 | Vacuum pump and filtration apparatus for eukaryotic plankton sampling >0.22 µm and filter containers (cryotubes)

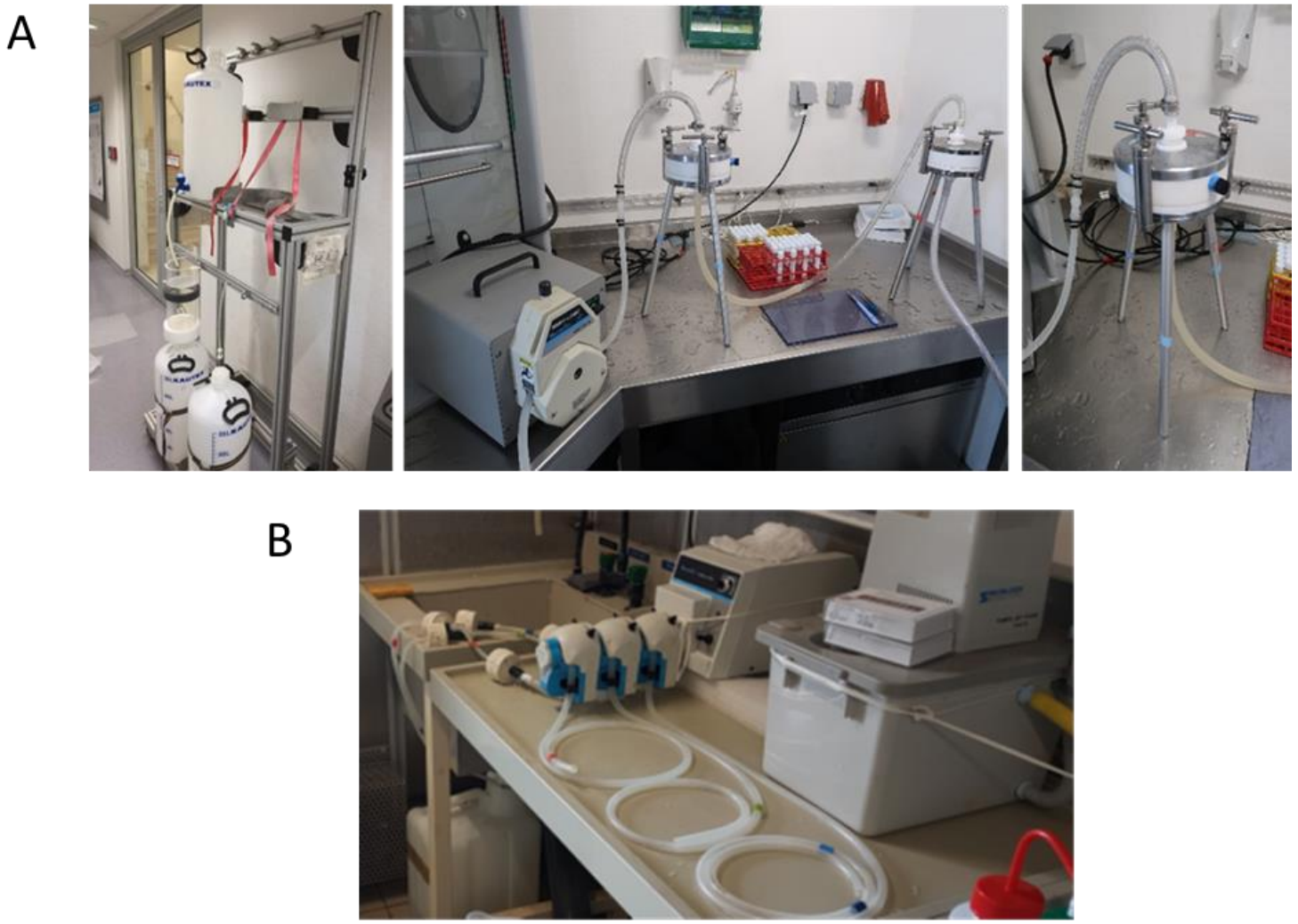


Fig. 4.3 | Upper panel (A). Left: filtration structure with 25 L containers and 200 µm and 20 µm filtration meshes. Mid: filtration set-up for 142 mm filters with a peristaltic pump and filtration tripods (3 µm and 0.22 µm) and filter cutting tray. Right: Filtration tripod 3 µm in detail. Lower panel (B) In-line filtration set-up for 47 mm filters with a peristaltic pump.

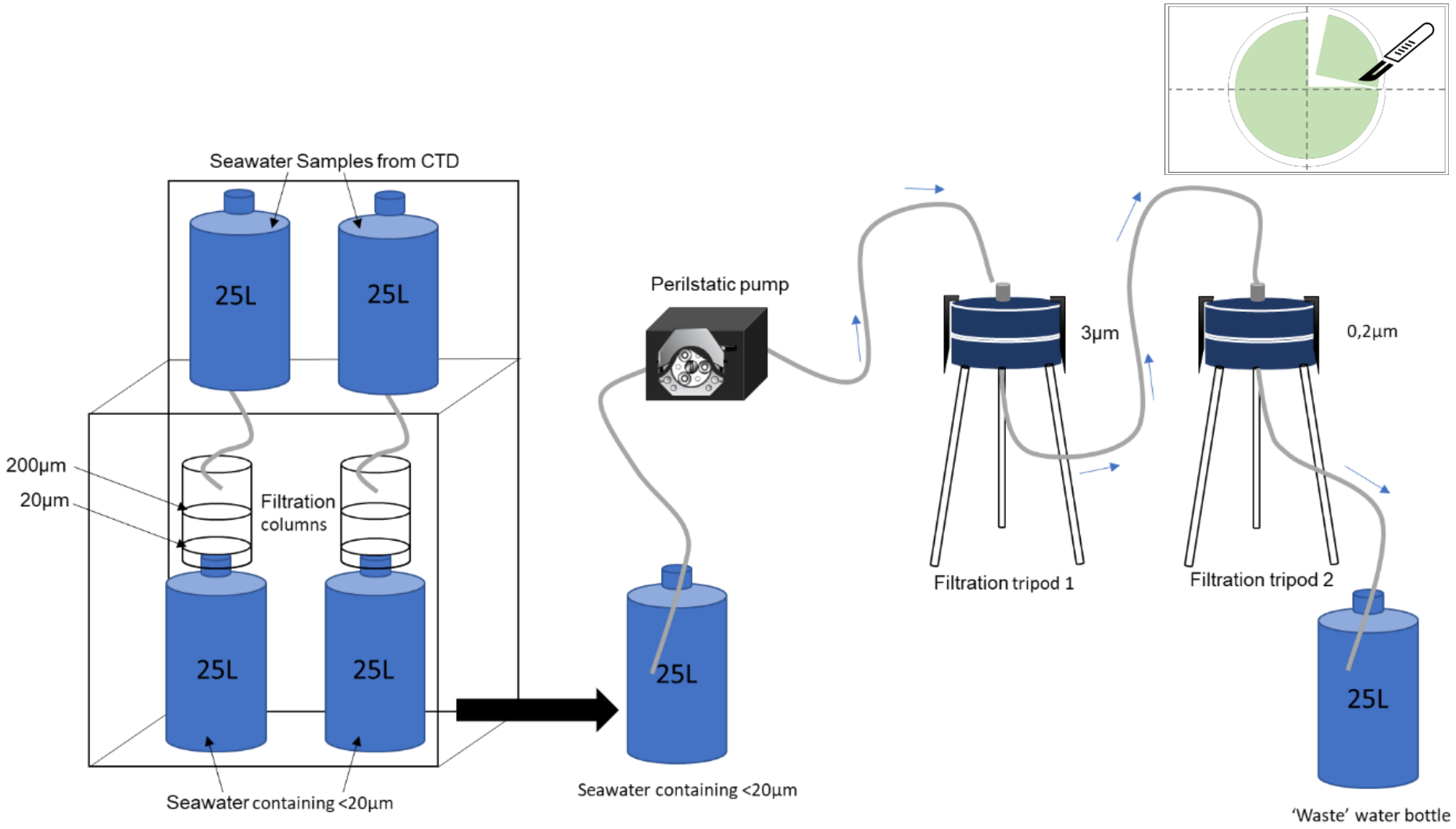


Fig. 4.4 | Schematical representation of the procedure (II) followed to size-fractionate mixoplankton shipboard. The metallic structure holds two series of 25 L bottles in parallel and gravity-filtration happens through 200 µm and 20 µm meshes. Pumping takes place next through 3 µm and 0.2 µm placed in series. Inset top right, shows the cutting of filter on a glass tray; both 3 µm and 0.2 µm filters can be cut and replicates obtained for different molecular purposes.

4.3.3 Consumables

Precautions for mixoplankton: No special steps are required; handle as per phytoplankton and/or protozooplankton.

For sample fixation

- Fixatives: acid Lugol´s solution, formaldehyde or glutaraldehyde solution; final concentration 2% v/v. Add Borax to the formaldehyde in excess (1-2% w/v) to keep stable at pH 7.
- Stains: Calcofluor (for polysaccharide; can be used to stain thecal plates in armoured dinoflagellates), DAPI (for nucleic acids), LysoTracker Green (acidotropic probe for food vacuoles).

For sample filtration

- x2 Filtration tripods or filter holder
- PVDF (Millipore Durapore) Membrane filters 0.22 µm (47 mm diameter)
- PVDF (Millipore Durapore) Membrane filters 0.2 µm and 3 µm (142 mm diameter)
- 15 mL cryotubes for filter storage
- x6 25 L plastic bottles with bottom tap
- Peristaltic pump and tubing
- Filtration mounts
- Tweezers
- Flat bladed Scalpel
- x2 Filtration towers (47 mm diameter) with 200 µm mesh
- x2 Filtration towers (47 mm diameter) with 20 µm mesh
- Vacuum pump

4.4 Sample Storage

Precautions for mixoplankton: No additional steps required; handle as per phytoplankton and/or protozooplankton.

Samples preserved with Lugol's solution should always be stored in darkness and ideally refrigerated (4-5°C; not higher than 10°C). Aldehyde samples should be stored frozen, preferably at -80°C, until staining. It is best to filter the environmental samples on the filters as soon as possible after sampling, unless the samples are quantified using a flow cytometer.

Samples filtered for genomic analyses should always be stored at -20°C. DNA samples may be stored longer prior to analysis, nevertheless the analysis within a year is recommended.

5 Identification and quantification of mixoplankton diversity and biomass

Precautions for mixoplankton: Section 5.2 gives some additional information for the researcher interested in specific mixoplankton groups.

5.1 General techniques and recommendations

Precautions for mixoplankton: No additional steps required; handle as per phytoplankton and/or protozooplankton though always err on the side of caution, handling samples gently.

Both light microscopy and genomic methods are described here to study planktonic diversity protist diversity (**Fig. 5.1**).

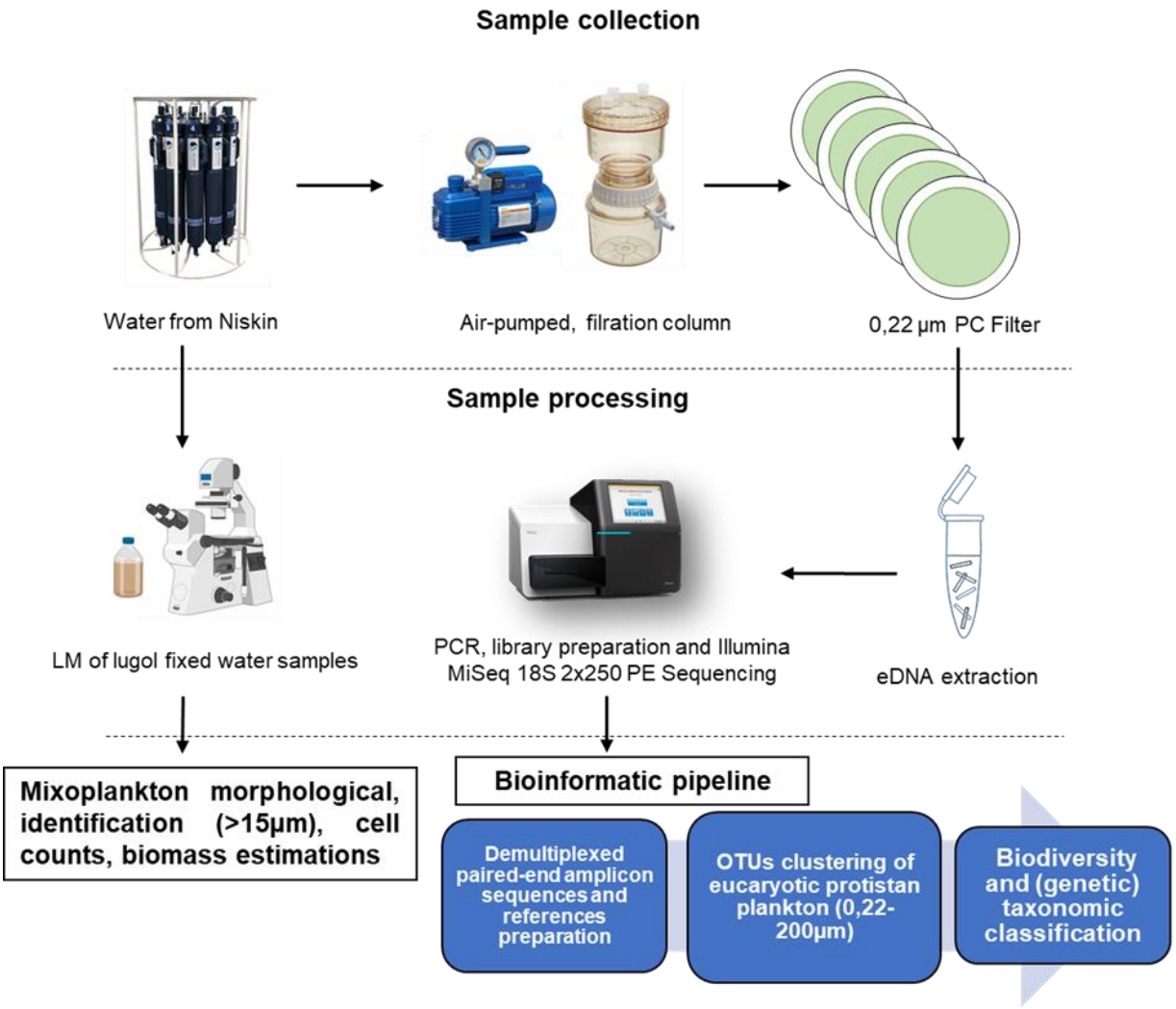


Fig. 5.1 | Schematical of traditional methods and genomic methods to study mixoplankton abundance and diversity from field sampling.

5.1.1 Light microscopy methods

Precautions for mixoplankton: As mixoplankton combine both phototrophy and phagotrophy in the same cell, protocols used for "phytoplankton" and for "protozooplankton" are required together. Identification (allocation) of the mixoplankton trophic mode for most species is incomplete; see Section 7.

Organisms >15 µm in diameter

These can be enumerated from the Lugol's preserved samples using plate counting chambers in accordance with Utermöhl (1958) on an inverted light microscope at 200x magnification. The volume of sample to be settled in the counting chamber is adjusted depending on the biomass richness of the water sample, so that a representative number of organisms can be enumerated (ideally, for statistical reasons, at least 200 cells).

The microscope should ideally be equipped with a camera to acquire images of the organisms to aid in the measurement of linear dimensions. Cellular linear dimension can be measured with image software such as CellSense or ImageJ, provided that the images are calibrated with appropriate reference scales. Cellular biovolumes are then calculated based on linear cellular dimensions using geometric formulas according to Hillebrand *et al.* (1999). This serves to convert cell counts (cells L^{-1}) into carbon mass (µg C L^{-1}) according to carbon to volume relationship recorded in the literature (e.g., Menden-Deuer & Lessard 2000; Mansour *et al.*, 2021).

The ability to identify members of the plankton relates directly to the experience of the operator and is, thus, typically not accurate beyond the genus level. It is strongly recommended that analyses are processed by the same person.

Further analysis of the glutaraldehyde preserved samples can be carried out. Glutaraldehyde preserved organisms can be collected on polycarbonate filters of 2 µm pore size and stained with Calcofluor (Andersen & Kristensen 1995) and DAPI (Porter & Feig 1980). These are then inspected with epifluorescence microscopy with light sources and filters of appropriate wavelengths to detect the fluorophores (UV light for DAPI and Calcoflour: excitation wavelength 350nm - emission 450 nm; blue or red light for chlorophyll: excitation wavelength 400nm or 700nm - emission 750 nm). This allows for a deeper characterization of the organisms morphotype than when observed in the Lugol's samples. Calcoflour stains thecal plates of armoured dinoflagellates helping in the morphological identification; DAPI stains cell nuclei helping the count of colonial organisms.

Chlorophyll emission would reveal the presence of chloroplasts in either CM which are not identifiable only based on morphology or NCM which are rarely distinguishable from their purely heterotrophic counterpart if only based on morphology (especially ciliates).

Samples on filters can be frozen (-20°C) and stored for several years without losing their quality, though storage at 4°C suffices if examined within few days. Samples preserved samples with Lugol's have to be examined within months to avoid cell loss. Lugol's fixation is preferable because of handling (less toxic for the operator and the environment) and the broad effectiveness on the different organism types in the population.

Another approach, mainly for NCM (especially ciliates), is to examine formaldehyde fixed samples under an inverted epifluorescence microscope (in a similar way as for Lugol's fixed samples, see above), because formaldehyde allows for the observation of the

autofluorescence of chloroplasts. Since both fixative methods have advantages and disadvantages, it is recommended to counter duplicate samples fixed with both fixatives (Karayanni *et al.* 2004; Romano *et al.* 2021).

Organisms <15 µm in diameter

These are more reliably enumerated with automated cell counters (e.g., flow cytometry), and are generally identified at class level, based on the pigments content detected via fluorescent emission under different excitation wavelengths (Anderson & Hansen 2020).

Samples for flow cytometry have to be preserved with transparent fixatives (like glutaraldehyde) and can be treated with LysoTracker Green (*a posteriori*) to stain acidic intracellular compartments, to detect the presence of (acidic) food vacuoles according to the protocol from Sintes & del Giorgio (2010). This allow the enumeration of organisms which were presumably actively feeding at the moment of sampling/fixation. Combining the chlorophyll fluorescent signal and the fluorescent signal of LysoTracker it is possible to enumerate CM species within the phytoflagellates population. If a flow cytometer is not available, samples fixed in glutaraldehyde or formaldehyde can be filtered onto Nuclepore filters and cells <15 µm enumerated using epifluorescence microscopy.

5.1.2 Genomic methods (DNA-barcoding; Illumina MiSeq)

Precautions for mixoplankton: No additional steps required; handle as per phytoplankton and/or protozooplankton. Identification (allocation) of the mixoplankton trophic mode for most species is incomplete; see Section 7.

Major mixoplankton groups and biodiversity can be assessed by targeting the V4 region of the 18S rRNA gene and using Illumina MiSeq 2 x 250bp paired-end sequencing. The small subunit (SSU) 18S rRNA gene is the most widely used marker for the detection and classification within the marine eukaryotic microbes. Indeed, the DNA-barcoding of different regions of this gene (V4-V9) has been proven to be a powerful and sensitive tool for large-scale biodiversity surveys, allowing comparison of studies rooted in taxonomy (Chain *et al.* 2016).

The NucleoSpin Soil extraction Kit (Machery-Nagel) has been tested and shown to be an efficient kit for DNA extractions from samples on PVDF (Millipore Durapore) membrane filters (following manufacturer's protocol). PCR reactions amplifying the 18S rRNA region can be performed using the following primers used to target the V4 region of the 18S SSU rRNA gene (Stoeck *et al.* 2010):

- TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′)
- TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′)

For PCR reactions a final volume of 25 µL can be used, containing 2.5 µL of sample DNA (5 ng/µL), 5 µL of both forward and reverse primers (1 µM) and 12.5 µL high-fidelity polymerase (Kapa Biosystems).

PCR plates should be sealed, and the following PCR-program run in a thermal cycler: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s; extension at 72°C for 30 s final extension at 72°C for 5 min. All PCR products (480 bp, ~383 bp + 97 bp of primers) should be verified on a 1.5% agarose gel.

The following library preparations of 18S ribosomal RNA gene amplicons are required:

- PCR clean-up 1,
- indexing PCR,
- PCR clean-up 2,
- library quantification,
- normalization and pooling following the 16S Metagenomic Sequencing Library Preparation guide (Illumina 2013).

Two different bioinformatic pipelines can be used to handle unprocessed but demultiplexed Illumina paired-end *fastq* files and generate the final amplicon sequences: q-zip and DADA2.

- **q-zip** (Hardge *et al.*, 2018)**:** A bash script that wraps and connects bioinformatics tools to treat demultiplexed Ilumina paired-end amplicon sequences**.** SWARM is used as a clustering method (Mahé *et al.* 2014). The pipeline filters and trims (and thereby reduces) the reference sequence set to obtain a more accurate annotation as well as more versatile chimera filtering (decreasing false positives); it also codes for the creation of an amplicon contingency and OTU table. Singletons are filtered out automatically and log and statistic files are provided.
- **DADA2** (Callahan *et al.* 2016, 2017)**:** An open-source software package (R) that denoises and removes sequencing errors from Illumina amplicon sequence data to DADA2 infers sample sequences exactly and resolves differences of as little as 1 nucleotide.

Operational Taxonomic Unit (OTU) table (q-zip) or ASV table (DADA2) containing the taxonomic information (reference data base Silva v.132 and NCBI BLAST) are created at the end of both bioinformatic pipelines. Each of the identified taxa can be assigned a trophic classification based on currently available databases.

5.1.3 Equipment

Precautions for mixoplankton: Mixoplankton are members of the protist plankton that have hitherto been characterised as “phytoplankton” or “protozooplankton”. Typically, the same equipment used for standard plankton analyses can be deployed.

<u>For traditional light microscopy methods</u>

- Inverted light microscope equipped with camera (and, ideally, also equipped for epifluorescence), with software for measurement of an organisms’ linear dimensions.
- Epifluorescence microscope
- Glass trays
- Counting and sedimentation chambers (Utermöhl and Sedgewick Rafter)
- Flow cytometer

<u>For genomic methods</u>

- Illumina MiSeq System
- Bioanalyzer: High-Resolution Automated Electrophoresis of DNA, RNA

- NanoPhotometer / Qubit Fluorometric Quantification: Measure DNA, RNA and proteins
- Gel Electrophoresis - Gels, tanks & gel plate, gel comb, microwave
- UV light, and camera
- Pipette set (µL)
- Pipette tips
- DNA Standard/Ladder

5.2 Specific recommendations for different mixoplankton functional groups

Precautions for mixoplankton: Mixoplankton are often fragile members of the plankton, and their cells are easily disrupted. This section provides additional information for handling these organisms.

The MixITiN proposal as well as various studies have further highlighted the importance of establishing effective protocols for field sampling of mixoplankton (Christaki *et al.* 2011; Anderson *et al.* 2017). In the case of biodiversity estimation studies, it is crucial to properly define the sampling effort in order to correctly interpret the final data (Leles *et al.* 2017, 2019). The plankton community shows different degree of sensitivity to sampling procedures, with some organisms being more robust than others. As a result, data (qualitative and quantitative) on the more fragile species is jeopardised. Special care should therefore be taken when sampling and cultivation-independent studies are also sometimes essential for the study of many mixoplankton organisms, in particular NCM.

Sampling methods should account for the fragility of these organisms that are otherwise difficult to study and allow follow-up analysis with sensitive molecular techniques such as transcriptomics, qPCR or Fluorescence in situ hybridisation.

5.2.1 Constitutive Mixoplankton (CM)

Constitutive mixoplankton for the most part equate to non-diatom protist phytoplankton. The greatest challenge will be presented in handling the relatively delicate larger flagellates (notably dinoflagellates), and in identification of the smaller flagellates (ca. <3 µm). In general, it is best to use similar protocols to those described below for GNCM and SNCM species in order to minimize physical damage to the cells.

5.2.2 Generalist Non-Constitutive Mixoplankton (GNCM)

If water samples are collected with Niskin bottles, it is preferable for those to be equipped with a silicon tube by which subsamples may be siphoned off to decrease organism loss due to hydro-mechanical disturbance (**Fig. 5.2**).

5.2.3 Specialist Non-Constitutive Mixoplankton (SNCM)

Cultivation-independent studies are essential for the study of many NCM species; this requires the collection of physiologically competent organisms from the field. For endosymbiotic uncultivatable mixoplankton (e.g., Acantharians, Collodarians), sampling using slow horizontal plankton net tows are proven to be the best compromise between ease of sampling and keeping cell quality (Graham *et al.* 1976; Mansour *et al.* 2020).

The method to acquire clean isolated cells for transcriptomic analysis is described below. This work is conducted under field conditions, where facilities are often not as good as in terrestrial-bound laboratories. Thus, sample preparations are typically not conducted working in a controlled air-stream, under a hood, nor has the work area necessarily been treated with RNAse inhibitors (or similar). Aseptic techniques and gloves, to minimise further contamination, should be used throughout all procedures, and especially those for molecular methods.

Fig. 5.2 | Silicon tube used to reduce organism loss when water samples are collected with Niskin bottles.

Cell isolation and sample preparation procedure for RNA extraction of field samples

- Prepare filter-sterilised seawater (FSW, 0.2 µm-pore-size).
- Isolate cells from plankton net samples into a Petri dish with FSW using a micropipette or modified Pasteur pipette.
- Incubate cells in FSW (1 h) and repeat the isolating procedure with a transfer to fresh FSW three times. This procedure allows for self-cleaning of particles attached to the cells and dilution to achieve effective extinction of any contaminating organisms accidentally taken with during isolation.
- Clean cells can be used for physiological experiments (see Section 6) before proceeding to the next step.
- Prepare 100 µL lysis buffer (from RNAqueous kit) in 0.2 mL PCR tubes in a frozen Alurack.
- Where possible continue to work on ice.
- Transfer one cell per lysis buffer filled tube and immediately freeze in liquid nitrogen.
- Store at -80°C. Immediate storing at -80°C, rather than freezing in liquid nitrogen, has proven to suffice, though flash freezing is still preferred when possible.

For further details see Mansour & Not (2021).

6 Identification and quantification of mixoplankton activities and fluxes

Precautions for mixoplankton: Because mixoplankton combine both phytoplankton-like and protozooplankton-like physiologies in the same cell, a full analysis of their ecophysiological function is greatly complicated and would ideally require concurrent measurements of phototrophy and phagotrophy. Section 6.3 provides information about specific mixoplankton functional types in this regard.

6.1 Traditional Physiological Methods

6.1.1 Prey ingestion

Methods to identify and quantify ingestion are different for organisms in the different size classes. To identify ingestion in nanoplankton (organisms of 2-20 µm) acidotropic probes are usually employed (see above, LysoTracker Green stain). Mixoplankton in this size class are usually represented by bacterivorous CM species. Bacterivory rates of these small CM can be measured via incubation of the natural population with surrogate prey, such as fluorescently labeled bacteria (FLB) with which it is possible to measure the uptake via a protocol introduced by Sherr *et al.* (1987) (see also Unrein *et al.* 2007, 2014).

Ingestion rates of microplankton (organisms of 20-200 µm) are generally measured via the dilution technique which was introduced by Capriulo & Carpenter (1980) and specifically applied on this group of organisms by Landry & Hassett (1982). The technique consists of the fractioning of the natural population in two different size classes via a mesh, which would separate the microzooplankton from its potential prey items (organisms smaller than 20 µm). A mixture of the smaller and larger size fractions are then prepared in different ratios (1:2- 1:3- 1:5) and incubated *in situ* or with similar conditions of light and temperature. Grazing rates are measured, relatively to a control (untreated sample), via disappearance of (potential) prey items or by decrease of chlorophyll in the small size fraction.

Warning: This technique does not allow to distinguish grazing associated to heterotrophic organisms, from grazing of CM or NCM species which are known to belong to the size class (microzooplankton). To do that, this approach can be supported via extrapolation of feeding relationships from laboratory determined grazing rates of known species (e.g., Heinbokel & Beers 1979), thus estimating the potential grazing based on predator taxa and biovolume (Hansen *et al.* 1997).

6.1.2 Primary production

Size fractioning can be also employed to estimate primary production in the different mixoplankton compartments: photosynthetic nanoplankton (bacterivorous CM) and photosynthetic microplankton (which include both CM and NCM species). Numerous techniques are generally employed to estimate primary production rates. Rough estimates can be derived from interpolation of chlorophyll a concentration and irradiance level in the water sample (Ryther & Yentsch 1957). However, rates are better estimated by measuring the development of the oxygen concentration in the illuminated water sample via O_2 sensors (Walker 1987) or the incorporation of carbon isotopes (^{14}C) into the biomass of the organisms (Steemann Nielsen 1952).

Warning: None of these methods can distinguish primary production associated with CM or NCM species from primary production of strict photo-autotrophic phytoplankton within the same population. However, if the measurements of such rates are associated with the identification and quantification of the organism functional types in the sample, then the specific contribution of mixoplankton to primary production may be roughly deducted from laboratory determined photosynthetic rates of known species, as mentioned above regarding ingestion.

6.2 Fluorometric analysis

The last two decades has seen the development, and increasingly common, deployment of instruments that exploit the fact that the *in vivo* fluorescence signature of chloroplasts, when conducted in a certain fashion, yields information about the photo-physiological status of the organisms, and can give a handle on primary production as well (e.g., Suggett *et al*. 2009; Robinson et al. 2014; Aardema *et al*. 2019). How these signatures vary in mixoplankton of different functional types, feeding in different ways in response to different environmental clues, is unknown. At present, it is assumed (and instruments tested against the assumption) that the methods are interrogating "phytoplankton" only. The same problem applies, of course, to measurements using ^{14}C or O_2 protocols. With the sensitivity of fluorometric methods, however, there is scope to deploy protocols at much higher spatial and temporal scales.

There is thus an urgent need to conduct laboratory and then field trials using fluorometric methods both to attempt to disentangle "phytoplankton" versus "mixoplankton" signals, and because of the potential of the methods to actually usefully separate those signals to get a better handle on the balance of photo- and phago- trophy.

6.3 Identification of potential activity by transcriptomics

There is a long-term aspiration (stemming from the 1990's; Caron *et al.* 1999) that molecular biology will be able to supply information on metabolic rates. For mixoplankton research, complicated as it is by the coupling of different trophic modes, such a goal would indeed prove revolutionary. Genomic approaches to identify inorganic nutrient uptake potential and to help disentangle heterotrophic processes such as phagotrophy from osmotrophy and phototrophic-induced anabolic activities, would be of clear utility. This information would then support cellular and metabolic modelling by better elucidating the physiological mechanisms and quantifying their importance in different scenarios.

Application of 'omics approaches to the groups of NCM, specifically eSNCM, would offer the potential to understand the active processes of the organisms at a given time (Burns *et al.* 2018).

However, at present (2021) there are no such protocols for deployment that could yield information other than a potential for activity. Given the known subtleties of photo-phago-trophic expressions in mixoplankton, differences in trait expression between species, and set against the need in modelling to group species together in functional groups, such methods for routine deployment still appear some way off.

6.4 Specific recommendations for different mixoplankton functional groups

For 'omics methods, detailed laboratory protocols for cDNA library preparations from single-cell samples, useful for all MFTs, are available in Mansour & Not (2021) and Mansour *et al.* (2021a, b).

6.4.1 Constitutive Mixoplankton (CM)

CM species are invariably classified traditionally as phytoplankton, and emphasis will be placed on phototrophy, using the usual suite of methodologies directed at C-fixation (^{14}C, O_2, PAM, etc.).

6.4.2 Generalist Non-Constitutive Mixoplankton (GNCM)

Photosynthetic rates of GNCM ciliates can be measured on single cells, that have been manually isolated from natural sea water samples with a drawn Pasteur pipette, applying the ^{14}C technique by Rivkin & Seliger (1981). Ciliate cells would have to be first rinsed in filtered sea water (FSW) and then incubated for some hours in the light in small volume of FSW (2 mL) spiked with $NaH^{14}CO_3$. At the end of the incubation, samples have to be acidified and dried so that only organic carbon would remain in the incubation vial. This allows measurement of the amount of isotope which has been incorporated into the ciliate biomass, and thus calculation of carbon incorporation rates as pgC $cell^{-1}$ h^{-1}.

6.4.3 Specialist Non-Constitutive Mixoplankton (SNCM)

As for GNCM, photosynthetic rates can be measured on manually isolated single cells. It is generally not possible to establish cultures of eSNCM species (such as Radiolaria) and maintain them in the laboratory; this makes the ^{14}C technique more difficult to implement due to regulations on conducting experiments using radioisotopes on small boats often used to sample these organisms.

Alternatively, though less sensitive (more cells needed), stable isotopes (^{13}C, ^{15}N) can be employed. This has the added benefit of also allowing the simultaneous measurement of nitrogen uptake rates in addition to carbon uptake rates. Similar to the ^{14}C methodology, cells are incubated in FSW spiked with $NaH^{13}CO_3$. After which the specimens are deposited individually (or filtered) on pre-combusted GF/F filters. Controls are needed of an unlabelled spike and dark incubation. Specimens on filters are dried at 60°C and kept in the dark for EA-IRMS analysis.

7 Mixoplankton functional type identifications

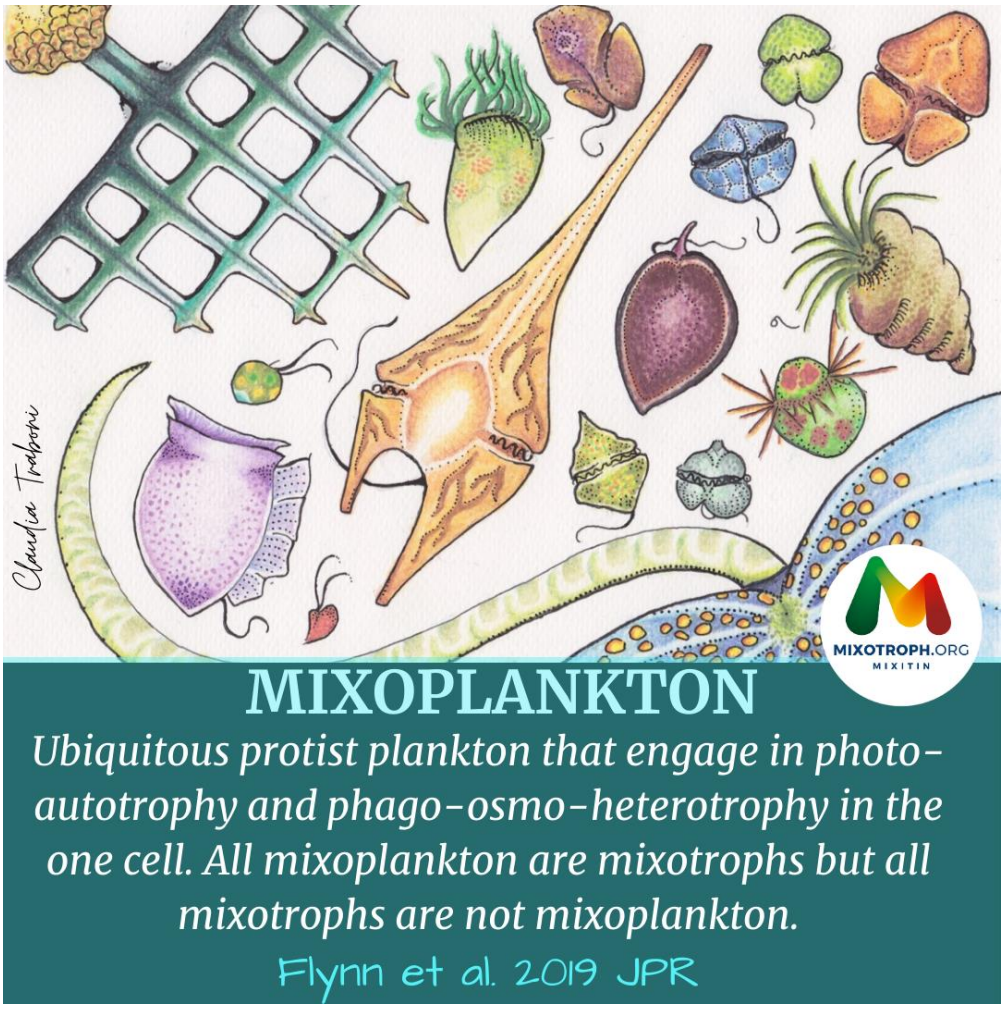
Precautions for mixoplankton: Functional type allocation within mixoplankton is an on-going activity. Rather few species have yet been well documented with respect to their photo- versus phago- trophic activities. This situation is expected to be improved as research continues over the next decade.

The mixoplankton functional type (MFT) classification was developed by Mitra *et al.* (2016). An updated version, with revised MFT names and with examples for harmful algal bloom species, is given in **Fig. 7.1**.

Mixoplankton functional type identifications for field samples were developed originally in support of the works of Leles *et al.* (2017, 2019). All the marine protist species that haMFTs have been incorporated within the World Register of Marine Species database (WoRMS documents Leles *et al.* 2017, 2019 as the primary data source). In order to access the MFT information from WoRMS, check the “attributes” tab for each species. Various coauthors from the Leles *et al.* (2017, 2019) and Faure *et al.* (2019) publications are currently (2021) involved in creating the first ever fully comprehensive database of mixoplankton species.

Within MixITiN these databases were used in Schneider *et al.* (2020) to allocate functional types to North Sea plankton. The assumption made in those works was that the genus identification could be used to allocate the trophic status. Subsequent analysis (Richardson, Mitra, Flynn & Widdicombe in preparation) has shown this is not a robust assumption; species of the same genus can be members of different protist functional types.

Information concerning the identification of MFTs will be updated over the coming years as more data come to light.



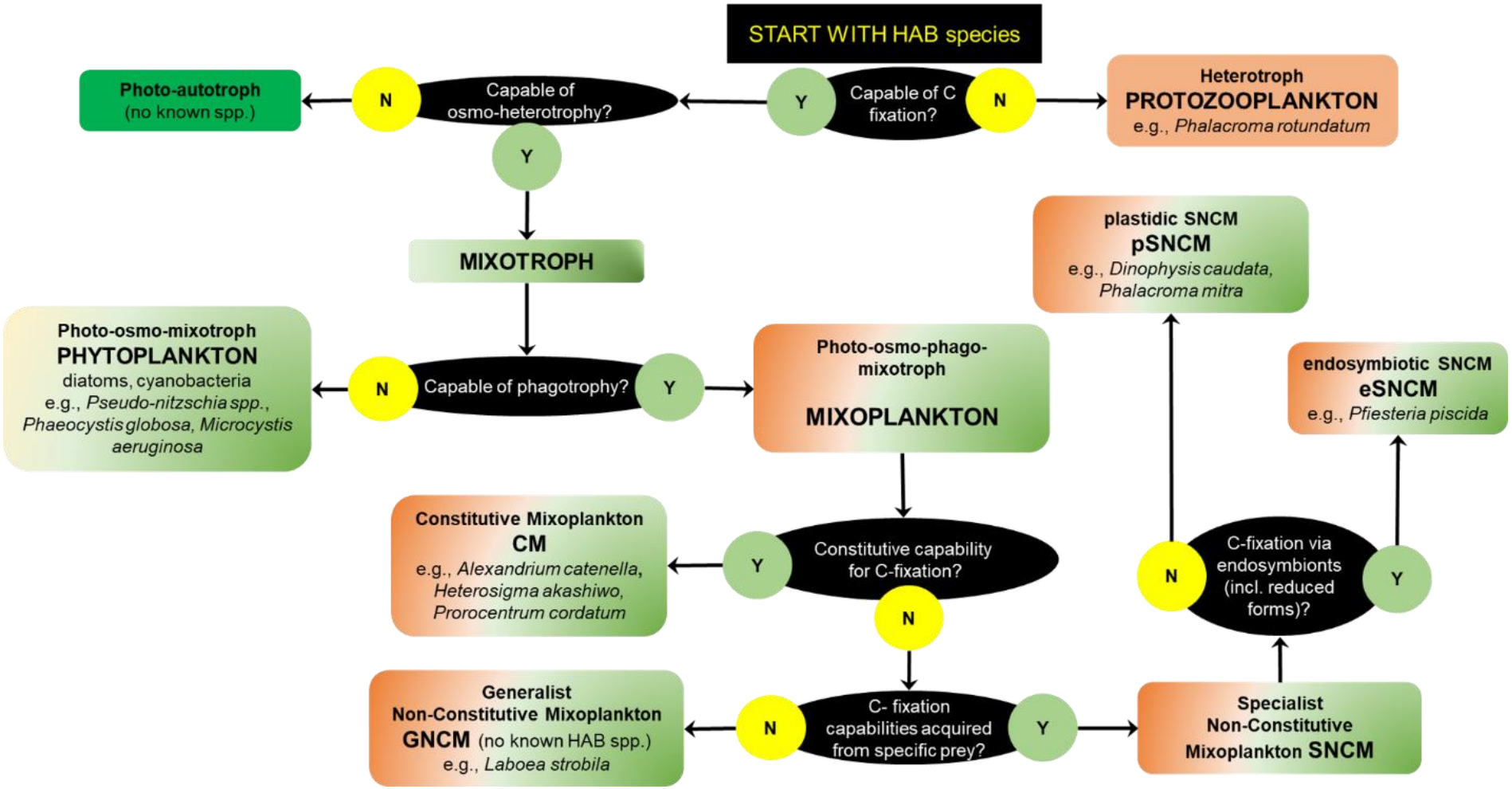


Fig. 7.1 | Functional group classification key for Harmful Algal Bloom (HAB) species developed from the protist functional group key in Mitra *et al.* (2016) with example species from the IOC-UNESCO HABs list aligned to functional groups according to the Mixoplankton Database (Mitra *et al.* in prep). Reproduced from Mitra & Flynn (2021).

8 Future perspectives; needs for progressing mixoplankton field-based research

8.1 Overarching challenges

Since we now recognize the importance of different kinds of mixoplankton in pelagic food webs in global oceans from the poles to the temperate and tropical waters (Leles et al. 2017, 2019; Faure et al. 2019), there is a clear need to develop techniques to measure *in situ* grazing and photosynthesis rates by mixoplankton. In some cases, single cell techniques can be applied, but these are often laborious and require specially trained scientists. Recent progress show that molecular techniques can be applied at least to give some indication of the presence of species in different environments, but these techniques are still only qualitative.

8.2 Constitutive Mixoplankton (CM)

The main challenges when it comes to the CM species are clearly related to the fact that these organisms do not always feed. Many CM species require light to feed, while others do not. Some CM do not feed, or feed very little when nutrient concentrations are high, while others feed irrespective of the nutrient availability (Stoecker *et al*. 2017 and references therein). And feeding may be confined to ingestion of just a very few cells per day, which may not be readily detected during incubation experiments (e.g., Zubkov & Tarran 2018). Thus, we lack general techniques that allow us to judge which CM in certain water masses are predominantly mixoplanktonic at any particularly point in time.

8.3 Generalist Non-Constitutive Mixoplankton (GNCM)

GNCM are mainly represented by aloricate ciliate species. These organisms are well known to be particularly fragile, thus measurements of their abundance risks misinterpretation in field studies due to the deployment of inappropriate sampling methods. Additionally, they are not morphologically distinguishable from purely heterotrophic species unless accurate examination is conducted through epifluorescence microscopy. Physiological rates are only known for few species under restricted laboratory conditions (Schoener & McManus 2017; Maselli *et al.* 2020), though it seems evident that the acquired ability to photosynthesise can give GNCM a competitive advantage over pure heterotrophic predators which is of particular relevance when prey concentrations are low (Schoener & McManus 2017). Acquired phototrophy also enables GNCM ciliates to better withstand periods of prey deprivation as compared to heterotrophic ciliate species (Stoecker *et al.* 1988; Schoener & McManus 2012) so sustaining higher trophic levels when the total biomass of the planktonic population is relatively low. Thus, GNCM activity appears relevant for the understanding of marine food webs and nutrient cycling, suggesting the need for it to be incorporated into ecosystem models of acquatic environments. To do so requires better and more routine field monitoring.

8.4 Specialist Non-Constitutive Mixoplankton (SNCM)

Work on the eSNCM, such as radiolaria and forams, presents particular challenges, such as small amount of available material, and the restriction of only studying their physiology in the field. Typically, these organisms are sampled and studied in “rough-and-ready” conditions. Improvements in sustaining the cells would greatly help physiological studies.

In contrast, the two pSNCM species - *Mesodinium* and Dinophysis – have received a lot of attention. Lessons learnt from these studies could help guide studies of other SNCM fieldwork.

8.5 Mixoplankton within the microplankton – the role of autonomous monitoring

Autonomous approaches are increasingly being developed to track essential ocean variables (Batten *et al.* 2019; Whitt *et al.* 2020). Other than the usual suite of chemico-physical properties, the main (if not the only) parameter measured for plankton remains chlorophyll, perhaps now with the addition of fluorometric measurements of photosystem health. Traditionally synonymous with “phytoplankton” and “primary production”, in mature ecosystems where mixoplankton can dominate, such pigment signatures can and must be interpreted in other or additional ways.

Ultimately, as for all plankton, we will only have a true picture of mixoplankton temporal and spatial distributions through deployment of autonomous instruments, equipped with artificial-intelligence supported screening methods for identifiable cell abundance and biomass. While that technology remains some way off, the first step, recognizing that these organisms actually exist as important components of the plankton and can in large measure be studied using coupled versions of traditional protocols for phytoplankton + protozooplankton, has been vital. After 100 or so years of dividing microplankton between phytoplankton and protozooplankton, mixoplankton are at last receiving the attention they deserve by mainstream marine science.

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