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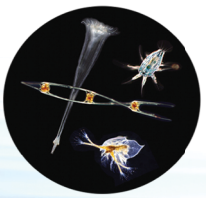

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HORIZONS

Mixotrophic protists and a new paradigm for marine ecology: where does plankton research go now?

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Many protist plankton are mixotrophs, combining phototrophy and phagotrophy. Their role in freshwater and marine ecology has emerged as a major developing feature of plankton research over recent decades. To better aid discussions, we suggest these organisms are termed "mixoplankton", as "planktonic protist organisms that express, or have potential to express, phototrophy and phagotrophy". The term "phytoplankton" then describes phototrophic organisms incapable of phagotrophy. "Protozooplankton" describes phagotrophic protists that do not engage in acquired phototrophy. The complexity of the changes to the conceptual base of the plankton trophic web caused by inclusion of mixoplanktonic activities are such that we suggest that the restructured description is termed the "mixoplankton paradigm". Implications and opportunities for revision of survey and fieldwork, of laboratory experiments and of simulation modelling are considered. The main challenges are not only with taxonomic and functional identifications, and with measuring rates of potentially competing processes within single cells, but with decades of inertia built around the traditional paradigm that assumes a separation of trophic processes between

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different organisms. In keeping with the synergistic nature of cooperative photo- and phagotrophy in mixoplankton, a comprehensive multidisciplinary approach will be required to tackle the task ahead.

KEYWORDS: mixotrophy; protist; mixoplankton; phytoplankton; protozooplankton; microbial loop; allometry

INTRODUCTION

Mixotrophy is the combination of autotrophy and heterotrophy in the same organism. The means by which this combination occurs in different organisms varies, but the form that has most fascinated scientists (and captured the public attention; Wyndham, 1951) has been that which combines photo(auto)trophy with carnivory, or as applied to protists, with phago (hetero)trophy. Long considered by most aquatic scientists to be of minor significance, or just a curiosity, we now recognize that protist plankton engaging in photo- and phago-mixotrophy are common and important members of the global plankton community (Leles *et al*., 2017, 2019; Faure *et al*., 2019).

Even though photo- and phago-mixotrophy in planktonic protists has been studied by many scientists over many decades in freshwater and marine systems (e.g. Biecheler, 1936; Blackbourn *et al*., 1973; Bird and Kalff, 1986; Estep *et al*. 1986; Laval-Peuto and Febvre, 1986; Sanders *et al*., 1990; Sanders, 1991; Jones, 1994; Stoecker *et al*., 1987; Jeong *et al*., 2010; Hansen, 2011), appreciation that these organisms play a core role in especially marine ecosystems has been slow to mature. From 2011 to 2013 a series of workshops (funded by the Leverhulme Trust, U.K.) were held in Sweden (Kalmar), UK (Swansea) and the USA (Horn Point), bringing together experts across the field of marine planktonic protists. Outputs from those meetings explored the false dichotomy of the traditional phytoplankton–zooplankton paradigm (Flynn *et al*., 2013), advantages of acquired phototrophy (Flynn and Hansen, 2013), the role of mixotrophy in shaping the biological carbon pump (Mitra *et al*., 2014b), stoichiometric implications for mixotrophy (Lundgren *et al*., 2016), the functional classification of planktonic protists (Mitra *et al*., 2016) and the biogeographies of the different types of mixotrophic protist plankton (Leles *et al*., 2017, 2019). Many additional publications have also raised the profile of photo- and phago-mixotrophic plankton over the last two decades (e.g. Stickney *et al*., 2000; Burkholder *et al*., 2008; Zubkov and Tarran, 2008; Carvalho and Granéli, 2010; Brutemark and Granéli, 2011; Sanders, 2011; Hartmann *et al*., 2013; Wilken *et al*., 2013; Saad *et al*., 2016; Selosse *et al*., 2017; Stoecker *et al*., 2017), and brought the subject to a wider audience (Mitra, 2016, 2018; Glibert *et al*., 2019).

Why is this subject important? Because the combining of primary and secondary production in a single organism radically changes biogeochemical and trophic dynamics involving those organisms (Hitchman and Jones, 2000; Mitra *et al*., 2014b; Caron, 2016; Ward and Follows, 2016; Ghyoot *et al*., 2017a, 2017b; Leles *et al*. 2018). It alters the flows of energy and materials in and out of organisms that form the base of the food chain, and changes the way that we understand and thence simulate processes from harmful algal blooms (HABs) and fisheries to global climate change.

In all areas of science, there comes a point when the weight of new evidence warrants a fundamental reassessment of the paradigm in which we express our understanding. Within marine ecology, while the components of the microbial loop had been known about for decades before the mid 1970s, the broad importance of the concept was only brought to wide attention with works such as those of Pomeroy (1974), Williams (1981) and Azam *et al*. (1983), with increasing acceptance following that (e.g. Pomeroy *et al*., 2007; Fenchel, 2008). We suggest that a similar reassessment point has now been reached concerning the importance of photo- and phago-mixotrophic plankton, and its potential to radically reshape marine ecology. To consider such a reshaping represents a very significant, and most likely controversial task, cutting across all sectors of plankton research. Here we present some ideas on how this task could be undertaken. However, we start by considering naming conventions and some misconceptions that surround the topic of evolution and functional traits of planktonic mixotrophs. We then continue by exploring the implications for marine ecology, and then consider various specific challenges for plankton research.

To aid this discussion we present a glossary (Table I).

WHAT IS IN A NAME?

Traditional descriptions of plankton most obviously comprise phytoplankton and zooplankton, with increasing inclusion of bacterioplankton from the 1980s. Often these descriptors attract add-on terms donating organism size (pico-, nano-, micro-, meso-; e.g. Sieburth *et al*., 1978). Over a century of marine research has been built upon terminologies largely of terrestrial origin, dividing primary and secondary production neatly between plant-like phytoplankton and animal-like zooplankton. Thus, phytoplankton may be referred to as the "grass of the sea",

Table I: Glossary to terms describing forms of nourishment and functional types of plankton

Term	Definition
Autotrophy	Nutrition involving the synthesis of complex organic substances using photosynthesis (*phototrophy*) or chemosynthesis. Typically associated with the use of inorganic nutrients.
Bacterioplankton	Bacterial (prokaryote) *plankton* acquiring nourishment via *osmo(hetero)trophy*, and some also via *chemo (auto)trophy*
Constitutive mixoplankton (CMs)	*Protist plankton* with an inherent capacity for *phototrophy* that can also exhibit *phagotrophy*
Cyanobacteria	*Bacterioplankton* (prokaryote) members of the *phytoplankton*
Generalists non-constitutive mixoplankton (GNCMs)	*NCMs* that acquire their capacity for *phototrophy* from general (i.e. non-specific) phototrophic prey (cf. *SNCM*)
Heterotrophy	Nutrition involving the consumption and interconversions of sources of organic carbon
Metazooplankton	Multicellular (i.e. non-*protist*) *zooplankton*
Mixoplankton	*Plankton protists* capable of obtaining nourishment via *photo(auto)trophy* and *phago(hetero)trophy*, as well as via *osmo(hetero)trophy*.
Mixotrophy	Nutrition involving both *autotrophy* and *heterotrophy*
Non-constitutive mixoplankton (NCMs)	*Protist plankton* that acquire the capability for *phototrophy* from consumption (via *phagotrophy*) of phototrophic prey
Osmotrophy	A mode of *heterotrophy* (i.e. osmo(hetero)trophy) involving the uptake and consumption of dissolved organic compounds
Phagotrophy	A mode of *heterotrophy* (i.e. phago(hetero)trophy) involving the engulfment of particles (often whole organisms) into a phagocytic vacuole in which digestion occurs
Phototrophy	A mode of *autotrophy* (i.e. photo(auto)trophy) involving the fixation of CO_2 using energy derived from light.
Phytoplankton	*Plankton* obtaining nourishment via *photo(auto) trophy* and *osmo(hetero)trophy*. They are incapable of *phagotrophy*; cf. mixoplankton.
Plankton	Organisms that cannot maintain a fixed location in the water column, and are thus moved by the tides and currents
Protist	Single-celled eukaryote organism
Protophytoplankton	*Protist phytoplankton*
Protozooplankton	*Protist zooplankton*
Specialist non-constitutive mixoplankton (SNCMs)	*NCMs* that acquire their capacity for *phototrophy* from specific phototrophic prey (cf. *GNCM*)
Zooplankton	*Plankton* obtaining nourishment via *heterotrophy*. They are incapable of *phototrophy*; cf. *mixoplankton*.

Terms in *italics* are further defined within this glossary. To these terms may be applied (as appropriate), a prefix indicating organism size according to **pico-** (0.2–2 μm), **nano-** (2–20 μm), **micro-** (20–200 μm) and **meso-** (>200 μm). Thus, nano-*protozooplankton* include organisms often termed "heterotrophic nanoflagellates", while meso-*metazooplankton* include copepods.

and their copepod predators as "herbivores" or "insects of the sea" (e.g. Cushing, 1975), with classic models (e.g. NPZ: Fasham *et al.*, 1990; Franks, 2002), their complex successors (e.g. DARWIN: Follows *et al.*, 2007; MEDUSA: Yool *et al.*, 2013; ERSEM: Baretta *et al.*, 1995; Butenschön *et al.*, 2016) and indeed climate change (Arora *et al.*, 2013) and fisheries management models (Plagányi, 2007), also deploying this classic dichotomy.

The growing realization that plankton communities often contain organisms that combine phototrophy and phagotrophy (i.e. primary and higher production), requires that we more readily and clearly discriminate between organisms that are actually or potentially photo- and phago-mixotrophic and organisms that are not. We need greater clarity during our conversations, in conferences, on posters, in scientific publications and critically also in teaching. The problem is that the terminology currently referencing these protist plankton is unwieldy and/or ambiguous. And the problem starts with the word "mixotroph" (the combining of autotrophy and heterotrophy in one organism; Table I).

For protist plankton, the mode of autotrophy is phototrophy, while heterotrophy may be enabled by osmotrophy and/or phagotrophy (Fig. 1; Table I). There is a profound difference between heterotrophy supported by osmotrophy versus that by phagotrophy, both for trophic dynamics and also for food web structuring. Phagotrophy typically involves the killing of other organisms through the process of capture, ingestion (either partially or totally) and digestion. In contrast, osmotrophy does not require the act of killing. Indiscriminate use of "mixotrophy" fails to draw attention to this important difference.

Osmotrophy, the ability to use dissolved organics such as amino acids and vitamins (auxotrophy; Droop, 2007), is common in plankton typically considered as phototrophs. In aquatic ecology the subject has a long and controversial history (see Flynn and Butler, 1984 for a review of older discussions on the ecological importance to plankton); for many species in nature the role of osmotrophy probably acts mainly to mitigate against metabolite leakage (Flynn and Berry, 1999).

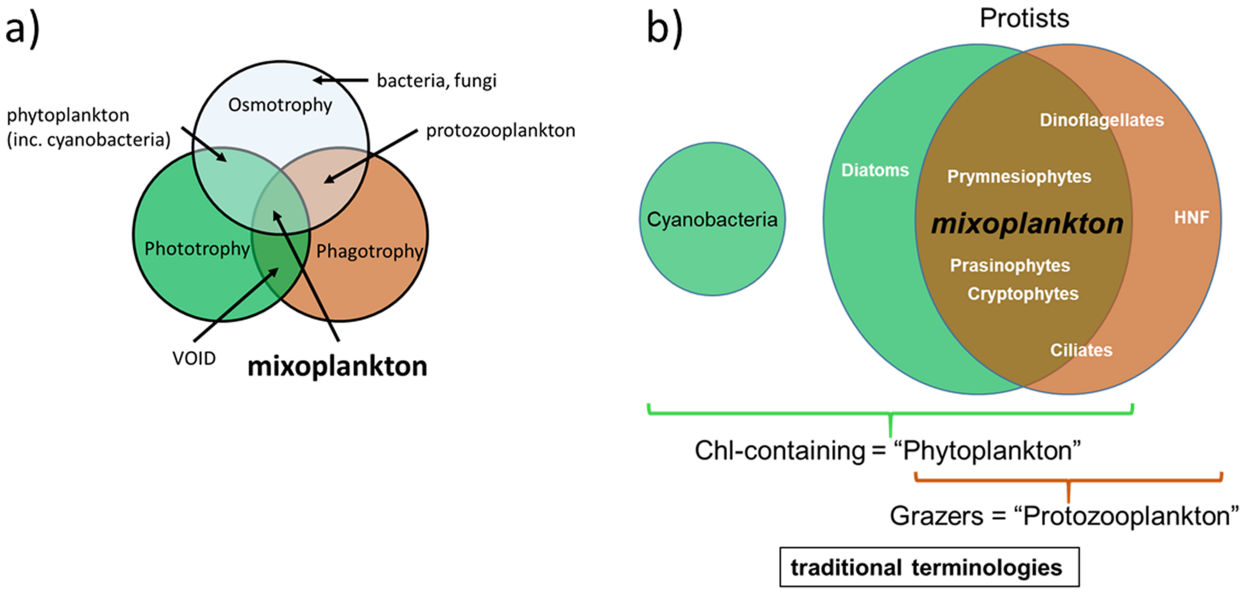

Fig. 1. Overlaps in the trophic capacity of different microplankton. (**a**) Venn diagram of overlaps between photo-, osmo- and phagotrophy. "VOID" indicates that no organisms exist in this sector. Mixoplankton are, by definition, capable of all three modes. Phytoplankton are mixotrophic by virtue of photo- and osmotrophy; they are non-phagotrophic. Protozooplankton are incapable of phototrophy. (**b**) Overlaps between traditional allocations of chlorophyll (Chl) containing organisms as "phytoplankton" and microbial grazers as "protozooplankton". Symbol sizes or area allocations do not apportion significance.

Nonetheless, phytoplankton are typically mixotrophic in this sense, and indeed various modelling explorations of mixotrophy also refer to photo- and osmo-mixotrophy (e.g. Våge *et al.*, 2013). This mode of mixotrophy (photo(auto)trophy + osmo (hetero)trophy) in protistan and cyanobacterial microalgae is also exploited for biotechnology (Chojnacka and Noworyta, 2004; Liang *et al.*, 2009; Bhatnagar *et al.*, 2011; Morales-Sánchez *et al.*, 2013). Confusingly the mode of mixotrophy being studied is neither always apparent from the title nor in the abstract of many scientific publications (e.g. Yelton *et al.*, 2016), and indeed both osmotrophy and phagotrophy may be implicated (Burkholder *et al.*, 2008). We need to be able to differentiate readily between generic "mixotrophy" and specifically that which involves photo-phagotrophy.

Because of the likely ubiquity of osmotrophy, use of this trait in the functional classification of protists is compromised (Flynn *et al.*, 2013). Accordingly, Mitra *et al.* (2016) presented a revised classification of the protist plankton based on functional types, centred on the potential for and the mode of expressing photo- and phago-mixotrophy. That publication differentiates between the "pure" photo(auto)trophs (phytoplankton), the mixotrophs that have a constitutive ability to photosynthesise ("constitutive mixotroph", CM), those that need to acquire their phototrophic capability from their prey ("non-constitutive mixotrophs", NCM) and the "pure" phago- and heterotrophs (protozooplankton). NCMs are then themselves split between generalists (GNCM), plastidic specialists (pSNCM) and endosymbiotic specialists (eSNCM) according to their mode of acquiring phototrophic capabilities. All these groups are fundamentally different functional types (Fig. 2). While the terms CM and NCM are gradually gaining increased usage (e.g. Johnson and Moeller, 2018; Hansson *et al.*, 2019; Naselli-Flores and Barone, 2019), what we are still missing is a term akin to the traditional terms—phytoplankton and zooplankton—to use to collectively refer to all photo- and phago-mixotrophic planktonic protists.

As such a short descriptive term, we propose that the word "mixoplankton" be used to specifically reference photo- and phagotrophic protists. This term already appears in various online teaching resources as well as on Fishbase website (https://www.fishbase.de/glossary/Glossary.php?q=mixoplankton), where mixoplankton are defined as "*Planktonic organisms that can be classified at several trophic levels. For example, some ciliates can be photosynthetic but also can ingest other plankton and are heterotrophic.*" Such a description perhaps does not make it clear that one and the same organism is engaging simultaneously or alternatively in photo-*plus* phago-'trophic activities. Nor does this description exclude reference to mixotrophs that are incapable of phagotrophy. We thus suggest that this definition is reworded to read:

"mixoplankton: *planktonic protist organisms that express, or have potential to express, phototrophy and phagotrophy.*"

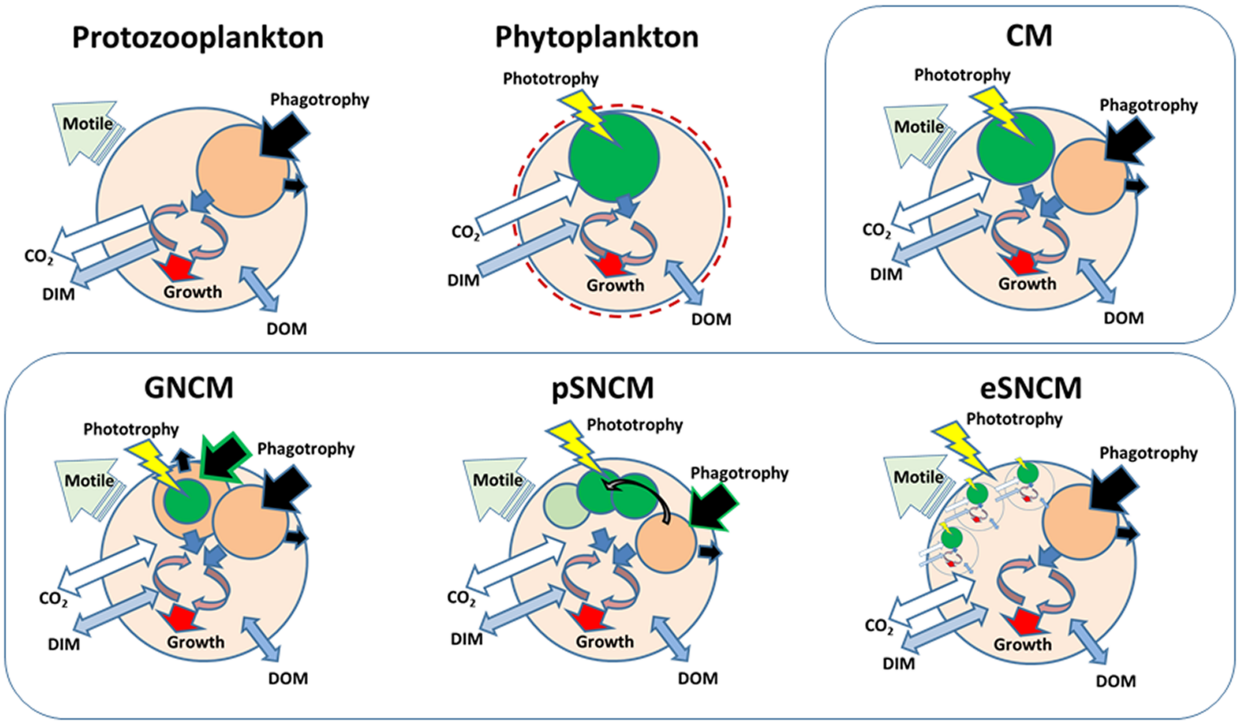

Fig. 2. Schematics showing the distinct differences between different protist plankton physiologies. The protozooplankton are osmo–phagotrophic; they are incapable of phototrophy. The phytoplankton are photo-osmo mixotrophic; they are incapable of phagotrophy. The constitutive mixoplankton (CM) and non-constitutive mixoplankton (NCMs) are all photo-, osmo- and phago-mixotrophic. However, some mixoplankton have life stages that are not photo- and phagotrophic, with nutrition aligning with that of "phytoplankton" or "protozooplankton". The generalist GNCMs acquire phototrophy from many phototroph prey types; pSNCMs are plastidic specialists acquiring phototrophy from specialist prey type(s); eSNCMs are endosymbiotic acquiring phototrophy by harbouring specific phototrophic prey. Note: illustrations are not to scale; in particular, eSNCMs are in relative terms ca. 10 to 100 times larger than the others (see Fig. 3).

The word "potential" in the definition of mixoplankton is also important, because while some mixoplankton are obligatory photo- and phago-mixotrophs (or have life stages that are), other species (specifically many CMs) may not show such an obligatory requirement (Sanders *et al*., 1990; Jones, 1997; Calbet *et al*., 2011; Berge and Hansen, 2016; Stoecker *et al*., 2017).

Usage of the overarching term "mixoplankton" has various advantages:

- It is short and not hyphenated, amenable to the addition of an allometric prefix (e.g. nano-mixoplankton).
- There is a clear linkage to plankton; mixotrophy as a mode of nutrition is distributed across many organism groups besides plankton (e.g. corals, the sea slug *Elysia viridis*, the Venus flytrap *Dionaea muscipula*).
- Importantly, "mixoplankton" discriminates between the mixotrophy expressed in protists unable to phagocytose (i.e. photo- and osmo-mixotrophy, as conducted by diatoms for example), versus that expressed by protists that can conduct phagotrophy (i.e. the mixotrophy of the CM and NCM functional groups described by Mitra *et al*., 2016). We can readily realign the original descriptions of Mitra *et al*., 2016 by modifying the meaning of the abbreviations CM and NCM to "constitutive mixoplankton" and "non-constitutive mixoplankton", respectively.
- Perhaps of equal importance to the above is that phytoplankton are then characterized as being incapable of phagotrophy, while protozooplankton are incapable of phototrophy (Table I; Fig. 2).

From here on, we shall use the term "mixoplankton" in reference to photo- and phago-mixotrophic planktonic protists, including where the original referenced paper termed such organisms as "mixotrophs".

MISCONCEPTIONS ON THE EVOLUTION AND TRAITS OF MIXOPLANKTON

To help understand why different organisms occupy the niches that they do occupy requires a full understanding of their functional traits, and also of those organisms

with which they interact. A common conception is that mixoplankton are inferior to their phytoplankton or protozooplankton counterparts with respect to their expression of traits such as photosynthesis, nutrient uptake and feeding (Dolan and Pérez, 2000; Litchman *et al*., 2007; cf. Calbet *et al*., 2011). This assumes that an organism that specializes in two nutritional routes is a generalist in its expression of each of those routes. This argument could be considered as a trait-trade-off (TTO), a concept which has found considerable support in the plankton research community, especially with respect to plankton modelling (Litchman and Klausmeier, 2008; Litchman *et al*., 2013). Such TTO arguments must only be applied to organisms from the same environment (Litchman *et al*., 2007), and TTOs are in any case questionable from an evolutionary standpoint (Flynn *et al*., 2015).

Clearly, we expect traits expressed by phytoplankton and protozooplankton to be advantaged in the environment in which they evolved, just as those species that combine photo- and phagotrophy must be advantaged by possessing both phototrophy and phagotrophy in other environments. Questions of which trait is better or not in a given environmental setting have been answered by evolution. To better clarify this matter requires a consideration of the evolution of the protists.

The genetic diversity of protists is vast, equalling that of all other eukaryotes combined (Burki 2014; Adl *et al*., 2019). The evolution of eukaryotes (eukaryogenesis) was based on the phagotrophic acquisition of an alphaproteobacteria into a host, which subsequently lead to the formation (evolution) of mitochondria (Hampl *et al*., 2019). The formation of mitochondria provided the cells with extra energy that allowed the evolution of eukaryotic cell structures (Martin *et al*., 2017). One prerequisite for this event is that the host must have had an actin filament system that enabled the formation of protrusions and the subsequent engulfment of bacteria (Yutin *et al*., 2009). Whether the last eukaryotic common ancestor was an Archea or not is still not resolved (Martijn and Ettema, 2013), but under any scenario phagotrophy evolved very early in life on Earth. Moreover, secondary endosymbiotic events gave rise to photosynthesis within eukaryotes, which appears to have been lost and (re)gained several times in many protist groups (Archibald, 2009). Extant protozooplankton thus include organisms that gained and then lost their scope for phototrophy (i.e. that had ancestors that were mixoplankton).

Protist evolution saw many cycles of gaining and losing capabilities (Figueroa-Martinez *et al*., 2015; Hampl *et al*., 2019). Mixoplankton did not arise as a coming together of protist phytoplankton plus protozooplankton traits into one organism, and protist phytoplankton did not fail to acquire the capability for phagotrophy. On the contrary, the most profound event in protist plankton evolution in some ways was the loss of the ancestral trait of phagotrophy. Not only did this remove an important nutritional pathway, but it also removed a major route through which protists have acquired genetic and physiological variety of importance for their evolution.

Ecologically the most important of those protist groups in contemporary aquatic environments that lost phagotrophy are the diatoms, which also evolved a non-cellulosic cell wall (frustule of silica) that was likely incompatible with phagotrophy from an early stage in their evolution. Planktonic diatoms are also effectively non-motile (they cannot swim) within the plankton. Motility is rather an important trait for a planktonic predator; the low motility of the mixoplanktonic Foraminifera and other Rhizaria are compensated for in this regard by the use of pseudopods for feeding. The environment in which diatoms flourish is also typically an immature one, of high turbulence, relatively high inorganic nutrient concentrations, and lacking in the prey that could otherwise provide nutrients. A similar set of arguments could be made for the often-assumed absence of phagotrophy in the ecologically dominant calcified form of coccolithophorids (cf. Rokitta *et al*., 2011). In contrast, mixoplankton dominate in more mature systems containing competitors that are also potential prey, and in systems in which nutrients (which can include light) are supplied in unbalanced proportions, if not limiting amounts (Mitra *et al*., 2014b). These are also the situations in which HABs occur (Granéli, 2006; Granéli *et al*., 2008; Glibert *et al*., 2018), where mixoplankton are often dominant species.

There is significant variation across different mixoplankton types with respect to their core physiology (Fig. 2), and allied variation in feeding types, and hence in the form of trophic interactions. Despite this, the traits of these organisms have often been referenced in the literature in a rather indiscriminate fashion, as if all mixotrophic plankton are effectively the same rather than being different (CM vs GNCM, vs SNCM etc.). This is especially so in the conceptual and modelling-orientated literature (e.g. Ward and Follows, 2016). The allometric "rule"-based analysis of Andersen *et al*. (2016), suggesting that mixoplankton are mechanistically aligned (optimized) at a position intermediate between smaller phototrophic phytoplankton and larger phagotrophic micro (proto) zooplankton, does not align with reality. In fact, mixoplankton span size ranges that encompass the whole breadth of protist phytoplankton and protozooplankton (Fig. 3); this range exceeds that similar to that of ant-to-cow. Equally important though, there appears to be important functional-allometric differences between the CMs, GNCMS and SNCMs, which is also seen in their biogeography (Leles *et al*., 2017, 2019).

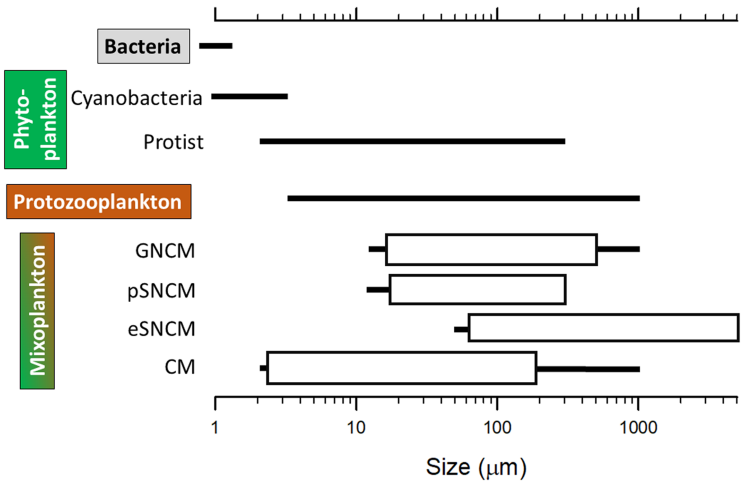

Fig. 3. Size ranges for different microbial plankton functional types. As labelled here, phytoplankton (prokaryotic cyanobacteria and allies, and eukaryotic protists) are photo- and osmo- mixotrophic; they are incapable of phagotrophy. Protozooplankton are osmo- and phagotrophic; they are incapable of phototrophy. For the mixoplankton, which all express or have potential to express photo-, osmo- and phagotrophy, the typical size ranges are as indicated by the boxes. Note that in total mixoplankton sizes span all other protist plankton types, though the CMs are the dominant examples in the sub 20 μm size categories. GNCM, generalist non-constitutive mixoplankton; pSNCM, plastidic specialist non-constitutive mixoplankton; eSNCM, endosymbiotic non-constitutive mixoplankton; CM, constitutive mixoplankton. The size ranges for mixoplankton have been taken from the data collected for Leles *et al*. (2017, 2019) and from personal observations made by authors. See also Figs 1 and 2.

In conclusion, mixoplankton should not be collectively viewed as inferior, nor indeed superior, to phytoplankton and protozooplankton. They are just different (and different within themselves—Fig. 2). They also, in consequence, have the potential to fulfil different functional roles within ecology, exploiting different niches. Before we appreciated the global abundance of mixoplankton we could perhaps justify ignoring their contribution. We can no longer afford to do so.

PLACING MIXOPLANKTON WITHIN MARINE ECOLOGY

Researching and understanding the role of mixoplankton within marine plankton systems has the potential to completely reform our understanding of marine ecology. However, the development of plankton science in this regard has been complicated by:

(i) the failure to appreciate the implications of different modes of mixoplankton functioning; the differing degrees with which mixotrophy is obligatory (Sanders *et al*., 1990; Jones, 1997; Stoecker *et al*., 2017; Gomes *et al*., 2018) even within the same or closely related species (Calbet *et al*., 2011; Berge and Hansen, 2016);

(ii) the afore-mentioned confusion in terminologies.

There is in consequence something of a legacy of published works that ignore or misrepresent mixoplankton and are in retrospect questionable as to whether they are best considered as incomplete, or explorations in theoretical biology or ecology rather than of reality. The implications of the advance in our understanding and appreciation of mixoplankton within plankton ecology thus have similarities with the rise to prominence of the "microbial loop" (Azam *et al*., 1983) and the microbial food web in the 1980s, and the inclusion of the "viral shunt" (Wilhelm and Suttle, 1999) in the 1990s. Before the 1980s, marine ecology saw at its base a system dominated by net-sized phytoplankton (i.e. cells retained in plankton nets (mesh size typically 20–35 μm) and meso-zooplankton (mesh size typically >200 μm; Cushing, 1975). The pre-1980s paradigm can be seen to have been augmented by the addition of microbial food webs; the form of this revised trophic structure is indicated in Fig. 4a. The whole web contributes in various ways to the "biological carbon pump" (Turner, 2015) and to the "microbial carbon pump" (Jiao *et al*., 2010; Legendre *et al*., 2015).

The enhanced appreciation of the importance of the microbial food web was brought about in large measure by discovering and identifying the importance of certain types of biological entities (e.g. *Prochlorococcus*, viruses, etc.), as well as the overdue recognition of the importance of known groups (bacteria, cyanobacteria, heterotrophic nanoflagellates etc.). The recent expansion of interests in mixoplankton could be argued as just the overdue recognition of facets of ecology known for over a century (e.g. Biecheler, 1936; Blackbourn *et al*., 1973; Jones, 1997; Stoecker, 1998; Jones, 2000). However, the inclusion of mixoplankton as a frequent and at times dominant

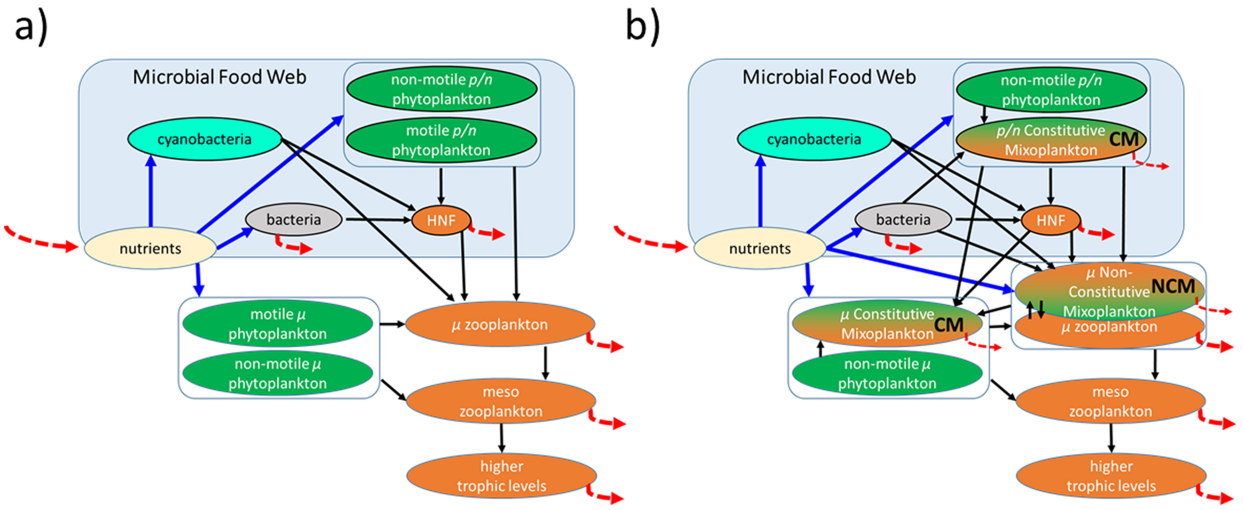

Fig. 4. Differences between the pre-2010 paradigm for plankton trophic dynamics, (**a**), and the post-2010 paradigm, (**b**), that acknowledges the significance and roles of mixoplankton. The microbial food web components are within the blue boxes; viruses impact all organism groups though (because of higher numeric host abundances) may impact smaller organisms more. Red dashed arrows indicate input and outputs of dissolved inorganic and organic nutrients, blue arrows are nutrient uptakes and black arrows indicate grazing routes. Non-motile phytoplankton include diatoms, and also non-motile life stages of others (notably organisms such as the nanoplanktonic coccolithorphorid *Emiliania*, and the microplanktonic colonial *Phaeocystis*). In configuring (**b**), motile phytoplankton in (**a**) are considered to be potentially mixoplankton, as are ca. half of the protozooplankton indicated in (**a**). CM: constitutive mixoplankton. NCM: non-constitutive mixoplankton. HNF: heterotrophic nanoflagellates. *p/n*: pico/nano-sized organisms (i.e. 0.2–2.0 and 2.0–20 μm diameter, respectively). *μ*: micro-sized organisms (i.e. 20–200 μm diameter).

activity in plankton biogeography (Leles *et al*., 2017, 2019; Faure *et al*., 2019; Gutierrez-Rodriguez *et al*. in preparation) and trophic dynamics (e.g. Hartmann *et al*., 2013) marks a radically different development in marine science compared to the inclusion of either the microbial loop or the viral shunt. It is different because it sees the bulk of the known members of the traditional trophic web description actually undertaking different (additional) trophic roles. Thus, most of the protist primary producers traditionally labelled as "phytoplankton", other than those in immature ecosystems (notably diatoms), are actually also part-time grazers, while half those organisms traditionally labelled as "micro (proto)zooplankton" in the photic zone are also photosynthesising (Fig. 4b; see review by Stoecker *et al*., 2009). This situation operates across the whole microbial plankton allometric scale, from picoflagellates (Zubkov and Tarran, 2008; Stoecker and Lavrentyev, 2018) to mm-dimension SNCMs (Decelle *et al*., 2012; Biard *et al*., 2017) (Fig. 3). The trophic linkages are redrawn and new links added.

One of the most frequent questions that arises in scientific discussions on mixoplankton is, how important is mixotrophy and when are these organisms functionally mixotrophic? Whether we need to know when they are eating, or whether it is sufficient just to know that they may do so if the need or opportunity arises, is a non-trivial question to answer. The ability to eat may supply small but vital amounts of nutrition to a mixoplanktonic organism that would be unavailable to non-mixoplankton competitors at critical stages. At the same time competitors are removed by a combination of phagotrophy and production of compounds with allelopathic effects (John *et al*., 2002, 2015; Skovgaard and Hansen, 2003; Tillmann, 2003); every difference is a difference. We suspect that many laboratory cultures of these organisms lose their ability to express different nutritional mechanisms over the years of them being held in constant light and high nutrient conditions (e.g. Blossom and Hansen in preparation). It thus follows that if organisms in nature can express a trait then that trait is, at least occasionally, exploited to advantage in nature.

Of the protist functional groups identified by Mitra *et al*. (2016), the NCMs must be regularly and significantly mixotrophic as many have to feed to acquire chloroplasts to support their obligatory need to undertake photosynthesis. The challenge is primarily with identifying when CM species are eating. Some CM species are perhaps only rare feeders in nature, while others (given the voracious behaviour of those brought into culture) are most likely regular feeders (reviewed by Jeong *et al*., 2010; Hansen, 2011). The former group include species such as *Tripos furca* (=*Ceratium furca*; Smalley *et al*., 2003, 2012), *Prorocentrum minimum* (Johnson, 2015) and *Heterocapsa triquetra* (Jeong *et al*., 2005), while the latter include species such as *Prymnesium* (Skovgaard and Hansen, 2003; Carvalho and Granéli, 2010), *Karlodinium* (Berge *et al*., 2008a and b; Calbet *et al*., 2011) and *Fragilidium* spp (Jeong *et al*., 1999; Hansen *et al*., 2000; Skovgaard *et al*., 2000).

As evidence of the importance of differentiating between the CM and NCM forms, it is perhaps noteworthy that the microbial food web interfaces directly with many CMs (Burkholder *et al*., 2008; Jeong *et al*., 2010; Hartmann *et al*., 2013; Stoecker and Lavrentyev, 2018), while the NCMs generally tend to be larger (eSNCMs up to mm dimensions, e.g. green *Noctiluca*; Saito *et al*., 2006; Stoecker *et al*., 2009) (Figs 3 and 4b). That said, some NCMs are nano-sized, at least in the oligotrophic ocean (Pitta and Giannakourou, 2000; Pitta *et al*., 2001).

Although we do not yet know the full significance of mixoplanktonic activity for community structure and dynamics, or indeed for the physiology and growth of most individual species, the structural difference between Fig. 4a and b appears so profound as to represent a paradigm shift. The mixoplankton paradigm is not a "loop", or a "shunt", appended to the pre-2010 trophic web (Fig. 4). Indeed, mixoplankton activity has potentially important implications for the functioning of the microbial food web (Fig. 4b). Is it perhaps a "multiplier", improving assimilation efficiency and the allometrics of trophic transfer? We do not know. Perhaps until it becomes much clearer, we should just refer to it as simply the "*mixoplankton paradigm*".

IMPLICATIONS OF THE MIXOPLANKTON PARADIGM FOR PLANKTON RESEARCH

The ramifications of the mixoplankton paradigm for plankton research are wide ranging. Some implications are perhaps more obvious than others, but taking a fresh look at everything may be the safer route forward. To really understand the implication of mixoplankton for marine ecology, future research will have to answer many questions such as: when and where are mixoplankton important? How and from where/who do mixoplankton acquire their energy and nutrients? What activity rates can be associated with mixoplankton? Which circumstances promote mixotrophy? Only a multidisciplinary approach can answer those questions; below we commence such a review, though it is readily apparent that the task will proceed for likely several decades.

Survey and fieldwork

Survey and fieldwork are ideally suited to answer questions such as when, where and how many organisms occur. These are still highly relevant questions regarding mixoplankton. However, survey methods, irrespective of the approach and technology (remote sensing, autobuoys, Continuous Plankton Recorder, etc.) have been designed and optimized essentially for organisms that are traditionally labelled as "phytoplankton" or "zooplankton" (both groups of which we now appreciate include mixoplankton), and typically for species that are nearest the surface, physically robust and relatively large. Many of these approaches are best suited to organisms with clear physiological traits (pigment signatures in cyanobacteria, reflective signatures in coccolithophorids, high structural integrity in diatoms). Many mixoplankton (flagellates and ciliates) are small, not structurally robust or easily sampled, nor amenable to identification. This means that long-term surveys (such as the CPR), focussed as they often are for specific groups or species, will unfortunately not provide historic data on most specific mixoplanktonic organisms. The work of Leles *et al*. (2017, 2019) shows clearly that there are serious gaps in our appreciation of the biogeography of, especially, the CMs stemming from such traditional methods. It may be possible, through suitable cross calibration with presence/absence of other plankton to glean some information. More profound, perhaps, is the need to detach a signal for chlorophyll from an automatic and exclusive association with primary production supported by inorganic nutrient acquisition, when in mixoplankton that same Chl signal is directly linked to primary production coupled to secondary production.

The paucity of suitably trained researchers who can readily identify mixoplankton not only hinders survey work directly, but will likely restrict the ground truthing of other technologies. Crucially this includes checking the veracity of molecular methodologies such as metabarcoding (who is there?) and metatranscriptomics (what might they be doing?); see discussion in Leles *et al*. (2019) and Faure *et al*. (2019). The challenge of exploiting molecular methods becomes even greater when one considers the presence of non-self genetic material within mixoplankton, as nucleic acids from their prey, in acquired plastids and/or in symbionts; more single-cell analyses are needed, including single-cell polymerase chain reaction (PCR) and genomic studies (Tai *et al*., 2013; Kolisko *et al*., 2014). Beisner *et al*. (2019) gives a review of the current states of the art in this subject arena, specifically for nano-sized mixoplankton. Molecular approaches have contributed much to elucidating patterns of mixotrophy in plankton (Burns *et al*., 2015; McKie-Krisberg *et al*., 2018; Faure *et al*., 2019). Nonetheless, "old fashioned" techniques, such as optical and electron microscopy, also have important continuing roles and have been, and still are, frequently deployed in research on mixoplankton (Anderson, 1978; Swanberg and Caron, 1991; Mafra *et al*., 2016; Kim *et al*., 2017).

Experimental work

Knowing what is there, is one thing. Knowing what it is actually doing there and at what rate is more challenging, and arguably more important (Flynn *et al*., 2018). Marine scientists have spent decades developing and deploying field and laboratory techniques for measuring primary production (photosynthesis, nutrient uptake) and, with markedly lesser success (Mitra *et al*., 2014a), for grazing activities and secondary production by zooplankton. Researchers have studied laboratory cultures with emphasis on using axenic strains, and most frequently using strains that have been maintained in culture for decades in high inorganic nutrient, constant temperature/light environments (and thus have likely evolved, e.g. Martins *et al*. 2004). These approaches, although undertaken for perfectly sound reasons under the traditional paradigm of considering these phototrophic protists as phytoplankton, can now be seen as simplified too far. Our "laboratory rats", as single strain isolates, have indeed not represented the true physiology of natural populations, which consists of a high heterogenic diversity, but for reasons far beyond just adapting to constant-temperature room conditions (e.g. Kremp *et al*., 2012; Alpermann *et al*., 2010; Brandenburg *et al*., 2018).

Simply determining whether a mixoplankton is operating at that instant as a photo- and phago-mixotroph, and what its rate of grazing is upon what prey organisms, presents a profound challenge that few researchers and laboratories are well placed to even attempt to confront. We need to know those rates of photosynthesis, grazing, nutrient regeneration, respiration, etc.; without such data we cannot verify the performance of simulation models. To complicate things further, we need similar data for their prey species as well (which could also be mixoplankton), growing in the same water at the same time. There are very few studies of mixoplankton physiology that are adequately executed to provide data describing the dynamics of growth and trophic activity (e.g. Skovgaard *et al*., 2000, 2003), and even fewer provide the necessary information on both the mixoplankton and its prey (Adolf *et al*., 2003, Lin *et al*., 2018); see discussion in Flynn and Mitra (2009). Simply catching these organisms in the act of eating is a problem (Anderson *et al*., 2017). Consider a nano-mixoplankton eating bacteria; it may only need to eat one bacterium a day to acquire its P or Fe quota. Did we observe that event in our shipboard incubation? Do we know whether the time of day is important for the event? Is the prey presented for possible consumption in an experiment an appropriate species, and in the correct nutritional state?

Training in phytoplankton physiological ecology was perhaps at its zenith during 1970–1980. Since the emergence of molecular biology, however, the literature has seen far fewer papers reporting all-engrossing experiments reporting time series incubations and cultures growth dynamics, etc. Molecular biology and genomics have played a pivotal role in elucidating the evolution of phagotrophy and eukaryogenisis, which is strongly linked to functionality of mixoplankton (Burns *et al*., 2015). Those approaches were particularly useful in revealing features of the trophic interactions between NCMs (Takishita *et al*., 2002). Emphasis has been placed upon who is there (at best only semi-quantitatively), and determining the scope for action (as implied from DNA and RNA data). Even meta-transcriptomic approaches can only indicate the potential contribution of genetic functionality at community level rather than the actual rate of action (Stewart *et al*., 2012; Wohlrab *et al*., 2018). The absence of genetic capability indicates an absence of functional ability; molecular biology can at best indicate potential capability for rate activity, but it (neither DNA sequences data, nor RNA data) cannot provide the actual rates. And will molecular biology be able to discriminate between a bacterial–mixoplankton interaction where the bacteria are external to the protist (as a contaminant or part of its associated microbiome) rather than internal having been eaten? There are many potential sources of conflicting signals even in cultured organisms. For example, membrane production and (auto)digestion processes occur in cells that are not phagocytic. So, to what extent molecular methods can provide help in establishing rate processes and relative contributions of photo- versus osmo- versus phagotrophy in mixoplankton is far from clear at present, though various studies have explored the topic (Santoferrara *et al*., 2014; McKie-Krisberg *et al*., 2018).

In short, there is much to do in the laboratory and in the field, and that applies whether these studies also involve reappraisals of species formally labelled as just phytoplankton or protozooplankton, or as prey, as competitors, or as predators. Critically, while phytoplankton are amenable to study in axenic conditions, or under conditions in which autecology dominates, studies of mixoplankton require a simultaneous study of their prey. This greatly complicates matters, generating problems comparable if not more complex, to those encountered in studies of zooplankton (Mitra *et al*., 2014a).

Conceptual and simulation models

Survey and fieldwork, as well as experimental studies, are very important for providing information needed to support successful conceptual and numerical modelling of plankton ecology; they provide data and understanding to aid the construction and validation of the models. The added value of models, especially of numerical

simulation models, is the scope for their use to explore cause-and-effect questions, such as why and how are the pathways in trophic dynamics as they are. What is apparent is that modelled mixoplankton activity has clear scope to radically alter simulated trophic dynamics and biogeochemical flows (Flynn and Mitra, 2009; Mitra *et al.* 2014b, 2016; Ward and Follows, 2016; Ghyoot *et al*., 2017b; Leles *et al*. 2018). The question is how closely those projections simulate reality, or whether they simply reflect the conceptual basis of the mixoplankton description. So, do we understand the physiology and functional roles of mixoplankton well enough to construct robust models to test hypotheses?

Mixotrophy in plankton has stimulated a curiosity-driven exploration of simulated ecology (Jost *et al*., 2004; Hammer and Pitchford, 2005). Mixotrophy in such models has often been photo- and osmotrophic, rather than photo-, osmo- and phagotrophic. Modelled mixotrophs have often had traits that have been "traded" to prevent them from becoming superior and dominating over their pure phototrophic or heterotrophic counterparts (Jost *et al*., 2004; Ward *et al*., 2011). The rationale behind TTO models describing phytoplankton activity (Bruggeman and Kooijman, 2007; Follows *et al*., 2007; Litchman and Klausmeier, 2008; Smith *et al*., 2016) comes under a different spotlight if we consider that these organisms are also predators. Factors that appear of benefit for phytoplankton (e.g. small size favouring nutrient acquisition) or for consumers (e.g. larger size than the prey, and to minimize predation), and the relative importance of bottom-up and top-down factors become confused when we consider mixoplankton ecology and physiology. Then there are also the arguments against TTO approaches mentioned above. For modellers who wish to exploit TTO concepts for pragmatic computational reasons, at present we simply do not have sufficient empirical evidence from which to construct robust TTO arguments.

Other approaches (Flynn and Mitra, 2009; Ghyoot *et al*., 2017a) have specifically sought to align model construction with the functional type descriptions that we observe (Mitra *et al*., 2016). It is important that we recognize the importance of different functional forms of mixoplankton (Fig. 2). We need to do so at least for the forms that are most frequently dominant in terms of biomass (i.e. CM vs GNCM), though pSNCM and eSNCM forms are also important groups in terms of HABs and biogeochemistry. All mixoplankton types appear to be of significance across large areas of the oceans (Leles *et al*., 2017, 2019). How does such knowledge shape a reappraisal of works considered as exemplars for global-scale plankton modelling, such as the DARWIN model approach of Follows *et al*. (2007), which sought to describe global "phytoplankton" productivities according to standard phototrophic trait approaches, dominated by bottom-up controls? Equally problematic, is that plankton models that do not describe mixotrophic activity have had their skill considered with reference to data collected for natural populations that will most likely have included, on occasion, significant mixoplankton presence and activity.

Over the last decade, plankton simulation science has gone from the extreme of ignoring mixotrophy, to the opposite extreme of assuming that all plankton are mixotrophic (Ward and Follows, 2016). Ward and Follows (2016) deployed an allometric-linked sliding scale of mixoplanktonic capability. However, as noted above, far from being confined as an optimal strategy for mid-sized protists (Andersen *et al*., 2016), significant levels of photo- and phago-mixotrophy operates in some of the smallest phototrophic flagellates (Hartmann *et al*., 2013; Hansen and Hjorth, 2002; Anderson *et al*., 2018), through 1 mm diameter green *Noctiluca* (Hansen *et al*., 2004; Gomes *et al*., 2018), to the very largest colonial Radiolaria (Collodaria; Swanberg, 1983; Biard *et al*., 2016). There is a clear difference in the alignment of allometry and different mixoplankton functional types as well (Fig. 3). We should perhaps guard against developing and exploiting simple explanations that may lead to premature conclusions.

For sure, the modelling of protist plankton activities requires a reappraisal. We need computationally efficient models that describe these different organisms (Fig. 2), in a fashion that is acceptable in the eyes of those scientists who study the physiology and ecology of mixoplankton, before we place them in ecosystem simulators. To do otherwise, to describe theoretical mixoplankton forms that do not simulate the behaviour of real organisms, is surely at least as questionable as to continue to ignore the existence of mixoplankton.

Conceptual models of planktonic ecology also extend far beyond the form indicated in Fig. 4, to more overarching concepts, which also warrant a revisit under the mixoplankton paradigm. Thus, we can reconsider the underpinning of Margalef's mandala (Margalef, 1978) and perhaps even revisit the conceptual basis of Longhurst (2007) provinces. When working on Leles *et al*. (2017, 2019) we sought to place biogeographies of CM and NCM protists within the Longhurst province descriptions aware that what we were doing was using a structure built in the era of the traditional paradigm, and allied concepts, to attempt to explain the biogeography of very different functional groups. It may come to pass that a merging of a mixoplanktonic centric revision of Margalef's mandala (Glibert, 2016) and the Longhurst-style provinces will see a reforming of biogeographic interpretations to provide an enhanced holistic understanding of ocean life.

CONCLUSION

There is clearly much work to do across the entire gamut of plankton research in the context of mixoplankton. It is possible that our holistic view of how planet-scale processes operate may ultimately be little changed. Maybe, to borrow from Williams (1984) commentary upon how the microbial loop was not impacting the science of plankton ecology as much as one may have expected, the mixoplankton paradigm will prove to be just another of the "Emperor's New Suit of Clothes" (i.e. *de facto* invisible). The microbial loop is still not included commonly in extant plankton models. While mixoplankton are ubiquitous, and we will eventually understand better how their food web operates, perhaps ultimately it is just a wheel spinning inside other wheels of greater consequence, with microbial primary, secondary and bacterial productions being so integrated that we would be better considering them as a whole (Flynn, 1988).

On the other hand, perhaps the mixoplankton paradigm will bring into sharper relief that the microbial loop and the allied bacterial food web, with its role in the microbial carbon pump generating refractory dissolved organic matter (DOM) (Jiao *et al*., 2010; Lechtenfeld *et al*., 2014), is indeed collectively worthy of more detailed inclusion in models. The important role of bacteria-mixoplankton interactions in oligotrophic systems is clear (Hartmann *et al*., 2013; Mitra *et al*., 2014b). By similar arguments, maybe our concepts for the management of HABs will need to be reappraised, recognizing the importance of alternative trophic interactions (including the needs of some species for certain prey) in addition to the primarily bottom-up light/inorganic-nutrient processes that are currently focussed upon (Glibert *et al*., 2018; Shumway *et al*., 2018).

If such events come to pass, then the mixoplankton paradigm will develop into more than just recognizing the importance of photo- and phago-mixotrophic protist plankton. We are only going to find out if we look, and we can only attain that goal by combining the different field of research in plankton ecology in a multidisciplinary approach. We will need a combination of laboratory and field rate determinations, biochemistry (stoichiometry) and molecular approaches, new methodologies for field and survey work and new models to test the mixoplankton paradigm. And, of course, we need to train the next generation of scientists to deliver this.

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