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Review

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Authors for correspondence:
Susanne Wilken
e-mail: s.wilken@uva.nl
Alexandra Z. Worden
e-mail: azworden@geomar.de

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The need to account for cell biology in characterizing predatory mixotrophs in aquatic environments

Susanne Wilken[1,2,3], Charmaine C. M. Yung[2,4], Maria Hamilton[2,5], Kenneth Hoadley[4], Juliana Nzongo[2,5], Charlotte Eckmann[2,5], Maria Corrochano-Luque[2], Camille Poirier[2,4] and Alexandra Z. Worden[2,4,5]

[1]Department of Freshwater and Marine Ecology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, 1090GE Amsterdam, Noord-Holland, The Netherlands
[2]Monterey Bay Aquarium Research Institute, Moss Landing, CA 95039, USA
[3]Department of Marine Biology and Oceanography, Institute of Marine Sciences (ICM)-CSIC, 08003 Barcelona, Catalunya, Spain
[4]Ocean EcoSystems Biology Unit, GEOMAR Helmholtz Centre for Ocean Research Kiel, 24148 Kiel, Schleswig-Holstein, Germany
[5]Division of Physical and Biological Sciences, UC Santa Cruz, Santa Cruz, CA 95064, USA

SW, 0000-0002-3852-557X; AZW, 0000-0002-9888-9324

Photosynthesis in eukaryotes first arose through phagocytotic processes wherein an engulfed cyanobacterium was not digested, but instead became a permanent organelle. Other photosynthetic lineages then arose when eukaryotic cells engulfed other already photosynthetic eukaryotic cells. Some of the resulting lineages subsequently lost their ability for phagocytosis, while many others maintained the ability to do both processes. These mixotrophic taxa have more complicated ecological roles, in that they are both primary producers and consumers that can shift more towards producing the organic matter that forms the base of aquatic food chains, or towards respiring and releasing CO_2. We still have much to learn about which taxa are predatory mixotrophs as well as about the physiological consequences of this lifestyle, in part, because much of the diversity of unicellular eukaryotes in aquatic ecosystems remains uncultured. Here, we discuss existing methods for studying predatory mixotrophs, their individual biases, and how single-cell approaches can enhance knowledge of these important taxa. The question remains what the gold standard should be for assigning a mixotrophic status to ill-characterized or uncultured taxa—a status that dictates how organisms are incorporated into carbon cycle models and how their ecosystem roles may shift in future lakes and oceans.

This article is part of a discussion meeting issue 'Single cell ecology'.

1. Introduction

Phagocytosis is an ancient trait and a uniquely eukaryotic form of nutrition [1] that allows a predatory lifestyle in protists—and additional roles in multicellular organisms [2]. It is a process initiated by an encounter with suitable prey, typically detected by receptors that trigger ingestion into cytosolic, membrane-bound food vacuoles [3,4] (figure 1). This requires restructuring of the actin cytoskeleton and coordination of vacuolar transport and fusion, eventually enabling digestion of prey by lysosomal enzymes under acidic conditions [5]. Nutrients and carbon thus made available are resorbed and either respired or assimilated in biosynthetic processes, while remaindered indigestible material is egested allowing membrane recycling. In aquatic environments, unicellular predatory eukaryotes feeding via phagocytosis have been estimated to consume 60% or more of primary production [6] and a large portion of bacterial production [7], and can thus control carbon flux through aquatic food webs.

The capacity for phagocytosis further shaped the dynamic evolutionary processes that gave rise to multiple eukaryotic supergroups and diversity within.

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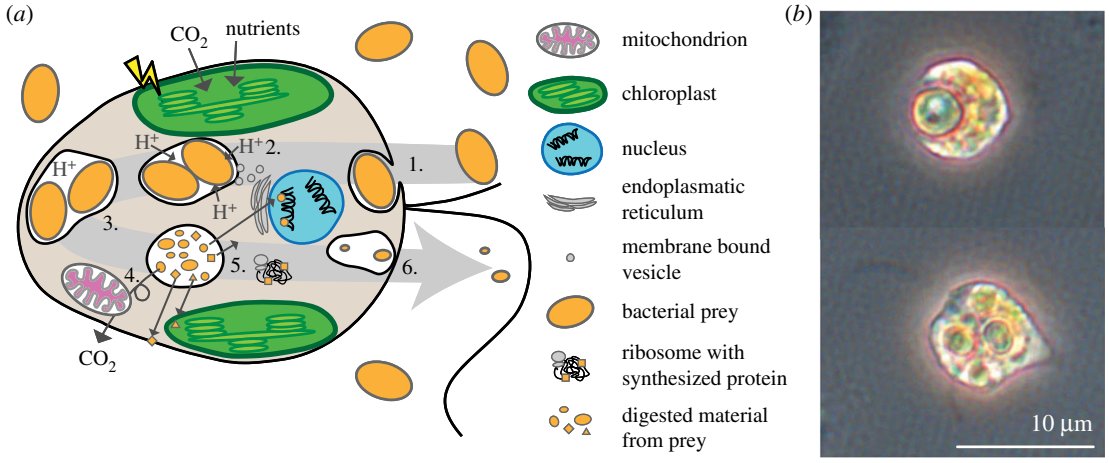

Figure 1. The phagocytotic process in a mixotrophic flagellate. The steps in phagocytosis (*a*) include detection and ingestion of a prey item (e.g. bacterium, orange) (1), modification of the food vacuole properties by fusion with vesicles originating from the endoplasmatic reticulum (ER) and acidification through proton pumping by V-type ATPases (2), creating the acidic conditions required for lysosomal enzyme activities and prey digestion (3). Molecules from digested prey are resorbed into the cytosol via diverse transporters and used to fuel respiration (4) or incorporated as building blocks in macromolecule biosynthesis (5). Remaining indigestible material is ultimately egested (6). Illustrated cellular structures are plastids with thylakoids (green), nucleus (blue) neighboured by ER (grey) and mitochondrion (purple). A freshwater eukaryote *Ochromonas globosa* feeding on the cyanobacterium *Microcystis aeruginosa* (*b*). Shown are a eukaryotic cell ingesting a cyanobacterial prey cell (top image) and a eukaryotic cell with multiple *M. aeruginosa* in food vacuoles (bottom image). Samples fixed with Lugol's iodine solution.

The primary endosymbiosis involving a photosynthetic cyanobacterium phagocytosed by a heterotrophic host, and ultimately integrated as the plastid [8], provided a stepping stone for the subsequent evolution of other photosynthetic eukaryotes. These evolved from multiple, evolutionarily distant groups of ancestrally heterotrophic protists via secondary and tertiary endosymbiosis events, wherein a heterotroph engulfed and retained an already photosynthetic eukaryotic cell [9–11]. Despite the intricate role of phagocytosis in the evolution of photosynthetic eukaryotes, the traditional view of aquatic protists entails a clear separation into purely photosynthetic protists forming part of the phytoplankton, and purely heterotrophic protists constituting part of the zooplankton. Yet we know that many photosynthetic protists from across the eukaryotic tree of life have retained their capacity for phagocytosis and hence combine photosynthetic activity with heterotrophic nutrition in a mixotrophic lifestyle [12] (termed here predatory mixotrophy).

Over evolutionary time, some members of these photosynthetic lineages have subsequently lost either photosynthetic or predatory capabilities, making it difficult to discern predatory mixotrophs from sequence data alone, unless a sequenced, cultured representative exists for the taxon. Further, heterotrophic protists can also temporarily acquire photosynthetic potential via kleptoplastidy wherein plastids are retained selectively from photosynthetic prey organisms [13]. Other forms of mixotrophy are osmotrophy wherein dissolved organic carbon is taken up to fuel a heterotrophic metabolism [14], and algal symbioses wherein a heterotrophic host harbours a photosynthetic endosymbiont (e.g. in Foraminifera, Radiolaria and dinoflagellates) [15] and for purposes of clarity these are not discussed further herein. Thus, the diversity of modern photosynthetic eukaryotes includes various combinations of nutritional forms, and has been shaped by their phagocytotic potential or that of their heterotrophic ancestors.

Evidence for the importance of unicellular predatory mixotrophs to bacterivory in aquatic ecosystems ranges from early studies in lakes [16], to subtropical ocean gyres [17,18] and polar seas [19], underscoring the need to understand their implications for elemental flow through planktonic food-webs [20]. For instance, predatory mixotrophs can acquire the nutrients needed to support photosynthetic growth by ingesting microbial prey [21]. This contrasts with assumptions regarding control of primary production by availability of dissolved inorganic nutrients and provides a competitive advantage of mixotrophs over purely photosynthetic protists in oligotrophic environments [22,23]. Other theoretical predictions on the consequences of a high proportion of predatory mixotrophs include stronger suppression of bacterial abundances [24–26], more efficient carbon transfer to higher trophic levels [23,27] and increased carbon export into deep waters [27].

A current goal is to gain knowledge of the molecular taxonomic identity of aquatic predatory mixotrophs. Additionally, their physiology, biogeography and contributions to ecosystem processes must be resolved so that there is empirical support for assumptions and parameters on which appropriate trait-based and other model types can be built [27–29]. The first step is to determine which taxa are predatory mixotrophs and their abundance in nature, and then of course we must develop understanding of the relative importance of photosynthesis versus phagotrophy to their nutrition [28–30]. This task is methodologically challenging and will require a combination of methods, some already existing [31] and some yet to be developed. Here, we discuss the reliability and weaknesses of current methods for studying predatory mixotrophs and implications for interpretation of experimental results. We include some primary data to highlight potential biases of broadly used methods. Finally, we highlight the promises of single-cell techniques for characterizing predatory mixotrophs and ultimately quantifying their rates of photosynthesis and phagotrophy in nature.

2. Using cultures to characterize mixotrophy

The most comprehensive characterization of a mixotrophic lifestyle is achieved in cultured isolates, where not only can

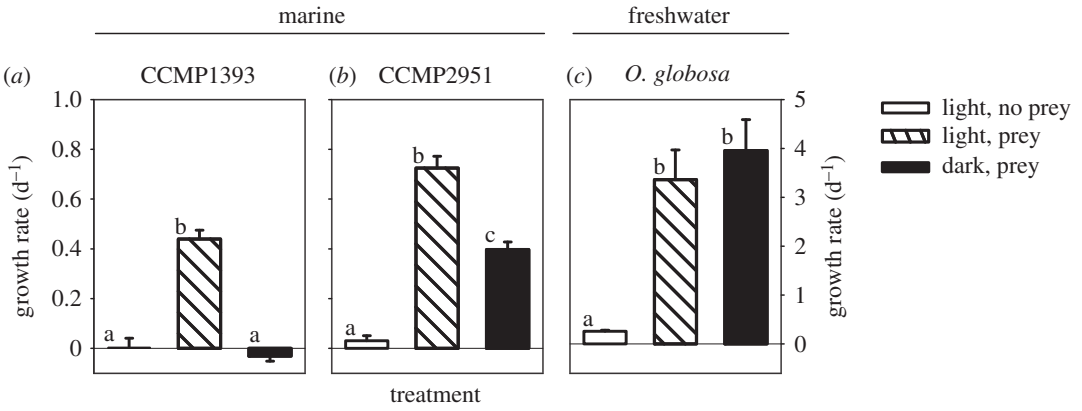

Figure 2. Growth rates of three mixotrophic species of the stramenopile genus *Ochromonas* under autotrophic growth in the light without bacterial prey, under mixotrophic growth on bacterial prey in the light, and under heterotrophic growth on bacterial prey in darkness. Data on marine isolates (*a*,*b*) and the freshwater *O. globosa* (*c*) are from [37] and [38], respectively. Note the difference in *y*-axis scaling in panel (*c*) versus (*a*) and (*b*). Error bars indicate standard deviation of three replicates and significantly different growth rates (one-way ANOVA) are indicated by letters.

ingestion of prey into food vacuoles easily be observed by light or transmission electron microscopy (TEM), but also digestion of prey can be visualized [32–34]. Predatory mixotrophs have marked differences in growth physiology and these differences can be robustly characterized when cultures are available [35,36]. For example, in our studies of three mixotrophic species of the stramenopile alga *Ochromonas* [37,38], the level of reliance on prey consumption can be complete (obligate; figure 2*a*) or only partial for different species, where there is a benefit to having prey available, but it is not required (facultative; figure 2*b*,*c*). Correspondingly, the reliance on photosynthesis can also be obligate or facultative. These growth differences as well as assimilation of prey into predator biomass have been clearly demonstrated in marine dinoflagellates and prymnesiophytes, as well as both marine and freshwater chrysophytes, such as *Ochromonas*, using experimental manipulations of prey and light availability [35,39–45].

In cultured mixotrophs, rates of photosynthesis can be measured by standard techniques, following one of three currencies: oxygen production, electron transfer rates through the photosynthetic apparatus, or carbon-fixation. In many mixotrophs rates of photosynthesis depend on the amount of prey, as they can either invest their resources into maintenance of the photosynthetic machinery, which can be expensive, or into structures required for feeding [46]. Such trade-offs are reflected in adjustment of the nutritional balance in response to resource availability [47]. In mixotrophs with facultative autotrophy, the cellular chlorophyll content can be strongly reduced when prey is abundant [41]. In mixotrophs with obligate requirement for photosynthesis, on the other hand, ingestion of prey can stimulate increased rates of photosynthesis if the prey supplies nutrients or other growth factors needed for an otherwise photosynthetic lifestyle [42,43]. Reflecting its role in purely photoautotrophic organisms, the photosynthetic machinery is typically assumed to mainly serve carbon fixation. However, access to an alternative carbon source through feeding might weaken the reliance on carbon fixation and allow more flexibility for the use of photosynthetically produced reducing equivalents and energy for other cellular processes. A preference for photoheterotrophic nutrition in which the photosynthetic machinery mainly supplies reducing equivalents and energy, while carbon is acquired through feeding, has been suggested for the freshwater chrysophyte *Ochromonas danica* [48]. Such flexibility in the use of the photosynthetic machinery could cause larger variation in the ratios among oxygen production, electron transport and carbon fixation than typically found in pure photoautotrophs and make conversions between these different currencies less reliable.

Understanding ecosystem impacts of mixotrophic predators further necessitates quantification of their ingestion rates. In culture experiments, this can be done by following disappearance of prey, if the prey mortality incurred by the mixotroph is high enough to clearly impact prey population dynamics [48,49]. In mixotrophs with low ingestion rates amendment with fluorescently labelled surrogate prey is used instead. The uptake of these tracers, such as fluorescently labelled bacteria or fluorescent microspheres, can be followed into predator cells over time by epifluorescence microscopy [42,49,50]. Culture experiments show that ingestion rates can range substantially. For instance, in the freshwater chrysophyte and obligate phototroph *Dinobryon cylindricum* ingestion of bacteria is negligible in darkness and ranges up to 8.5 bacteria cell^{-1} h^{-1} at a high light intensity of 400 µmol quanta m^{-2} s^{-1} [42], while the obligate phagotroph *Poterioochromonas malhamensis* showed twofold higher ingestion rates in the dark than in the light [41]. Quantification of ingestion rates is prone to some biases. To follow prey disappearance, it is crucial to exclude indirect effects of the predator on prey growth, for instance, via organic exudates that support bacterial growth. The use of fluorescent prey as tracers relies on the assumption that no other processes cause increased co-association of prey and predator over time. For small cells such as the picoeukaryotes (less than 2 µm cell diameter), it is difficult to distinguish whether a microbe is inside a picoeukaryote or rather just physically co-associated owing to attachment on the eukaryote's cell exterior or even coincidental overlap of prey and predator cells that occurs when the sample is filtered for mounting and visualization. Even with cultures, mixotrophic processes can thus be difficult to study, especially for cells that are too small to clearly be visualized by microscopy [51].

It remains an open question whether photosynthetic picoeukaryotes are capable of phagocytosis. *Micromonas polaris* CCMP2099, an important picoeukaryotic primary producer in polar waters, has recently been proposed to consume bacteria based on experiments with fluorescent microspheres and low percentages of co-occurrence observed on a filter by microscopy [51]. Here, we test the impact of growth conditions

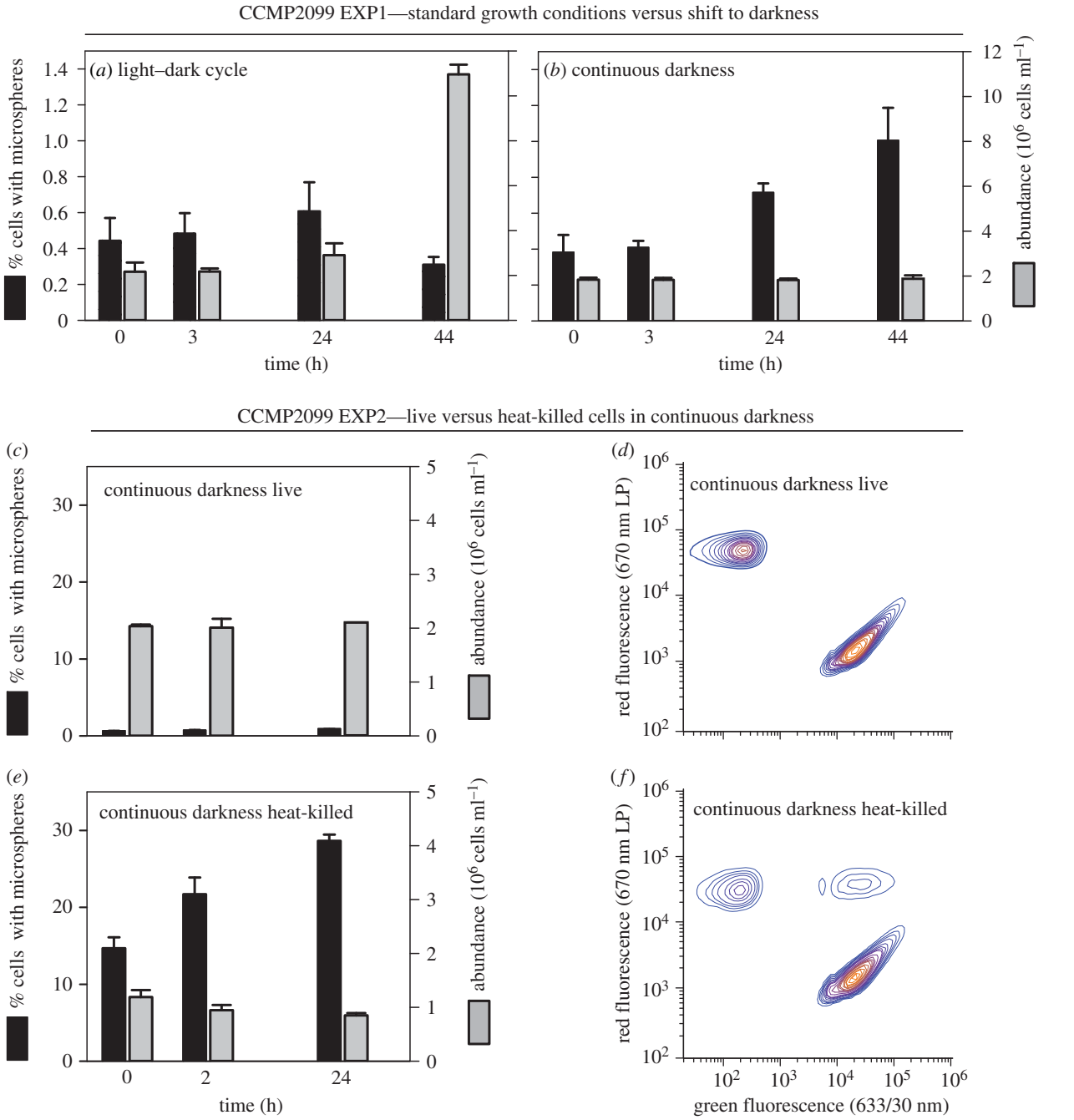

Figure 3. Percentage of *Micromonas polaris* (CCMP2099) cells with co-associated fluorescent polystyrene microspheres and the abundance of CCMP2099 in an experiment (EXP1) comparing exponentially grown cells incubated (*a*) under a light–dark cycle or (*b*) shifted into darkness, and in an experiment (EXP2) comparing (*c*,*d*) living versus (*e*,*f*) heat-killed cultures with cells having been pre-acclimated to darkness for 4d prior to experimental treatments. Error bars indicate the standard deviation of three biological replicates. Contoured density plots of flow cytometric data from (*d*) living and (*f*) heat-killed cultures from EXP2 show three populations: yellow-green fluorescent microspheres (carrying green fluorescence, baseline red fluorescence), CCMP2099 cells (carrying red chlorophyll-derived fluorescence, baseline green) and CCMP2099 cells (*f* only) with co-associated microspheres showing both red and green fluorescence. (Online version in colour.)

and cell viability of *M. polaris* on its tendency to form co-associations with fluorescent microspheres by flow cytometry (see the electronic supplementary material for methods). Because feeding could represent a strategy to survive long periods of darkness in polar regions, we first tested CCMP2099's tendency to form co-associations with fluorescent microspheres when grown in exponential phase on a light–dark cycle and maintained like this (figure 3*a*) or shifted into complete darkness causing cell division to cease (figure 3*b*). The experiment was performed with axenic cultures of *M. polaris* at an abundance of 2×10^6 cells ml^{-1} offering 3×10^6 microspheres ml^{-1} as surrogate prey. In both treatments, a low proportion of *M. polaris* was associated with microspheres under the nutrient replete conditions tested, and this proportion did not increase during short-term incubations (0–3 h, figure 3*a*,*b*), but did increase after 24 h in darkness (RM-ANOVA effect of light: $p = 0.572$, effect of time: $p < 0.001$; Holm–Sidak pairwise comparison of T0 and T24 in dark-treatment $p = 0.013$). To further examine if the cells with co-associated microspheres were indeed senescing, as might occur in a photoautotroph in darkness, we performed an additional experiment. Specifically, microspheres were incubated with 'senescent cells' represented by heat-killed cells (figure 3*e*,*f*) versus living cultures kept in darkness (figure 3*c*,*d*). In the heat-killed treatment, 14.7% of the CCMP2099 population formed co-associations with microspheres, as visualized by flow cytometry (figure 3*f*) and the

percentage increased during the incubation period (figure 3*e*; RM-ANOVA effects of treatment and time: $p < 0.001$). These results emphasize how co-associations can be influenced by physiology via processes independent of phagocytosis, probably cell surface properties and stickiness related to culture health (or death).

Apart from variation in ingestion rates, predatory mixotrophs and heterotrophs show surprising flexibility in feeding—also in how they detect and ingest particles. Although predatory nanoflagellates (2–20 µm in size) are traditionally considered as bacterivores [7,52], many of them have wide prey spectra and feed as omnivores on multiple trophic levels [53]. Behavioural and biochemical aspects of prey selectivity can best be studied in cultured organisms and include prey searching, prey capture, initiation of ingestion and finally digestion [53,54]. Feeding behaviour studied by videomicroscopy showed switches in swimming patterns of the marine heterotrophic dinoflagellate *Oxyrrhis marina*, from interception feeding on bacterial prey to raptorial feeding on larger eukaryotic cells [54,55]. Also the mixotrophic lifestyle of two marine prymnesiophytes was found reflected in their swimming behaviour, as analysis of flow fields generated by individual cells indicated that their feeding currents are insufficient to support purely heterotrophic growth on natural bacterial assemblages [56]. Videomicroscopy further showed that prey selectivity can even occur after ingestion, as in the mixotrophic freshwater chrysophyte *Ochromonas globosa*, which unselectively ingested both microspheres and bacteria but rapidly egested the microspheres [57]. The response of predatory protists to chemo-attractants originating from prey [58] and selective digestion behaviours as described above indicate the importance of prey recognition at multiple phases of feeding from prey searching to after ingestion; nevertheless, the biochemical basis of prey recognition is not well understood.

The process of phagocytosis is mediated by receptors recognizing cell surface molecules of prey and a mannose binding lectin has been identified as a receptor involved in prey recognition in *Oxyrrhis marina* [59]. Also integrins have been suggested as receptors potentially occurring in protistan predators [54] based on their established role in metazoan phagocytosis [60] and occurrence in several protist lineages [61]. Next to suitable prey organisms many predatory mixotrophs also ingest fluorescent, polystyrene microspheres that do not carry cell surface recognition sites [57,62,63], indicating receptor-independent initiation of phagocytosis. In mouse phagocytes, receptor-independent phagocytosis can be initiated by plasma membrane deformation and has been suggested as an evolutionarily ancient form of phagocytosis [64]. So far, available information stems from only a few model organisms that do not represent the eukaryotic diversity found in aquatic ecosystems. More is still to be learned on the receptors and initiation of phagocytosis in environmentally relevant protists. Such information will be required to study the interaction between biochemical and behavioural aspects of feeding through, for instance, experimentation with receptor stimulation. Also the mediation of feeding behaviour by photosynthetic resource acquisition in mixotrophs has hardly been studied.

Full characterization of a predatory mixotroph requires both visual observation of ingested prey and quantitative experiments on growth benefits resulting from photosynthesis and prey assimilation. However, growth physiology is difficult to trace in natural assemblages and therefore several different methods have been developed to establish mixotrophic status, each with their own advantages and biases [31,65]. Available techniques include basic identification as a predatory mixotroph via visualization methods such as microscopy and flow cytometry, sometimes in connection with stains targeting acidic food vacuoles [66] and incubations with labelled prey [18,51,67,68]. Stable isotope probing (SIP), wherein a prey population carrying an isotopic signature is incubated with wild communities to detect prey assimilation into nucleic acids [69,70], can be used to detect predatory activity in putative mixotrophs. Rate measurements of both ingestion and photosynthesis in individual mixotrophs in nature are urgently needed, but currently still lacking.

3. Characterizing mixotrophs in nature

Once in the field, one relies more on assumptions about basic biology so that results can be assigned to a specific process. Characterization of a protist as predatory mixotroph requires detection of both photosynthesis and phagotrophy or at least the potential for both processes. Even if all predatory mixotrophs manifested a similar balance in dependence on prey, their contributions to photosynthesis and predation remain mired with those of pure photoautotrophs and bacterivores in the field. The broadly used methods for estimating primary production in aquatic environments quantify bulk rates of the entire community and thus do not delineate between the contributions of photoautotrophic and mixotrophic taxa. Likewise, there are multiple methods for estimating bacterial mortality owing to predation by bacterivores, most of which do not delineate between pure phagotrophs and predatory mixotrophs. To understand predatory mixotrophs, ideally, quantification of both processes is needed at either the level of populations or even individual cells.

(a) Use of fluorescent surrogate prey

Identification of protists as predatory mixotrophs is often based on detection of putative prey (either naturally occurring prey or fluorescently labelled prey surrogates) in a pigmented eukaryote [71,72]. Epifluorescence microscopy and flow cytometry are commonly used to capture these co-associations. Microscopy typically allows verification and imaging of the prey being positioned inside food vacuoles in predatory heterotrophic [50] and mixotrophic protists (e.g. figure 1*b*) large enough (greater than 3 µm) to resolve sufficient morphological detail, including the plastid, based on fluorescing natural pigments for the latter [33,73]. Microcopy can be used to distinguish between ingestion and other forms of physical co-association; however, visualization is challenging for small cells, such as the pico- and nanoflagellates, as discussed above. Flow cytometry more generally detects pigmented eukaryotes (as a population of potential mixotrophs) containing a co-associated signal from putative prey (or microbes representing another form of co-association)—if fluorescent—and can be used to select and sort these cells by fluorescence activated cell sorting (FACS). The resulting sorted cells can be characterized along with the co-sorted putative prey through amplicon sequencing (see below), or by scintillation counts of radioactively labelled prey [18,74]. Flow cytometry circumvents statistical issues with microscopic imaging if cell concentrations are low and offers faster analysis times.

Fluorescence *in situ* hybridization (FISH) further refines identification of the predators and verification of the position

of prey inside the predator because the FISH signal localizes to the cytoplasm, and thus facilitates the localization of prey inside the predator [73]. FISH provides one of the most solid approaches to visualizing natural mixotroph–prey interactions when used with fluorescent prey or dual probes, one hybridizing to the mixotroph and the other hybridizing to the prey [75]. This technique has demonstrated prymnesiophyte algae as important bacterivores, followed by chrysophytes and morphologically identified dinoflagellates, in the North Atlantic Ocean [76] and Mediterranean Sea [73], respectively. The dual FISH labelling approach requires prior knowledge of the predator and prey, if specific probes are desired, unlike FACS and amplicon sequencing, or hand picking cells under a microscope and sequencing, as has been done for predatory heterotrophs [77].

Most rate measurements on mixotrophs in nature follow ingestion rates by use of surrogate fluorescent prey as tracers, a technique first introduced for quantifying ingestion rates by heterotrophic flagellates [78] in natural communities. Multiple studies have since quantified ingestion rates by mixotrophs and shown their important and often dominant contributions to overall bacterivory in many freshwater and marine environments [16,17,67]. Nevertheless, the use of surrogate prey is subject to known biases involving prey selectivity and diel variations of ingestion rates by mixotrophs [31,65]. Furthermore, there are other processes that might lead to co-association of bacteria with photosynthetic eukaryotes that can be difficult to distinguish from ingestion. Bacteria benefit from higher nutrient concentrations available through co-association with photosynthetic eukaryotes in an area known as the 'phycosphere', which describes an envelope surrounding each phytoplankton cell where leaked chemical compounds and metabolites from the host cell are elevated relative to that of the surrounding water. However, the formation and stability of these associations are still not well understood [20,79]. As shown above, the surface properties of phytoplankton cells (and likely many cell types) are influenced by growth state, and senescing cells (figure 3*e,f* and later timepoint in darkness treatment, figure 3*b*) or even zygotes can be much 'stickier' than healthy cells [80]. Importantly, in nature, phytoplankton stickiness is known to promote aggregation [81] that can result in export from the photic zone. It is quantified by following associations of phytoplankton cells with fluorescent microspheres or other planktonic species over time [82], and thus relies largely on the same technique as measurement of ingestion rates, but assuming co-associations to be caused by stickiness. The tendency to aggregate is mediated by cellular excretion of organic molecules that form exopolymers, and some species show the highest excretion of organic matter in nutrient-stressed and senescent cells [83]. Such non-phagocytosis-related stickiness could lead to overestimation of ingestion rates, especially when low ingestion rates need to be distinguished against a background of coincidental overlap of bacterial (prey) and predator cells (that occurs on filters). Quantification of surrogate prey associated with putative predators at the start of the incubation is an important but rarely reported control, and can be significant compared to the numbers found associated with predators at termination of an incubation experiment (figure 3; [84]). In oligotrophic marine waters mixotrophs showed 50–75% lower ingestion rates than heterotrophs, but owing to their high numerical abundance relative to other potential bacterivores they can

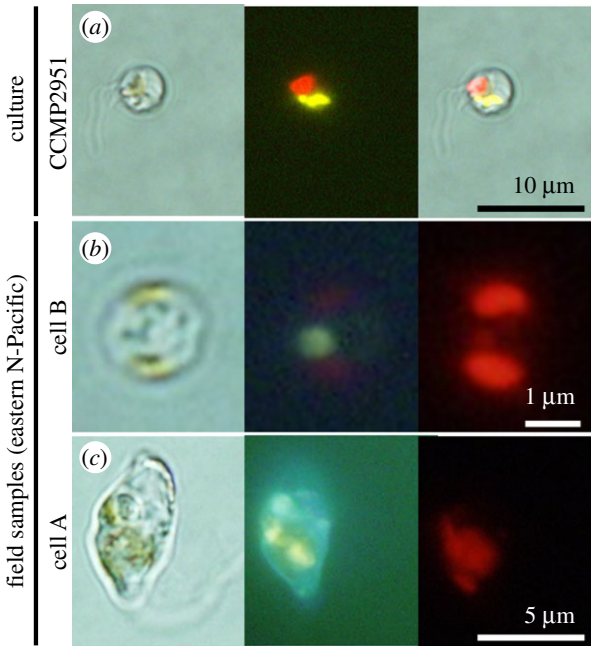

Figure 4. Pigmented flagellates with acidic compartments stained. Cultured *Ochromonas* CCMP2951 stained by LysoTracker (green DND-26) (*a*), and cells from an oligotrophic eastern North Pacific Ocean site (Station 67–125; 34.287°N; 127.355°W) sorted by FACS based on LysoSensor (yellow–blue DND 160) staining (*b,c*). Shown are images from bright-field light microscopy (left), epifluorescence microscopy showing chlorophyll and yellow–green LysoTracker fluorescence (*a*, middle), or yellow LysoSensor fluorescence and red chlorophyll fluorescence individually (*b,c*, middle and right). For CCMP2951 an overlay of bright field and epifluorescence microscopy is also shown (*a*, right). Images in (*a*) and (*b*) represent mixotrophic cells with signals from acidic food vacuoles, while the signal in (*c*) likely stems from an acidic thylakoid lumen and thus does not demonstrate mixotrophy.

dominate community bacterivory [18,67]. A small bias in rate estimates is thus multiplied by their high abundance and might result in substantially overestimated contributions to community bacterivory. The use of fluorescent surrogate prey is a relatively simple method available to most laboratories and applicable in remote field locations, and it therefore will continue to be widely used to study bacterivory by mixotrophs. Better understanding its biases and limitations will hence be important, including potential solutions such as the use of surfactants after fixation and prior to flow cytometry [76], and comparisons with other techniques.

(b) pH-sensitive fluorescent dyes

Like most approaches to detect phagotrophic feeding, staining of acidic vacuoles, as an indicator of food vacuoles, was first applied to heterotrophic protists [66]. Acidotropic dyes like LysoTracker and LysoSensor accumulate in acidic compartments of the cell, and flow cytometric counts of heterotrophic flagellates based on food-vacuole staining with LysoTracker were shown to be comparable to counts by epifluorescence microscopy [66]. Furthermore, a strong correlation between the activities of the digestive enzyme β-glucosaminidase with population average green fluorescence (derived from the acidotropic dye) suggested this technique could be used as a proxy for feeding activity [85]. Subsequently, acidotropic dyes have been applied to quantify the fraction of feeding cells in populations of the mixotrophic dinoflagellate *Dinophysis norvegica* [86] and the prymnesiophyte *Prymnesium parvum* [87] for which specificity of the stain was

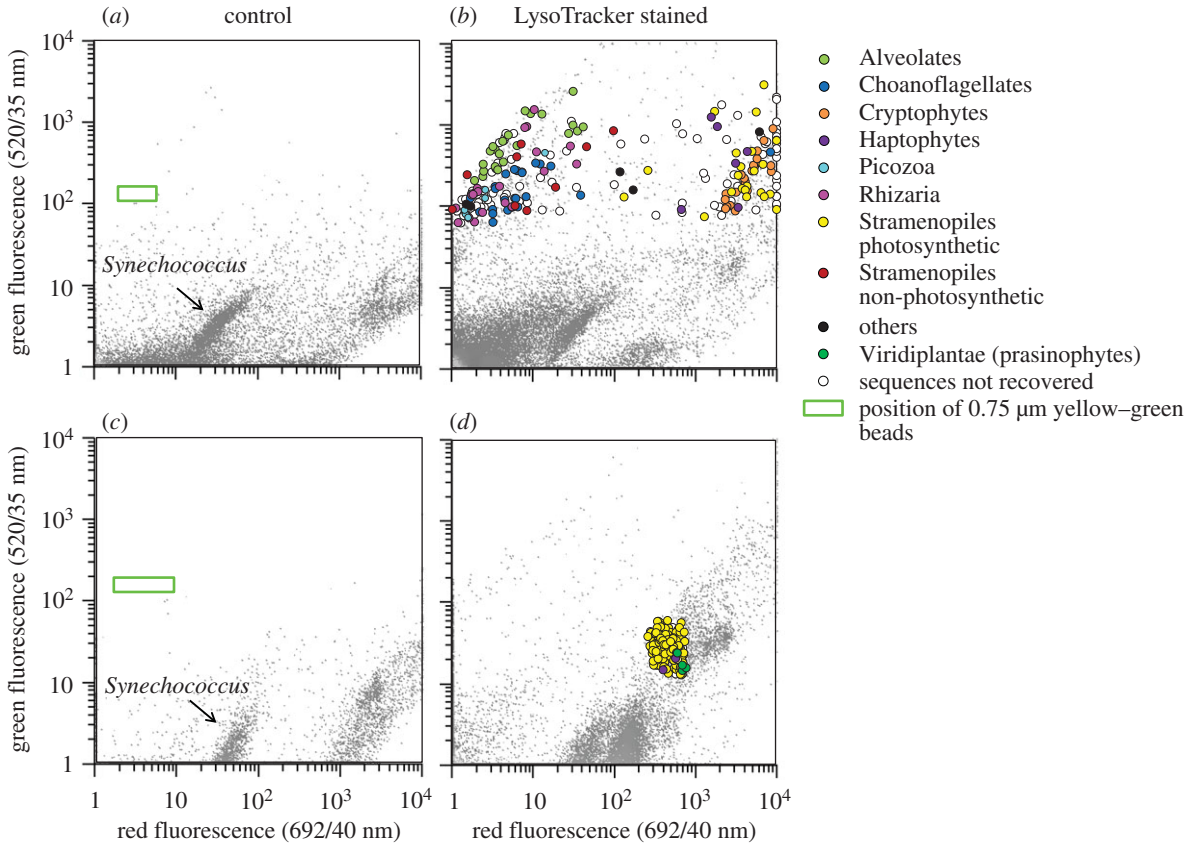

Figure 5. Cytograms of water from an eastern North Pacific coastal site (Station M1; 36.754°N; 122.021°W) on 5 May (*a*,*b*) and 20 March (*c*,*d*) 2014 reveal the complexity of interpreting signals from acidic vacuole stains. FACS targeting putatively phagotrophic protists used green LysoTracker staining (*b*,*d*) compared to an unstained control (*a*,*c*). Circles indicate the position of individual sorted cells and colours show taxonomic affiliations for each cell based on the 18S ribosomal RNA gene. Detailed taxonomy and flow cytometry data for each sorted cell can be found in the electronic supplementary material, files S3 and S4.

verified microscopically (localization to a food vacuole) or through co-association with a fluorescent signal from putative prey. Likewise, when applied to the mixotrophic marine chrysophyte *Ochromonas* CCMP2951, the food vacuole is clearly visible after staining (figure 4*a*). Importantly, photosynthetic eukaryotes contain multiple acidic structures, including the thylakoid lumen that can reach values below pH 5 under stressful conditions [88]. Thus, verification of the intracellular position of acidic compartments is important, and without such verification results can be misleading.

Although acidotropic dyes have already been employed to report on mixotrophs in natural communities [65,89,90], careful verification of their reliability is lacking. Here, we sorted populations, or individual cells, of potentially mixotrophic protists from sites in the eastern North Pacific by FACS (see detail of methods in the electronic supplementary material) using acidotropic probes. For example, cells stained with LysoSensor and sorted by FACS based on that signal from an oligotrophic site in the eastern North Pacific show different scenarios. Both show localized staining resembling food vacuoles (figure 4*b*), but one also shows signal localization within the plastid (figure 4*c*) that likely results from staining of an acidic thylakoid lumen. Staining by acidotropic probes therefore does not ensure the presence of food vacuoles and non-feeding related staining is more likely to occur in photosynthetic (due to lumen acidity) compared to heterotrophic protists. To assess the taxonomic identity of putative predatory protists, we also sorted individual cells based on the presence of green staining by LysoTracker, with the cells also showing red (chlorophyll) fluorescence representing putative mixotrophs (figure 5). Multiple displacement amplification (MDA) was used to amplify DNA in the sorted cells, followed by 18S and 16S rRNA V4 amplicon sequencing. The putative mixotrophs sorted in May 2014 and identified by either their nuclear (18S) or plastid (16S) rRNA sequence consisted of cryptophytes, prymnesiophytes and several photosynthetic stramenopile groups, including chrysophytes and dictyochophytes, but also diatoms (figure 5*b*, electronic supplementary material file S3). While most of these groups include mixotrophic species and their recovery is therefore in line with the assumption that the acidotropic dye signal originates from food vacuoles, diatoms do not. Moreover, water collected from the same station six weeks earlier showed a distinct population of LysoTracker positive, pigmented eukaryotes, which consisted entirely of photosynthetic stramenopiles, more specifically the diatom genus *Minidiscus* (figure 5*d*). Diatoms have been studied extensively, but not found capable of predation, and the signal most likely originated from the acidic silica deposition vesicles during the formation of their frustules [91]. Although the dye signal was somewhat weaker compared with the putative mixotrophs sorted later, it was higher than the control. Notably, some members of the groups captured in May also make siliceous structures, which could be the source of signal. The question remains open whether it is possible to reliably define a threshold to distinguish signals derived from food vacuoles versus other acidic compartments, and so far, the risk of protists falsely identified as mixotrophs needs to be considered when applying acidotropic probes in natural communities. Thus, the gold standard here would be to visualize the cells after staining to ascertain that they contain a clear (stained) food vacuole and presumably prey cells (figure 4). Complementing the higher throughput attained by flow

cytometry with high-resolution imaging techniques to verify co-associations detected with labelled prey, or staining of acidic vacuoles, could fully use the strengths of these techniques in future studies.

(c) Detection of prey assimilation

Detecting benefits of ingestion for growth is key to establishing mixotrophy; however, such data are nearly impossible to acquire from field studies. Alternatively, assimilation of carbon from stable-isotope or radioactively labelled prey can also be tracked and compared with carbon derived from photosynthetic carbon fixation. In culture experiments, the primarily heterotrophic growth strategy in the freshwater chrysophte *Ochromonas* sp. strain BG-1 has been confirmed by detection of assimilated carbon and nitrogen-stable isotopes by NanoSIMS [92], while the use of radioactively labelled prey revealed preferential assimilation of prey-derived carbon into proteins in the dinoflagellate *Karlodinium micrum* [40]. To distinguish prey assimilation by mixotrophs in nature from mere co-association of labelled putative prey with a mixotrophic predator, the label should be detected in molecules specific for the potential mixotroph. Protistan predators of the picocyanobacteria *Prochlorococcus* and *Synechococcus* were identified by RNA stable isotope probing (RNA-SIP) in the oligotrophic North Pacific and included presumably mixotrophic members of the Prymnesiophyceae and stramenopiles, specifically Dictyochophyceae and *Bolidomonas* [70]. Also grazers feeding on *Micromonas pusilla* CCMP1545 that were identified by DNA-SIP included mixotrophic dinoflagellates in a productive site in the eastern North Pacific [93]. Both RNA- and DNA-SIP rely on physical separation of heavy and light nucleic acids by density gradient ultracentrifugation, which requires relatively high isotope enrichment. This might result in a bias against detection of mixotrophs owing to dilution of stable isotope signals from assimilated prey by inorganic carbon acquisition via photosynthesis. A potentially more sensitive alternative is the use of bromodeoxyuridine (BrdU), a thymidine substitute, as a label in bacterial prey. BrdU uptake from bacterial prey and incorporation into mixotroph DNA can be detected by immunoprecipitation followed by amplicon sequencing [94]. While less sensitive to label dilution, this technique might be more prone to giving false positives. Both RNA/DNA-SIP or BrdU only detect predatory nutrition by following prey assimilation. Knowledge on the photosynthetic potential of mixotrophic predators usually comes from sequence similarity to known phototrophs, but could also be acquired by targeting the plastid-derived 16S rRNA gene of phototrophs for amplicon sequencing. While all of these methods are qualitative, quantitative methods for measuring prey assimilation in nature are urgently needed, but currently not available.

(d) Outlook: the need for single-cell techniques and reference data for understanding predatory mixotrophs and their roles in future oceans and lakes

The challenge to detect both photosynthesis and phagocytosis within the same cells calls urgently for single-cell approaches. The predicted increases in temperature of the surface ocean mixed layer, or lake epilimnia, and reduced nutrient injection from deeper waters owing to strengthened stratification, alongside longer periods at high light intensity owing to a shallower mixed layer depth [95,96], will all affect phytoplankton communities [97–99] including mixotrophs (figure 6). Elevated temperatures can cause shifts in the nutritional balance of mixotrophs, observed in two freshwater mixotrophic chrysophytes so far. The more heterotrophic *O. globosa* shifted further towards heterotrophy with increasing temperature under saturating light, nutrient and prey availability [38], while the more phototrophic *Dinobryon sociale* became less heterotrophic at higher temperatures, when grown with ambient bacterial densities present in non-axenic cultures [63]. Strengthened nutrient limitation could further stimulate higher ingestion rates, to compensate for the reduced availability of dissolved nutrients in the future surface ocean or oligotrophic lakes. On the other hand, if phagocytosis mainly serves as a route for carbon acquisition, increased light intensity in future surface mixed layers could reduce the requirement to feed [25]. A first step is to know who has the capacity for predatory mixotrophy and who does not. Building on 18S rRNA gene screening of sorted individual cells (figure 5*b*), with staining of specific attributes such as acidic vacuoles, alongside confirmation of food vacuoles' presence through assessment of localization (figure 4*b*), it should be possible to retrieve genome and transcriptome data from predatory mixotrophs. Partial genome assemblies have already been generated from population-based sorts of photosynthetic eukaryotes [100] and from merged data from individual cells stained with LysoTracker, but lacking chlorophyll [101–103]. These types of data promise to inform us on the potential for predation.

One important consideration is that, while oxygenic photosynthesis is well characterized at the protein level, and strongly conserved, the situation is more difficult for phagocytosis. Although phagocytosis is an ancient eukaryotic trait, many of the proteins required are poorly conserved, poorly known or not specific to phagocytosis. The Arp2/3 complex is an example of the latter case: it initiates the formation of branched actin filaments necessary for the phagocytotic ingestion of prey, but is also necessary for other modulations of cell shape [104]. Similarly, many proteins involved in vesicle trafficking and prey digestion might be shared in the process of autophagy, which is responsible for recycling of ageing cellular components in both multicellular organisms and protists [105]. Moreover, proteins comprising phagosomes from members of different eukaryotic supergroups have been shown to be divergent [106], although this finding may also be influenced by insufficient detection of the proteomic approach employed. Thus, prediction of phagotrophic potential from genomic data would be best supported by reference information from multiple eukaryotic Supergroups—and by necessity, by genomic data from uncultured mixotrophs for which relevant cultures do not exist.

Single cell amplified genomes (SAGs) can further provide the necessary references for meta-omics approaches, for instance, for mapping of metatranscriptomic [107] or metaproteomic data to specific taxa based on available SAGs. In this way, they can help to acquire information on activities of individual taxa in nature, and in the future might even be used for more quantitative stable isotope approaches, such as protein-SIP in which isotopic enrichment of peptides can be accurately quantified by mass-spectrometry [108,109]. In contrast to RNA/DNA-SIP, already low amounts of isotope incorporation can be quantified, promising suitability of protein-SIP for rate measurements, which—with the necessary reference

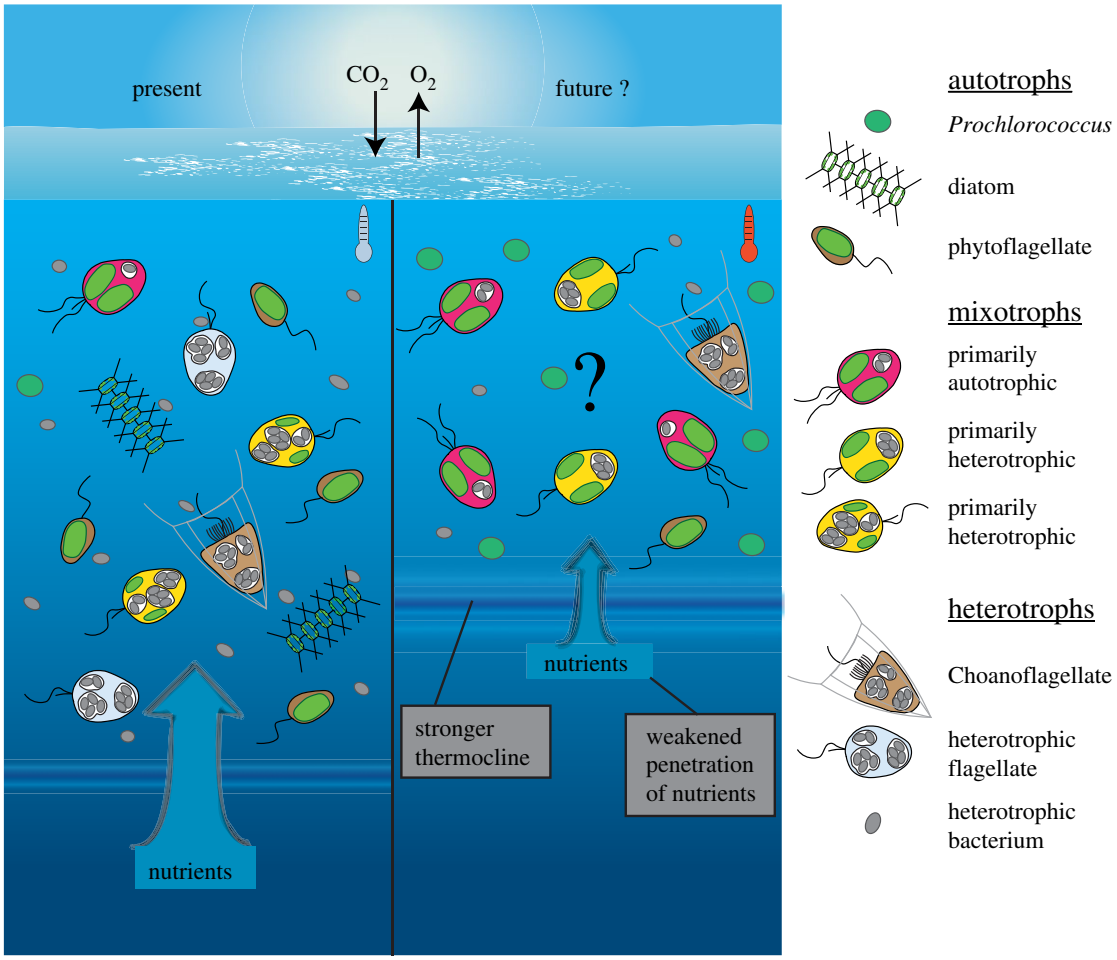

Figure 6. Cartoon of predicted changes in community composition in future oceans. The stronger stratification and shallower mixed layer depth expected in the future ocean (right) result in warmer, more oligotrophic waters subject to higher average light intensity. Protists are shown with photosynthetic potential (plastids in green) and/or with phagocytotic potential (food vacuoles containing bacterial prey). Future conditions are expected to favour strong competitors for nutrients like *Prochlorococcus* [97], but effects on mixotrophs remain unclear and might be species-specific. Some mixotrophs might shift towards increasing importance of phagotrophy (in pink), while others might shift towards increasing importance of photosynthesis (in yellow). Overall, mixotrophs are expected to be favoured under future conditions compared to specialist autotrophic or heterotrophic competitors.

sequences in hand—can be assigned to individual taxa. The use of both stable-isotope labelled prey and inorganic resources for photosynthetic uptake in separate incubations could thus allow quantification of the nutritional balance of mixotrophs with high phylogenetic resolution.

Single-cell methods can advance understanding of predatory mixotrophs in ways that extend beyond acquiring genome data. For example, chlorophyll fluorescence (referred to from here on as CF) measurements of live organisms can provide information regarding the absorption of light and flow of electrons through the photosynthetic apparatus [110,111]. A common component of CF, known as the maximum quantum yield of PSII (Fv/Fm), estimates the proportion of light absorbed by chlorophyll that is then used by the PSII reaction centre during photosynthesis. The maximum quantum yield of PSII provides a useful proxy for the overall health of the chloroplast and its photosynthetic ability. Chlorophyll fluorescence is increasingly used to estimate primary productivity from bulk water samples [112–114]. At the single-cell level, microscopy-based measurements can provide a powerful tool for exploring how various photosynthetic properties differ across species or populations [115], and have already been applied in cultured kleptoplastidic mixotrophic dinoflagellates [116]. Importantly, higher throughput options can facilitate studies of relatively low concentrations of predatory mixotrophs in the field. Custom-built flow cytometers capable of measuring Fv/Fm have been used to study impacts of iron enrichment in the open ocean [117,118]. In these studies, low signal-to-noise ratios limited reliable measurements to larger cell sizes (greater than 5 µm) with higher chlorophyll content. Recently, a similar approach has been used to look at the role of nitrate limitation in photosynthetic poise and lipid bioaccumulation (through staining) in the diatom *Phaeodactylum tricornutum* [119], highlighting the power of coupling live CF measurements with traditional cell phenotyping via flow cytometry. CF-enabled flow cytometers are not yet commercially available and additional reference studies on diverse cultured mixotrophic protists are clearly needed. These must characterize the physiological shifts associated with predatory behaviour, modes of nutritional acquisition, photophysiological signatures and the genomic repertoire of phagosomes from across eukaryotic supergroups. This includes robust experiments that characterize cell states that potentially interfere with the reliable detection of phagotrophy, such as outer membrane stickiness or presence of acidic compartments that are not food vacuoles, as well as variable conditions under which these occur. Collectively, such studies will provide the framework for interpretation of data recovered from individual uncultured predatory mixotrophs in the field and their activities in the environment.

Direct effects of changing environmental conditions on the physiology of mixotrophs will differ across species and shifts towards an increased importance of phagocytosis or photosynthesis to their nutrition are both possible [38,63] (figure 6, pink and yellow cells). Additional indirect effects will likely alter prey availability and competitive interactions with specialist autotrophic or heterotrophic protists. While the combined effects of different environmental drivers on mixotrophs remain unstudied, the generally more oligotrophic conditions predicted with increased surface ocean stratification are expected to favour mixotrophs over purely autotrophic or heterotrophic competitors [25,30]. Apart from the picocyanobacterium *Prochlorococcus*, which is well adapted to ultraoligotrophic conditions owing to its small size [97], the abundance of larger, purely autotrophic eukaryotes might decline, as might purely heterotrophic flagellates that could be outcompeted by mixotrophs (figure 6). The past developments of oceanographic proxies for primary productivity were grounded on a solid cell biological and physiological understanding of photosynthesis. Similarly, reliable proxies for marine mixotrophy will require a solid understanding of the process based on the cell biology and physiology of taxa from each of the major eukaryotic supergroups. Because of the uncultured nature of many putatively mixotrophic lineages, quantitative methods for assessing the abundance, diversity and metabolic activities of mixotrophs will require cross-scale studies. These by necessity must incorporate single-cell techniques combined with targeted imaging and stable isotope probing to gain baseline information on uncultured mixotrophs in the wild. Additionally, refined modeling efforts supported by differentiated traits representing varied mixotrophic strategies will be key in understanding transitions in plankton communities.

Data accessibility. Sequences reported in this paper have been submitted to the GenBank Sequence Read Archive under Bioproject #PRJNA540178.

Authors' contributions. S.W. and A.Z.W. conceived the study. M.H., J.N., M.C.-L. and A.Z.W. designed the *Micromonas* experiments; M.H., M.C.-L., J.N., C.E. performed experiments; and M.H., M.C.-L., J.N., C.C.M.Y. and S.W. analysed data. A.Z.W., C.P. and S.W. designed the FACS-sorting initiative, C.P. performed the sorting and C.C.M.Y. analysed sequence data. S.W., A.Z.W. and K.H. wrote the manuscript, and C.C.M.Y., M.H., C.E. and C.P. edited the manuscript, and all authors gave final approval.

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