

Critical Review

Importance of integrating mixoplankton into marine ecosystem policy and management—Examples from the Marine Strategy Framework Directive

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Abstract

Marine plankton capable of photosynthesis and predation (“mixoplankton”) comprise up to 50% of protist plankton and include many harmful species. However, marine environmental management policies, including the European Union Marine Strategy Framework Directive (MSFD) and the USEPA, assume a strict dichotomy between autotrophic phytoplankton and heterotrophic zooplankton. Mixoplankton often differ significantly from these two categories in their response to environmental pressures and affect the marine environment in ways we are only beginning to understand. While the management policies may conceptually provide scope for incorporating mixoplankton, such action is rarely implemented. We suggest that the effectiveness of monitoring and management programs could benefit from explicit implementations regarding the ecological roles and impact of mixoplankton. Taking the MSFD as an example of marine management guidelines, we propose appropriate methods to explicitly include mixoplankton in monitoring and marine management. *Integr Environ Assess Manag* 2024;20:1366–1383. © 2024 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

KEYWORDS: Coastal and marine management; HAB risk assessment; Marine Strategy Framework Directive; Mixoplankton; Plankton monitoring guidelines

INTRODUCTION

The importance of monitoring plankton production and biodiversity is globally recognized for maintaining and achieving good environmental conditions in marine habitats (Batten et al., 2019; Bedford et al., 2018; Bueno et al., 2017; Gowen et al., 2011; Lipssewers & Spilling, 2018; López-Jurado et al., 2015; McCormick & Cairns, 1994; McQuatters-Gollop et al., 2015; Scherer et al., 2016; Smayda, 1998; Strong et al., 2015; Varkitzi et al., 2018). Traditionally, coastal surveys report and classify plankton according to the relatively simple dichotomic view of the marine food web structure based on the photoautotrophic “phytoplankton” and their heterotrophic grazers, the “zooplankton” (Bresnan et al., 2015; Lima-Mendez et al., 2015; López-Jurado

et al., 2015; McQuatters-Gollop et al., 2015; Scherer et al., 2016; Strong et al., 2015). Examples of marine environmental management policies assuming this functional dichotomy are the European Union (EU) Marine Strategy Framework Directive (MSFD) and the USEPA.

However, many, if not most, of the “phytoplankton” and “zooplankton” protists are now recognized as being capable of both photoautotrophy and phagoheterotrophy, with these processes often taking place simultaneously and sometimes even depending on each other (Flynn et al., 2013; Mitra et al., 2016; Stoecker et al., 2009). These organisms are collectively termed “mixoplankton” (Flynn et al., 2019). The mixoplankton trophic mode is a broad spectrum between phytoplankton and zooplankton. Combined photoautotrophic and phagoheterotrophic growth in mixoplankton redraws the conceptual framework of marine plankton ecology. Mixoplankton increase the trophic levels of their grazers compared to strict phytoplankton while competing with them for resources, thus influencing the trophic structure of food webs and the resulting particle flux in the ocean. Furthermore, many

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marine ecosystem services such as harvestable fish biomass, climate regulation, and water quality maintenance are heavily impacted by plankton species (Costanza et al., 1998; Hays et al., 2005; Ryther, 1969) that are now defined as mixoplankton (Leles et al., 2017, 2019).

While strict photoautotrophic organisms such as diatoms and cyanobacteria can also form nuisance blooms, most harmful algae bloom (HAB) events in the marine environment are caused by mixoplankton (Blauw et al., 2006; Glibert, 2017), for example, *Alexandrium* spp., *Phaeocystis* spp., *Dinophysis* spp., and *Karenia* spp.). Mixoplankton HABs are often triggered by phagotrophic activity (Díaz & Reguera, 2023; Park et al., 2006). These outbreaks have caused major hazardous effects on ecosystems, local economies, and public health (Anderson et al., 2001; Davidson et al., 2014; Erdner et al., 2008; Hoagland & Scatista, 2006).

On a more positive note, photophagotrophy may improve stoichiometric food quality and promote nutrient transfer to higher trophic levels, especially under inorganic nutrient shortages, with beneficial effects on trophic transfer efficiency (Balzer et al., 2023; Ptacnik et al., 2004; Traboni et al., 2021). The dual contribution to trophic transfer in food webs and the detrimental effect on ecosystems and public health are the two main reasons why mixoplankton should be treated separately from phytoplankton and zooplankton in marine management.

Our search for literature on the platform Scopus for publications from 1970 to 2021 using the keywords “marine,” “plankton*,” “mixotroph*,” and “toxic*” revealed a growing body of scientific research that recognizes the importance of mixotrophic activity in plankton, especially in toxic species (Figure 1). Meanwhile, neither a targeted monitoring plan for mixoplankton nor routine techniques to

detect photophagotrophy have been implemented. Indicators that are valid proxies of photoautotrophic metabolism (further discussed in the following sections) prove inadequate to foresee the proliferation of mixoplankton. Mixoplankton are currently identified based on taxonomic records rather than their trophic mode (Abad et al., 2016; Beaugrand, 2005; HELCOM, 2017; McQuatters-Gollop et al., 2009; Paerl et al., 2003).

For instance, several mixoplankton species are addressed by the Bathing Water Directive (European Commission, 2003) and World Health Organization guidelines (World Health Organization, 2018) due to the detrimental effect that these species can have on public health when causing HABs. Aside from certain HAB species, information on the diversity in trophic modes through taxonomy remains scarce (see data sets by Schneider et al. [2020] and Mitra et al. [2023]). As a result, species are often not recognized as mixoplankton.

Since 2008, the MSFD has been the central EU directive for marine ecosystem assessment and management (European Commission, 2008). In the MSFD, 11 descriptors (abbreviated as “D,” Figure 2) define the criteria to assess the status of the marine environment, providing a robust and standardized qualitative description (European Environment Agency, 2014).

Phytoplankton and zooplankton are included in two main descriptors: Biological Diversity (D1) and Food Webs (D4). In addition, Eutrophication (D5) includes references to (harmful) phytoplankton. However, there are criteria associated with other descriptors that can relate to planktonic protists (Figure 2 and Table 1).

Furthermore, four Regional Sea Conventions (RSCs) for countries bordering each of the seas in the EU (Figure 2B) aim to harmonize criteria regionally under the MSFD. Within

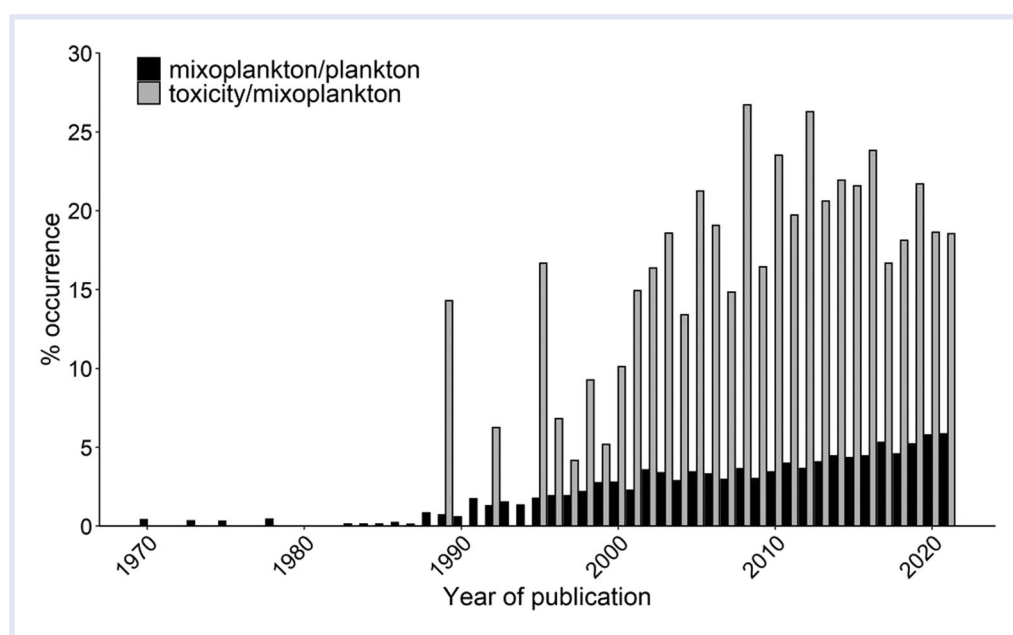


FIGURE 1 Proportion of scientific publications on plankton ecophysiology describing mixotrophic plankton from 1970 until 2021. Black bars indicate the increasing acknowledgment of mixoplankton throughout plankton literature. Gray bars reflect the trend of mixoplankton species involved in toxic outcomes

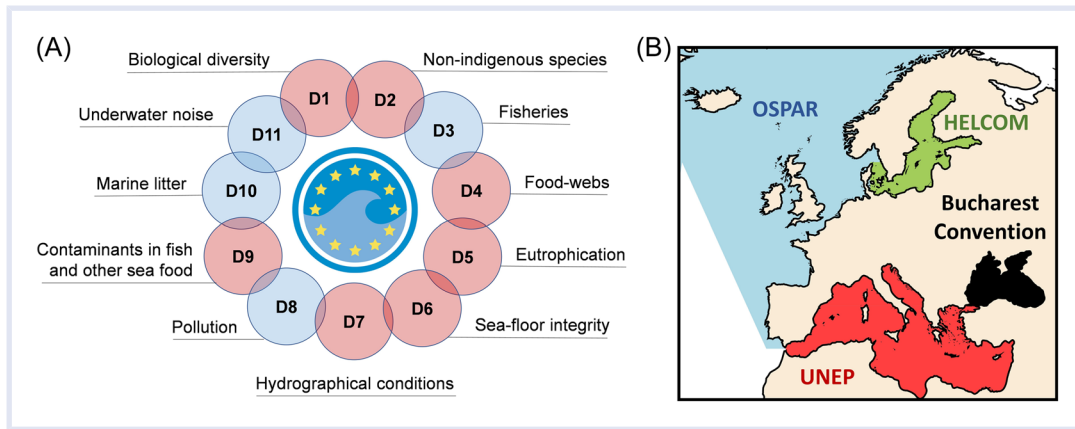


FIGURE 2 (A) Schematic representation of descriptors used within the MSFD structure for the achievement of “good environmental status” by 2020. The descriptors highlighted in red are expected to interface with the mixoplankton paradigm and their implementation on a national level might thus warrant revision. (B) European sea regions identified within the Regional Sea Conventions, with four different commissions responsible for the implementation of the MSFD: blue = OSPAR, green = HELCOM, red = UNEP (Mediterranean Sea), black = Bucharest Convention. MSFD, Marine Strategy Framework Directive

each RSC, the different member states operate for the execution of the MSFD and ensure that the different stages of the MSFD implementation are reviewed every six years.

Recent revisions of potential indicators for the MSFD only marginally mentioned mixotrophy (Bedford et al., 2018; Caroppo et al., 2013; Caruso et al., 2016; Gowen et al., 2011; Jaschinski et al., 2015; López-Jurado et al., 2015; McQuatters-Gollop et al., 2015; Varkitzi et al., 2018).

Literature that acknowledges the importance of considering mixoplankton activity in coastal management (Davidson et al., 2014; Lehtinen et al., 2016) seldom distinguishes between mixoplankton functional types nor suggests methodological approaches to implement changes in monitoring this group.

The goal of this article is to highlight the importance of including mixoplankton as a separate functional group in policy, management, and monitoring. The section

TABLE 1 European Union Marine Strategy Framework Directive descriptors and the criteria potentially associated with planktonic protists' abundance, diversity, and activity

Descriptors (D)	Criteria (and subcriteria)
1 Biodiversity	6. Habitat condition (1) Condition of the typical species and communities (2) Relative abundance and/or biomass (3) Physical, hydrological, and chemical conditions 7. (1) Ecosystem structure
2 Non-indigenous species (NIS)	1. (1) Trends in abundance of NIS 2. Environmental impact of NIS 2. (1) Ratio between invasive NIS and native species
4 Food webs	3. (1) Abundance trends of functionally important selected groups and/or species
5 Eutrophication	2. Direct effects of nutrient enrichment (1) Chlorophyll concentration (2) Water transparency (4) Shift in floristic species composition
6 Sea-floor integrity	6.1.2 Extent of seabed affected
7 Hydrographical conditions	2. (2) Changes in habitats, in particular, the functions provided by it
9 Contaminants in fish and other seafood	1. Levels, number, and frequency of contaminants (1) Levels of contaminants in seafood (2) Frequency of levels exceeded in seafood

“Mixoplankton differs from other functional groups in trophic functioning” illustrates the ecophysiological traits specific to mixoplankton and how they affect food web processes. The possible ecological and economic impact of mixoplankton is described in the section “Economic impacts of mixoplankton relevant to coastal resources and management,” while “Methodological challenges and suggestions to improve mixoplankton monitoring” showcases which anthropogenic pressures affect mixoplankton growth (including raising the potential of mixoplankton HABs) and their ecological impacts. The Conclusion provides an overview of the methodological limitations that have hampered the inclusion of mixoplankton in monitoring programs and suggests how to implement such methods. These suggestions are based on the European MSFD, but they have universal applications in marine and coastal water management.

MIXOPLANKTON DIFFERS FROM OTHER FUNCTIONAL GROUPS IN TROPHIC FUNCTIONING

Among mixoplankton, two major groups are discerned, termed “constitutive” and “nonconstitutive,” which greatly differ in their metabolic strategies. Nonconstitutive mixoplankton (NCM) acquire phototrophy from ingested prey and thus require a continuous supply of phototrophic prey. Constitutive mixoplankton (CM) have their own chloroplasts and occasionally resort to predation on bacteria and other protists as a source of nitrogen, phosphorous, or both (Brutemark & Granéli, 2011; Edwards et al., 2023; Skovgaard, 1996; Stoecker, 1998), particularly when inorganic nutrient levels are suboptimal (Millette et al., 2017; Smalley et al., 2003). This partly suggests a species- or genus-specific tolerance to resource limitation among mixoplankton. Mixoplankton are therefore less affected by light and/or inorganic nutrient limitation compared to autotrophic phytoplankton and less affected by prey availability than heterotrophic protozooplankton (Edwards et al., 2023; Péquin et al., 2022; Princiotta et al., 2023; Ward, 2019). Thus, mixoplankton can be expected to be stoichiometrically more stable than phytoplankton under fluctuating inorganic nutrient ratios as they might be able to buffer shortages of inorganic nutrients with phagotrophy. This can be an advantage in coping with the selective riverine input of nitrogen or phosphate deriving from agriculture and/or aquaculture (Balzer et al., 2023; Seitzinger et al., 2010) and a strategy to overcome nutrient depletion in oligotrophic regions (Hartmann et al., 2012).

In fact, experimental evidence highlights that mixoplankton decrease the variability in seston stoichiometry when compared to nonmixotrophic food webs mostly dominated by phytoplankton (Moorthi et al., 2017). Some species promote the growth and reproduction of zooplanktonic grazers such as marine calanoid copepods and freshwater daphnids (Katechakis et al., 2005; Ptasnik et al., 2004). Furthermore, mixotrophy may enhance the transfer of biomass up the food web (Balzer et al., 2023; Stoecker et al., 2016; Traboni et al., 2021), causing the

sinking carbon flux to increase by ~35% (Cohen, 2022; Ward & Follows, 2016).

Thus, mixotrophy could increase carbon uptake from the atmosphere and counteract ocean acidification. Hence, excluding mixoplankton from trophic assessments may lead to biased and only approximated estimates of phenological trends, competition patterns, and nutrient fluxes (Figure 3).

As mixoplankton are still understudied and undersampled, we are still only beginning to understand their response to climate change and thus their role in it. Ocean acidification may reduce photosynthetic activity in some mixoplankton species, making them more heterotrophic (Xu et al., 2023). Mixoplankton with a fast response to environmental factors tend to be more autotrophic than species with a slower response (Archibald et al., 2023). Recent modeling results suggest that warming shifts mixotrophs from carbon sinks to carbon sources and that this could make mixotrophs critical factors in the response of ecosystems' functioning toward climate change (Wieczynski et al., 2023).

A significant fraction of mixoplankton species produces toxins, leading to HABs (Burkholder et al., 2008) having an impact on public health and the blue economy. This may happen when mixoplankton experience the selective nutrient limitation mentioned above, but also prey availability can influence mixoplankton toxicity (Gao et al., 2017; Hernández-Urcera et al., 2018). For example, the CM *Karlodinium veneficum* has a higher toxin content when actively feeding (Adolf et al., 2009).

Toxicity enables mixoplankton to feed on a wider range of prey than may be expected for their cell size (Tillmann, 2003). For example, swarms of toxic mixoplankton *Karlodinium* spp. have been observed in field samples in Denmark to paralyze, kill, and feed on copepods hundreds of times larger than themselves (Berge et al., 2012). Thus, at high cell abundances, mixoplankton can gang up on competitors and predators and exercise decidedly negative control on higher trophic levels. In this way, HAB events and trophic impacts may go hand in hand.

Consistent with such empirical observations, model simulations conducted with explicit inclusion of mixoplankton have shown that they grow and change the surrounding environment in ways different from strict autotrophs and heterotrophs regarding, for instance, nutrient uptake, productivity, and carbon flow (Anschütz & Flynn, 2020; Ghyoot et al., 2017; Hammer & Pitchford, 2005; Mitra et al., 2014). There are severe economic issues arising from the activity of mixoplankton that would require a clear distinction of these organisms as a separate trophic mode from their strictly photoautotrophic counterparts in monitoring programs and marine management.

ECONOMIC IMPACTS OF MIXOPLANKTON RELEVANT TO COASTAL RESOURCES AND MANAGEMENT

Between 1987 and 2000, US and European coastlines were impacted by HABs, causing losses of several million dollars in different sectors, mostly tourism, fisheries, and public health

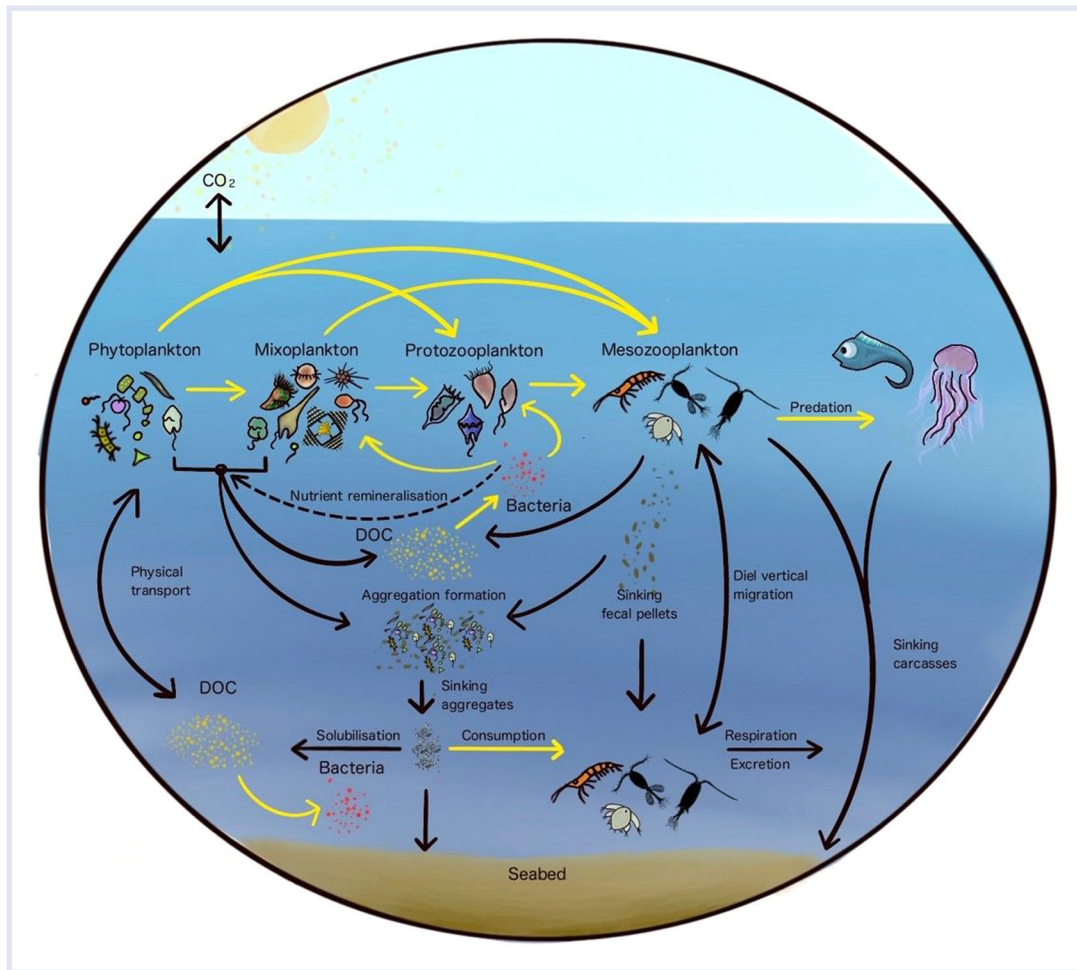


FIGURE 3 Schematic representation of the biological C pump including phyto-, mixo-, and protozooplankton as protistan representatives and mesozooplankton as direct metazoan consumers. The yellow lines indicate trophic interactions, whereas the black lines indicate the other physicochemical processes. Modified with permission from the *Annual Review of Marine Science*, Volume 9 © 2017 by Annual Reviews, <http://www.annualreviews.org>

(Davidson et al., 2014) (Figure 4 and Table 1). In most reported cases, HAB events led to the closure of shellfish fisheries and the price of shellfish sharply increased because of a drastic cut in seafood supply (Davidson et al., 2014). In Spain, Denmark, and Norway, harmful bloom outbreaks led to the establishment of monitoring programs due to public health and industry loss (Anderson et al., 2001).

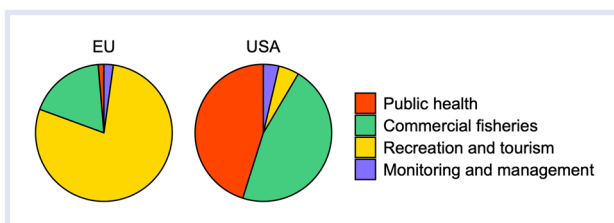


FIGURE 4 Averaged contribution of economic effects of harmful algal bloom events on several sectors in Europe and the United States between 1989 and 2000. Credit: Hoagland and Scatasta (2006). Percentages calculated are relative to total annual losses of \$813 M for the EU and \$82 M for the United States (monetary estimates were converted into 2005 dollars). Different methodologies were applied in the computation of the presented economic loss; therefore, quantitative comparisons should be considered with caution. EU, European Union

Table 2 shows a selected list of mixoplankton HAB events recorded in European marine waters. Norway, in particular, was severely affected in the late 1990s as massive salmon kills were reported upon blooms of (now recognized as mixoplankton) *Chrysochromulina* spp. and *Prymnesium* spp. for a total annual loss of US \$3 M (Anderson et al., 2001). In a recent Norwegian HAB event (May 2019), caused by *Chrysochromulina leadbeateri*, the estimated salmon loss accounted for US \$69 M, with over 10 000 dead fish and an economic drop in the fish market (www.fiskeridir.no/English, 2019). It is noteworthy that the biomass in the latest reported *Chrysochromulina* bloom is one order of magnitude higher than the one registered in 1995.

Since the observation of HAB event effects, regular toxin bioassays and nutrient and Chl_a analyses are routinely performed in most of the countries at risk and “phytoplankton” monitoring is being conducted to aid in predictions of harmful events. However, categorizing these organisms as phytoplankton is a mistake since mixoplankton respond to anthropogenic pressures in unique ways (see section “Methodological challenges and suggestions to improve mixoplankton monitoring”). Although mixoplankton HABs

TABLE 2 A selected list of HAB events caused by mixoplankton species that were reported in different EU countries

Year	Country	Mixoplankton species	Cells/L	Shellfish vector	Toxin/syndrome	Impact
1994	Norway	<i>Prymnesium</i> spp.	2×10^6			Threatened fish farms cleared the area.
1995	Norway	<i>Chrysochromulina</i> spp.	5×10^6			Gill damage and osmoregulatory problems.
1998	France	<i>Alexandrium tamarense</i>	90,000	<i>Ruditapes decussatus</i> , <i>Mytilus</i> sp.	STX/PSP	The lagoon was closed to harvesting shellfish.
1989	The Netherlands	<i>Gyrodinium aureolum</i>	1900	<i>Mytilus</i> sp.	DSP	Closure of mussel production.
1999	Germany	<i>Prorocentrum triestinum</i>				High biomass.
2001	Denmark	<i>Dinophysis acuminata</i>	1300	<i>Mytilus edulis</i>	OAV/DSP	Harvest was prohibited.
2006	Italy	<i>Alexandrium pacificum</i>	2240	<i>Mytilus galloprovincialis</i>	STX/PSP	Water discoloration—visual nuisance for locals.
2010	Slovenia	<i>Dinophysis caudata</i>	330		DSP	Closure of shellfish harvesting.
2014	Finland	<i>Alexandrium ostenfeldii</i>	6×10^6		STX-GTX-GYM	Media alert to local inhabitants and vacationers.
2014	Greece	<i>Karlodinium</i> spp.	3.3×10^6			Ichthyotoxicity and visual nuisance for locals.
2016	United Kingdom	<i>Dinophysis</i> spp.	90,274	<i>Mytilus edulis</i>	OAV/DSP	Closures enforced at multiple sites around the site.
2017	Norway	<i>Alexandrium tamarense</i>	3760	<i>Mytilus edulis</i>	STX/PSP	Harvesting was locally banned.
2018	Spain	<i>Dinophysis acuminata</i>	3080	<i>Donax trunculus</i> , <i>Mytilus galloprovincialis</i>	OAV/DSP	Compromised shellfish industry.
2019	Portugal	<i>Gymnodinium catenatum</i>	6440		PSP	Production area interdicted to bivalve harvesting.
2019	Norway	<i>Chrysochromulina leadbeateri</i>	1.5×10^7			Gill damage and osmoregulatory problems; mass salmon mortality.

Note: Related causative species and cell concentrations, bivalve vectors, and toxins analyzed, impacts on economy, and management decisions are reported when available. Data are extracted from the Harmful Algae Information System (<http://www.haedat.iode.org/index.php>).

Abbreviations: DSP, diarrhetic shellfish poisoning; EU, European Union; GTX, gonyautoxin; GYM, gymnodimines; HAB, harmful algae bloom; OA, okadaic acid; PSP, paralytic shellfish poisoning; STX, saxitoxins.

represent a significant threat to coastal economies, there are additional ecological consequences resulting from mixoplanktonic activity (see “Mixoplankton differs from other functional groups in trophic functioning” and “Economic impacts of mixoplankton relevant to coastal resources and management”). Yet, these ecological impacts have not been properly assessed mostly due to methodological limitations, leading to the incorrect identification of mixoplankton (see Conclusion).

Anthropogenic pressures on mixoplankton growth and their impacts

In general, mixoplankton dominate in mature ecosystems (Mittra et al., 2014; Schneider et al., 2020): systems that contain a complex and diverse structure in which most nutrients are bound as organics. In temperate marine systems, the spring bloom occurs in conditions of ample inorganic nutrient supply that supports plankton species with fast growth rates. In summer, low nutrient availability in combination with stratification favors mature planktonic systems with low growth rates and low dissolved inorganic-to-organic nitrogen ratios. However, several anthropogenic pressures might alter this natural seasonality and favor mixoplankton growth (Figures 5 and 6). These pressures and their impacts on mixoplankton are discussed below.

High nutrient inputs cause eutrophication (MSFD Descriptor D5) and light attenuation, which lead to decreased water quality and anoxia due to high respiration (Figures 5 and 6; European Commission, 2008). Due to better water treatment, nutrient inputs have decreased greatly. Decreasing inputs of inorganic nutrients may lead to a higher proportion of mixoplankton, as they are typical for mature systems with lower nutrient availability.

However, the nutrient composition may be more important than the overall concentration in the case of mixoplankton (Anderson et al., 2008). For example, elevated ammonium versus nitrate promotes growth and toxicity levels of some mixoplankton (Hattenrath-Lehmann & Gobler, 2015), with a very limited ability to use nitrate (García-Portela et al., 2020). Yet, a distinction in monitoring

between nitrogen sources (inorganic, organic, dissolved, and particulate) is not always made. Changes in mixoplankton N:P, and thus by inference nutrient input ratio, are also associated with increased toxicity (Granéli & Flynn, 2006; John & Flynn, 2000).

Aside from nutrient addition from rivers, a recent study found that their high metabolic plasticity may make some mixoplankton species more resistant to heavy metal pollution than strict autotrophic protists (Zhang et al., 2023).

Lastly, mixoplankton can outcompete autotrophs in estuaries (Figure 4), with low light and very low dissolved inorganic-to-organic nitrogen ratios (Balzer et al., 2023; Gobler et al., 2011; Millette et al., 2017). In some cases, mixoplankton may balance the effects of changes in light and nutrient supply on the transfer efficiency of energy to higher trophic levels (Katechakis et al., 2005; Traboni et al., 2021).

Aquaculture (MSFD Descriptor D3) often simultaneously induces inorganic and organic eutrophication and light limitation (Figure 5). Mixoplankton may take advantage of these conditions for the reasons mentioned above and give rise to HABs and associated economic effects in this sector (Glibert et al., 2005).

Increasing temperature and stratification, due to global and local warming (but also due to anthropogenic modifications of hydrodynamics on local scales, MSFD Descriptor D7), enhance communal respiration over gross primary production, decreasing the relative effectiveness of carbon sequestration from the atmosphere (Regaudie-De-Gioux & Duarte, 2012). On an ecological level, this can cause a simultaneous shift from a diatom-based community to dinoflagellates (of which most phototrophic forms are mixoplankton) (Gomes et al., 2018; Klais et al., 2011) and most likely also to other smaller mixoplankton flagellates that are usually not monitored. Mixoplankton are, indeed, potentially favored in conditions of stratification of the water column (Edwards et al., 2023) (Figure 5), and their toxicity may be related to biotic and abiotic factors (prey, dissolved nutrients (Burkholder et al., 2008). These factors can be altered indirectly by human activities conducted on land and at sea, such as building structures for tourism and commercial interest (harbor and wave-breakers, etc.).

Compromising sea-floor integrity (MSFD Descriptor D6) by dredging or dumping works can lead to the resuspension of resting stages of mixoplankton types into the water column (Figures 5 and 6), leading them to reappear and persist in seasons in which they are not expected (Balkis et al., 2016; Nehring, 1996). Germination events from mixoplanktonic dinoflagellate cysts outnumber those from their heterotrophic counterparts throughout the year (Balkis et al., 2016). The HAB-forming mixoplankton (e.g., *Alexandrium tamarense*, *Protoceratium reticulatum*, *Heterocapsa triquetra*, *Gymnodinium catenatum*, *Scrippsiella trochoidea*, and *Lyngulodinium polyedrum*) appear to be most abundant and persistent in sediments and showed the highest experimental germination success (Balkis et al., 2016). In some instances, the resting stages of toxic species may contain a higher toxin load than

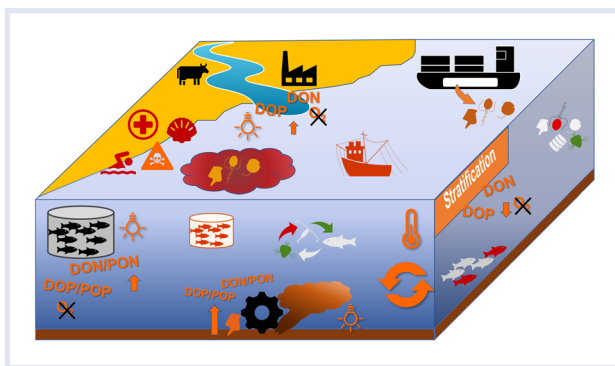


FIGURE 5 Schematic of the effects of anthropogenic activities upon marine ecology and mixoplankton and their subsequent effects on human health and economy. Black = anthropogenic pressures, orange = bio/ecological effects, and red and green = eco-human health and socioeconomic impacts. See also Figure 6 for further description of linkages to mixoplankton

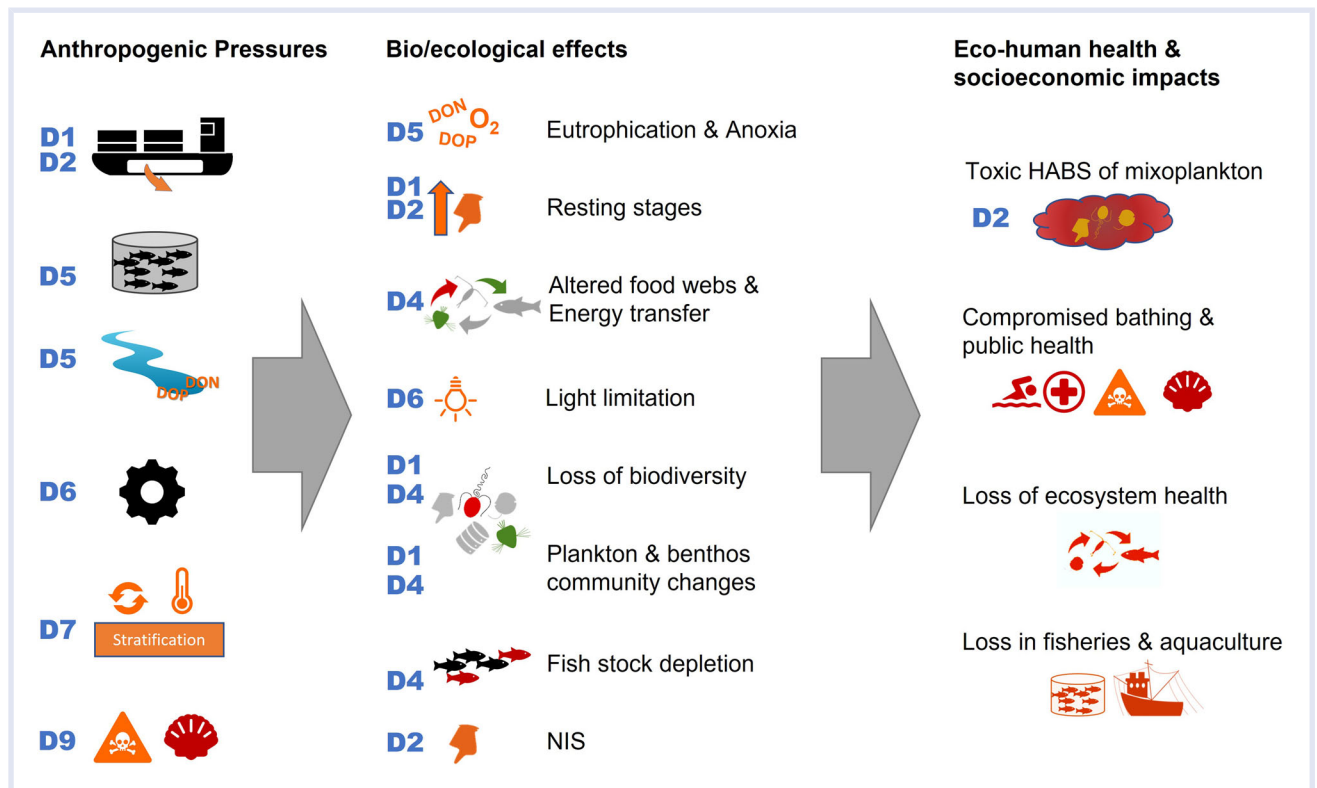


FIGURE 6 Linkage between anthropogenic pressures and mixoplankton, related bioecological effects, and eventually eco-human health and socioeconomic impacts. The combination of the letter “D” and a number refers to the MSFD descriptor that applies to the respective point. Icons match those in the schematic shown in Figure 5; icons in black symbolize pressures, icons in orange symbolize the effects, and icons in red and green symbolize possible impacts. The chart does not feature all possible relations between mixoplankton and their environment, only those considered most relevant for achieving good environmental status. NIS - non-indigenous species. MSFD, Marine Strategy Framework Directive

the vegetative forms (Oshima et al., 1992). In addition, dredging and dumping works can cause resuspension of sedimented nutrients but also higher turbidity at the same time and modify the local hydrographic features (D7). These are conditions that mixoplankton could grow in but are unfavorable for autotrophs due to the lack of light (Anschütz & Flynn, 2020). Similarly, mixoplankton appeared to be less affected in growth by glacial flour increasing turbidity than autotrophic microplankton (Maselli et al., 2023).

Non-indigenous species (NIS) (MSFD Descriptor D2) put ecosystems at risk of biodiversity loss and HAB formation (Hallegraeff & Gollasch, 2006). Katsanevakis et al. (2014) recognized that the introduction of NIS can play a role in HAB events. Due to dual metabolism and flexible physiology, allochthonous mixoplankton can outcompete strict auto- and heterotrophs in some instances. The spread of toxic *Gymnodium catenatum* (Hallegraeff & Gollasch, 2006) and of “green *Noctiluca*” (Harrison et al., 2011) are examples.

METHODOLOGICAL CHALLENGES AND SUGGESTIONS TO IMPROVE MIXOPLANKTON MONITORING

As mentioned above, the MSFD comprises 11 descriptors, each defined by several criteria and indicators. While criteria are targets or thresholds set to evaluate the

necessary measures to achieve management goals, indicators are qualitative and quantitative elements describing a biological or ecological system that provide information on the state of an ecosystem (Link, 2005). Table 1 summarizes the criteria that can be associated with planktonic protists and processes deriving from their activity. We identified these in seven out of the 11 descriptors in the MSFD.

According to reports on the implementation of the MSFD (OSPAR, 2017), plankton are now increasingly included in monitoring by the RSC. On a regional scale, some good examples of indicators that enable a more thorough assessment of the planktonic community already exist, such as the ratio between the abundance of diatoms (pure phototrophs) and dinoflagellates (phototrophic forms and mixoplankton) adopted by EU commissions HELCOM and OSPAR (Figure 1) as an indicator of good environmental status. While this indicator's purpose is not to capture functional diversity in plankton, it distinguishes between exclusively phototrophic organisms (diatoms) and those with a variety of functional types (dinoflagellates). However, this distinction is too general due to the diversity in functional types that can be found among dinoflagellates and still, very little consideration is given to mixoplankton.

As illustrated in the previous sections, our understanding of mixoplankton has developed substantially (Flynn et al., 2019;

Mitra et al., 2014; Stoecker et al., 2016) after the MSFD came into effect in 2008 (European Commission, 2008). As a result, conceptual and methodological approaches to plankton ecology warrant some reconsideration. Therefore, we thoroughly address the point-to-point methodological limitations that impede the integration of mixoplankton into monitoring programs and propose a series of actions to better include these organisms. The ultimate goal is a better assessment of the environmental state and highlighting how the MSFD could benefit from their integration as a criterion in ecological descriptors (Table 3).

In general terms, there is a need to improve the detection, classification of trophic mode, and enumeration of a wider range of plankton species with a higher temporal resolution, merging multiple approaches. This will require research and monetary efforts that will likely lead to the development of robust autonomous methodologies in the long term.

Identification of species

Currently, unicellular plankton communities are monitored by a variety of technical approaches, with microscope counting and identification traditionally being the most common and inexpensive methods. More recently, the use of molecular identification as a tool in taxonomy has been revealed as a global approach in marine sciences allowing the identification of still-unknown or less-represented plankton species (de Vargas et al., 2015). Both morphological and molecular approaches serve to monitor biodiversity (D1), the potential introduction of NIS (D2), the abundance of selected functional groups (D4, criteria 3.1), and eventually, the shift in the composition of bloom-forming species (D5, criteria 4). However, recognition is often limited to the genus level or even higher taxonomic categories. Hence, to identify the abundance of mixoplankton, there is a need to define plankton at the species level and continuously update a comprehensive list of species that now, and in the future, are recognized as mixoplankton and also the type of mixoplankton (CM or NCM), for example, as done in the data sets by Schneider et al. (2020) and Mitra et al. (2023).

Mixoplankton comprise delicate ciliates, several athecate (unarmoured) dinoflagellates, and other fragile flagellates and rhizarians (Stoecker et al., 2016). As a consequence, there are still gaps when accounting for the relative contribution of plankton groups in plankton monitoring. In fact, most of the emphasis in the above-mentioned research is on dinoflagellates. There are other important mixoplankton taxa, including the similarly sized raphidophytes (relatively large species and thus easily monitored) but also the smaller mixotrophic flagellates such as the haptophytes *Prymnesium* spp. and *Chrysochromulina* spp., which are common but often ignored until they cause a bloom event (Edvarsen & Paasche, 1998; Jones et al., 1993). For small fragile and/or cryptic flagellate and NCM ciliate species, sampling and microscopic identification may be difficult and are neither routinely done, nor is their physiology easy to measure

(Anderson et al., 2017). In addition, even if not toxic, high concentrations of the kleptoplastidic ciliate *Mesodinium rubrum* are the precursor to the proliferation of its predator, the toxic *Dinophysis* spp., which has species-specific dietary and plastid requirements (Reguera et al., 2012), and the dinoflagellates' performance has been linked to prey availability (Díaz & Reguera, 2023). In addition, the ingestion of their prey *Mesodinium* affects the fluorescence of *Dinophysis* and thus their detectability by imaging-in-flow cytometers (Houliez et al., 2023). Therefore, an accurate assessment of potential prey–predator pairs like *Mesodinium* and *Dinophysis* may help in forecasting undesired HAB events, which eventually affect food webs (D4) and cause the contamination of fish and other seafood (D9). Methods for automated detection such as imaging-in-flow cytometers need to be corrected for the variability that comes with mixotrophic traits as even small abundances of certain HAB species (e.g., *Dinophysis* spp.) can cause damage to public health and economies. Due to the above-mentioned reasons, we propose the following implementations:

- *Collection of water samples* using Niskin bottle samplers, rather than net sampling. This may help to overcome challenges derived from the underestimation of fragile and less-represented organisms and/or groups, hence contributing to better defining D1 and D2.
- *Use of molecular (DNA metabarcoding) techniques*: Metabarcoding is the analysis of a short region of one to a few genes (DNA barcodes). This allows for the analysis of large volumes of DNA samples and therefore yields information on the taxonomic diversity of an ecosystem. The design of more primers (DNA barcode identification) that are more selective to known mixoplankton species and the targeting of more than one genomic region (Piredda et al., 2017) could allow amplifying with high-resolution several plankton groups at a time, enhancing both relative abundance and diversity. The use of molecular techniques for the identification of species such as eDNA sequencing are being gradually incorporated as routine tools and have been proven to be useful in identifying mixoplankton from natural seawater samples (Lapeyra Martin et al., 2022). The technique could increase the detection of many fragile mixoplankton species that are often undersampled with common techniques such as net samples (Stoecker et al., 2016) or those that are hard to distinguish through microscopic visualization alone. Metabarcoding and eDNA could thus increase the resolution of the plankton biodiversity (D1).
- *Multiple cost-effective approaches to tackle protistan diversity*. Fluorescence in situ hybridization is a technique that allows the detection of particular taxa through selective binding to a specific oligonucleotide probe. This methodology has been used to assess and quantify protistan plankton diversity (Not et al., 2007) and functional roles, and often in combination with the methods of high-performance liquid chromatography and ChemTax (Gameiro et al., 2007; Mackey et al., 1996),

TABLE 3 Recent knowledge advances on mixoplankton in relation to the MSFD descriptors that imply the monitoring of planktonic protists and how monitoring could be adapted based on such implications

Aspects requiring more resolution	Approach	Methodology	Purpose/aim	Linked MSFD descriptors
Species diversity	Microscopy, molecular tools, pigment analysis	<ul style="list-style-type: none"> Collection of entire water sample (Niskin). Design more selective DNA primers. Fluorescence in situ hybridization, Chemtax, HPLC. 	<ul style="list-style-type: none"> Optimize sampling of underrepresented/delicate species. Increase resolution in taxonomic terms to the species level. 	D1, D2, D4
Species metabolism and toxicity	Literature search, microscopy, transcriptomics	<ul style="list-style-type: none"> Literature evidence. Messenger RNA expression. 	<ul style="list-style-type: none"> Assign species to trophic categories. Identify molecular markers of phagotrophy. 	D4
Contribution of mixoplankton to ocean production	Microscopy, flow cytometry, pigment analysis	<ul style="list-style-type: none"> Pulse-amplitude-modulation fluorometry. Dilution technique. Fluorescently labeled algae, FLB. Digestive vacuole staining. 	<ul style="list-style-type: none"> Determine biomass and primary productivity. Test phagotrophy. Measure feeding rates. 	D4, D5
Early warning signals of mixoplankton growth	Stable isotope analysis	<ul style="list-style-type: none"> Stable isotope labeling (¹⁵N, ¹³C). Incubations with zooplankton. Isotope composition of amino acids. 	<ul style="list-style-type: none"> Origin of nutrient acquisition. Enrichment factor and trophic level. 	D4
Modeling mixoplankton ecophysiology	Analytical chemistry, microscopy, molecular biology	<ul style="list-style-type: none"> Measure organic N and ammonium. Collection of sediment samples. Include hydrodynamics. Monitor precursors of toxic species. 	<ul style="list-style-type: none"> Aid forecast future germination events, high nutrient inputs, turbidity, potential toxic blooms. 	D5, D6, D7, D9
Modeling mixoplankton ecophysiology	Literature search, ecological modeling	<ul style="list-style-type: none"> Consider mixoplankton as a state variable. Include toxicity factors in models. Consider anthropogenic change of hydrodynamics. Explicitly describe putative prey and various N forms. Include mixoplankton in food networks. 	<ul style="list-style-type: none"> Discriminate mixoplankton from phyto- and protozooplankton. Predict the extent and damage of mixoplankton HABs on ecosystems. Understanding how mixoplankton benefit from sediment resuspension. Better define blooming potential and trophic linkages. 	D4, D5, D6, D7, D9

Abbreviations: FLB, fluorescently labeled bacterial prey; HAB, harmful algae bloom; HPLC, high-performance liquid chromatography; MSFD, Marine Strategy Framework Directive.

thus representing a fast and cost-effective identification tool (D1, D2).

Assessment of trophic mode

This represents the most challenging step. Normally, when species are known and capable of being kept in culture, trophic modes are assigned based on the experimental observation of their “behavior” or their cellular compartments (plastids, food vacuoles). Following this, for decades, the presence of Chl_a has become synonymous with photoautotrophy (specific to phytoplankton), while the occurrence of digestive vacuoles inside the cell has been associated with phagoheterotrophy (typical of protozooplankton). Yet, only a limited array of mixoplankton species has been trophically classified. As a result, most protists are still uncertain in metabolic terms and are often misplaced in the wrong trophic category together with their congeneric species. As an example, the genus *Mesodinium* includes both mixoplanktonic (*M. rubrum*) and obligate heterotrophic ciliates (*M. pulex*). Henceforth, diversity in taxonomic terms reflects diversity in metabolism, pinpointing the importance of differentiating between species when addressing marine biogeochemistry. Hence, to identify the trophic mode in mixoplankton, it is necessary to combine methods for the identification of phototrophy and heterotrophy simultaneously. Including a general group of mixoplankton in monitoring would already be a significant step until suitable methods become available to identify the mixoplankton type (CM vs. NCM) routinely. To overcome these limitations, we propose the following:

- *Literature data gathering and update.* Collect evidence from studies and experiments regarding the metabolic nature of protists, highlighting the abiotic conditions driving the metabolic strategy, prey selectivity, and feeding behavior. This would help predict the role of these organisms in different ecosystems on geographical and seasonal scales (D4, D5). Keys for taxonomical identification could be implemented with putative trophic mode indication as done by (Schneider et al., 2020), which should be indicated together with the presence or absence of the species (enhancing resolution on biodiversity assessment [D1] and the role in food webs [D4]).
- *Transcriptomics to reveal metabolism and toxicity* using cultured organisms to identify molecular markers of phagotrophy and toxins to be applied on a broader scale on natural samples. Despite the higher cost and the high messenger RNA diversity across lineages, transcriptomic approaches have been used to identify molecular markers associated with cellular metabolism in plankton in both laboratory (Lie et al., 2018) and field studies (Labarre et al., 2020). Also, genes encoding toxin-like transcripts have been revealed by using similar RNA-based methodologies in mixoplanktonic dinoflagellates (Cooper et al., 2016). Therefore, in the absence of a detailed DNA-based taxonomic diversity, transcriptomic

data can still inform us about (a) the trophic state of the planktonic community (D4) and (b) the physiological status of the involved organisms (Zhang et al., 2019) with possible consequences on other trophic levels (D5, D9).

Quantification of primary and secondary production

On a global scale, Chl_a fluorescence is commonly used as a proxy for biomass of autotrophic plankton and primary production estimates, measured under the eutrophication descriptor (D5) (criteria 2.1). Yet, Chl_a concentration does not always directly translate to biomass production, probably also because it does not allow for a distinction between rates given by CM and strict autotrophs. Attempts to experimentally “isolate” CM from pure autotrophs have been made with food vacuole staining detected through epifluorescence microscopy and/or flow cytometry (Beisner et al., 2019; McQuatters-Gollop et al., 2015, 2017; Muylaert et al., 2006). This does not prove to be always efficient because (a) if prey components have been digested already, there is no remnant of previous feeding history; (b) if the species does not feed on the prey in its entirety (e.g., tube feeders), it is more difficult to detect intact confined structures; and (c) acidotropic staining can selectively bind to acidic organelles in the cytoplasm other than food vacuoles, biasing our interpretation (Hansen, 2011).

Even among organisms with recognized phagotrophic abilities, this technique does not discriminate between an NCM that has just ingested its prey and a strict heterotroph, limiting our capability to quantify secondary production driven by either protist functional type. Efforts have been made to discriminate mixoplankton grazing from that of pure heterotrophs with the use of rotenone, but results seem to vary depending on the species and its growth phase (Ferreira & Calbet, 2020). Grazing estimates in protists have often been obtained with the use of fluorescently labeled algae (FLA) and fluorescently labeled bacterial prey (FLB); yet, the feeding mechanism and the duration of the incubations render this technique not always representative enough to account for the entire prey spectrum accessible to protists in natural samples (Ferreira et al., 2022). A possible solution that we propose is the combination of these techniques in a methodological pipeline suited for both laboratory and field experiments, to provide temporal estimates of primary and secondary production rates.

- In vivo measurement of photosynthetic activity at a local scale using, for example, a pulse-amplitude-modulation fluorometer or fast repetition rate fluorometry would be a better approach to assess primary production, mixoplankton proliferation, and the potential prey landscape that may give rise to mixoplankton bloom supported by phagotrophy (Higo et al., 2017).
- In addition, the *dilution technique* (Landry & Hassett, 1982) allows estimation of the overall grazing by nano- and microprotists, and it can be merged with the use of FLB and FLA to more easily visualize prey cells through

epifluorescence microscopy. This combined approach would provide estimates of photosynthesis and ingestion by the protistan community, with the possibility to test different prey species and/or size classes of FLA–FLBs. Flow cytometry can also be applied in combination with microscopy to detect the signal of Chl_a, FLA–FLB, and stained food vacuoles (Anderson et al., 2017). In laboratory experiments, also fast automated enumeration can be achieved if prey and predators fall into two distinct size classes (using particle counters) or retain specific pigments (using flow cytometry).

Contribution of mixoplankton to higher trophic levels. Mixoplankton represent a food source for metazoan consumers. Yet, their nutritional contribution to zooplankton diet and C export is difficult to measure as the signal is blurred by the presence of several phytoplankton and protozooplankton in the prey landscape of their predators. Manual counting of prey species can be a challenging task to perform on natural community samples, and it would require highly developed taxonomic expertise. Hence, perhaps, the most accurate and controlled technical procedure for the estimation of bulk nutrient flow and origin, and thus useful in the study of food webs (D4), is the use of stable isotopes (Landry & Décima, 2017). Both carbon and nitrogen can be traced by tagging the nutrient source with an alternative isotopic form and inferring whether mixoplankton transfer to predators the essential nutrients acquired from the prey or produced via photosynthesis (Michener & Kaufmann, 2007).

- Hence, we suggest performing incubations with zooplankton feeding on protists with a combination of labeled ¹⁵N prey and ¹³C bicarbonate. In doing so, it would be possible to infer the origin of nutrient acquisition by protists and dietary preference by zooplankton.
- The ratio of specific amino acids labeled with ¹⁵N between trophic levels can give us an estimate of the enrichment factor, enabling us to measure the trophic position of an organism in the food web (Décima et al., 2017).

The stable isotope approach does not always fit the criteria for a viable monitoring method (see Zampoukas et al., 2013) due to the cost and time involved, the variability of the isotopic baseline, or the suitability of specific labeled compounds used to track nutrient transfer across trophic levels (Décima et al., 2017; Gutiérrez-Rodríguez et al., 2014); nevertheless, it yields a valuable set of information that can increase the resolution of trophic interactions and trophic efficiency (D4).

Consider early warning indicators for mixoplankton blooms

Abiotic changes in the environment determine the grazing or photosynthetic effort in protists and modulate toxicity in

several protistan species. Henceforth, we recommend more systematic monitoring of noncommonly sampled parameters:

- *Particulate organic nitrogen and ammonium* should be included as key indicators of the trophic regime and possible nutritional base for mixotrophic protists, along with the usual monitoring of inorganic nutrients (nitrate, nitrite, silicate, phosphate). Most protists prefer ammonium over nitrate and, in some cases, ammonium represents the only nitrogen source that can support the growth of toxic species (e.g., *Karlodinium armiger*) (Binzer et al., 2020).
- *Collecting sediment samples and analyzing the presence of mixoplankton resting stages* could represent an early warning indicator of blooms upon sediment resuspension. Sea-floor integrity (D6) may be of relevancy for mixoplankton regarding dormant cysts of HAB-forming mixoplankton species (Giannakourou et al., 2005).
- Extension of monitoring to putative prey may in some instances help detect an early sign of HAB, as happens to be the case for the *Teleaulax–Mesodinium–Dinophysis* complex (Anschütz et al., 2022).

The increase in resolution on plankton monitoring toward the above-mentioned directions needs to be accompanied by the implementation of information about conditions that favor mixotrophy in pelagic habitats. This will enhance the evaluation of the impact of anthropogenic activities on the ecosystem (D7) and the identification of early indicators for the occurrence of eutrophication (D5) and HABs (D9).

Modeling mixoplankton

Many aspects of mixoplankton ecology are still unknown, and basic research is required. The lack of information on mixoplankton identity, trophic mode, and environmental drivers of their growth is one of the reasons why most food web models still provide inaccurate production values. Thus, we propose to incorporate the following:

- Gathering experimental evidence reporting the trophic interactions and effects of mixoplankton on other trophic levels and/or functional groups must be compiled to develop predictive models of food web productivity based on observations. Update literature in terms of diversity, functional trophic mode, invasiveness, and toxin production will enable parametrization of ecosystem models of regions especially impacted by local toxic blooms.
- Relate the blooming potential of mixoplankton to *environmental factors in models* (Edwards et al., 2023). Some mixoplankton species have advantages over strict autotrophs in conditions of stratification (e.g., *Dinophysis*), while others are particularly vulnerable to turbulence (mixoplanktonic ciliates). Therefore, the inclusion of hydrodynamic conditions (D7) of sediment resuspension, causing turbidity and sudden high nutrient pulses, provides a more detailed scenario for understanding complex causal relationships, leading to better programs of

measures to control eutrophication (D5) and possible HABs (D9). Depending on the aim of predictive models, these factors may need to be included when describing plankton behavior.

- Consider the trophic mode of toxic species and monitor potential prey. Many causative species for toxic blooms (D9) are mixoplankton. While the responsible species are being monitored, the fact that they are mixotrophic is generally not considered. In the case of NCM that rely on certain prey species to proliferate, monitoring these prey species and including them in predictive models may considerably improve forecasting reliability for related HABs (D9).
- The description of mixoplankton interactions in trophic networks (D'Alelio et al., 2016) could eventually integrate the key acquired knowledge and may serve as a tool to help in forecasting future changes in the main ecosystem services related to plankton (i.e., for D4 and D7).

All the proposed methodologies will lead to a more accurate description of the planktonic community assemblage (D1) and its functioning (D4). This would provide the basis to build historical data on which to assess the likelihood of the successful introduction of mixoplankton species by human activities into regions of species that are not indigenous (NIS, D2), which is supposed to be enhanced by their physiological plasticity (Figure 3). At present, neither protist species nor plankton are considered in the list of Invasive Species of Union Concern held by the EC-related directive (European Commission, 2017). Such data series could also aid in detecting changes in the trophic community as observed in the Tagus Estuary, where the ratio of strictly autotrophic diatoms to predominantly mixotrophic cryptophytes shifted toward the cryptophytes (Brito et al., 2015). Such information, complemented with the identification of early warning indicators of mixotrophy (D5, D7), may be highly relevant to assessing food web productivity (D4) and forecasting detrimental ecological effects of toxin production (D9).

CONCLUSION

Without a doubt, integrating mixoplankton into marine management and monitoring programs poses many challenges. However, set against the backdrop of climate change, there is a need to understand the functioning of marine ecosystems in depth, and that includes the role of mixoplankton. The descriptors discussed above using the MSFD as an example contain features that pertain to mixoplankton but do not explicitly include them. Target thresholds of quantifiable biological indicators in European waters had to be achieved by 2020 through a process set over a decade earlier (European Commission, 2008). There is considerable inertia in all management programs, but we argue that the mixoplankton paradigm (Flynn et al., 2019) represents such a fundamental improvement in our understanding that its explicit inclusion in monitoring programs should occur as soon as possible.

We propose possible methodological pipelines to progressively incorporate mixoplankton into mainstream monitoring. Research efforts coupled with coordinated policy-making have the potential to safeguard future economic losses resulting from inaccurate predictions. Thus, the cost of improving monitoring techniques for mixoplankton will likely be balanced if specific measures are taken to reduce the impact that undesired mixoplankton blooms may have on the economy, public health, tourism, and society.

Given the above, we argue that it requires a more holistic view of plankton ecology than just placing all “phytoplankton” in a “box” in a model. Mixoplankton should emerge as different functional groups, revising the conceptualization of monitoring programs.

AUTHOR CONTRIBUTION

Anna-Adriana Anschütz: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. **Maira Maselli:** Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. **Claudia Traboni:** Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing—original draft; writing—review and editing. **Arjen R. Boon:** Conceptualization; supervision; writing—review and editing. **Willem Stolte:** Conceptualization; investigation; supervision; writing—original draft; writing—review and editing.


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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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