Deuterium in marine organic biomarkers: toward a new tool for quantifying aquatic mixotrophy

Summary

The traditional separation between primary producers (autotrophs) and consumers (heterotrophs) at the base of the marine food web is being increasingly replaced by the paradigm that mixoplankton, planktonic protists with the nutritional ability to use both phagotrophy and photo(hetero)rophy and photo(auto)rophy to access energy are widespread globally. Thus, many ‘phytoplankton’ eat, while 50% of ‘protozooplankton’ also perform photosynthesis. Mixotrophy may enhance primary production, biomass transfer to higher trophic levels and the efficiency of the biological pump to sequester atmospheric \( \text{CO}_2 \) into the deep ocean. Although this view is gaining traction, science lacks a tool to quantify the relative contributions of autotrophy and heterotrophy in planktonic protists. This hinders our understanding of their impacts on carbon cycling within marine pelagic ecosystems. It has been shown that the hydrogen (H) isotopic signature of lipids is uniquely sensitive to heterotrophy relative to autotrophy in plants and bacteria. Here, we explored whether it is also sensitive to the trophic status in protists. The new understanding of H isotope signature of lipid biomarkers suggests it offers great potential as a novel tool for quantifying the prevalence of mixotrophy in diverse marine microorganisms and thus for investigating the implications of the ‘mixoplankton’ paradigm.

Introduction

Marine ecosystems play a pivotal role in global photosynthetic carbon fixation (Field et al., 1998; Falkowski et al., 2000). Their activity contributes to maintaining the balance between \( \text{O}_2 \) and \( \text{CO}_2 \) in the atmosphere and consequently to keeping climate relatively stable. The biological gravitational pump exports between c. 4.0 and 9.1 Pg of particulate organic carbon from surface waters annually (Boyd et al., 2019). Most current marine biogeochemical models assume that the plankton community is clearly divided into autotrophic phytoplankton and heterotrophic zooplankton (Duarte et al., 2013; Flynn et al., 2013; Williams et al., 2013; Leles et al., 2018). Increasingly, it is recognised that there is no strict separation between producers and consumers (Fig. 1) and that photo(auto)rophic and phag( hetero)rophic behaviours are not mutually exclusive. Indeed, most of the protist unicellular organisms at the base of the plankton food web cannot be regarded strictly as producers or consumers (Flynn et al., 2013, 2019; Mitra et al., 2014). Modelling suggests that mixoplanktonic activity (i.e. nutrition involving both autotrophy and phagotrophy) enhances primary production, biomass transfer to higher trophic levels and the biological carbon pump by up to 35% (Mitra et al., 2014; Ward & Follows, 2016; Leles et al., 2021). To this, we add the renewed interest in mixotrophy supported by a combination of photosynthesis and osmotrophy in diatoms and also in oceanic prokaryote phytoplankton (e.g. Yelton et al., 2016; Benavides et al., 2017; Muñoz-Marín et al., 2020). The mixoplanktonic behaviour of many toxic protists also likely explains their ecological success and the occurrence of harmful algal blooms (HABs) (Burkholder et al., 2008) that can severely affect coastal ecosystems and their services. Changing the prevalence of mixotrophic behaviours (involving osmotrophy and/or phagotrophy) within the plankton community could have a large impact on the global carbon cycle and thus on the climate. For example, a large >50-yr time series analysis suggests an increase in the relative abundance of diatoms vs dinoflagellates (Hinder et al., 2012). Another example, using quantative niche models, suggests that oceanic cyanobacterial communities will experience complex changes as a result of projected future climate conditions (Flombaum et al., 2013). These changes may result in different mixotrophic activities in the surface ocean and affect the efficiency of the biological pump. Thus, there are a number of reasons for having a better understanding of the role of mixotrophy in marine microbial ecology.
Even though mixoplankton (i.e. photosynthetic microbes capable of phagotrophy or ‘photo-osmo-phago-trophic’ plankton) are a key component of marine ecosystems (Leles et al., 2017, 2019), with all other phytoplankton capable of mixotrophy via osmophagy (Flynn et al., 2019), no tool currently exists to infer, both spatially and temporally, the trophic mode and the level of heterotrophic growth within different plankton groups. Tracking protist trophic modes, without an appropriate tool, in modern oceans is challenging as their species-specific metabolism and trophic adaptability remain largely unknown. Tracking their behaviour over geological time presents even greater challenges. For example, it was recently suggested that protists, mainly lopa-phytes, ‘turned to hunting’ in order to survive the end-Cretaceous impact (Gibbs et al., 2020). However, due to the absence of any direct proxy, only indirect lines of evidence, such as eco-evolutionary modelling and microfossils, are available to support this hypothesis. Only a fraction of protist taxa produce an observable microfossil in sediments (e.g. Radd et al., 2013; Cormier et al., 2016; Sophie et al., 2021). Such difficulties, combined with the need for data to better constrain biogeochemical models, drive the need for a means of tracking protists’ metabolic behaviour (particularly the prevalence of mixoplanktonic lifestyle) in response to global environmental changes (Chavez et al., 2011). This would facilitate the incorporation of mixotrophy into marine ecosystem models that are used to examine the future effects of anthropogenic perturbation on biogeochemical cycling in the global ocean.

Stable isotope ratios

Variations in the relative abundance of stable isotopes of diverse elements (e.g. hydrogen, carbon, nitrogen and oxygen) give important information about (bio)geochemistry and paleoclimate. The measurement of their relative abundance, as preserved in geological and biological archives (e.g. sediment cores, tree rings, plankton and herbaria), is ubiquitous over a broad range of earth science studies. Generally expressed with a delta notation (Werner et al., 2016), vascular plants (Luo & Sternberg, 1992; Cormier et al., 2018), in particular orchids (Gebauer et al., 2016; Schiebold et al., 2018), and parasitic plants (Cormier et al., 2019). Despite these indications, the application of the δ2H content in organic biomarkers has been largely limited to (palaeo)hydrological or salinity studies. Indeed, there have been various studies focused on using δ2H values of organic material from terrestrial plants to reconstruct hydrology (Sachse et al., 2012), and a handful of studies on marine phototrophs, with a focus almost entirely on the effects of salinity on lipid δ2H values (Kasper et al., 2014). Nonetheless, the effect of metabolism on δ2H values provides evidence that δ2H values can offer other valuable biogeochemical information (Estep & Hoering, 1980; Ziegler, 1989; Luo & Sternberg, 1992; Zhang et al., 2009; Meer et al., 2015; Gebauer et al., 2016; Cormier et al., 2018, 2019). Here, we suggest that δ2H values of marine organic material could become a useful tool to study the role of mixotrophy in marine ecosystems.

Two main factors determine the δ2H values of compounds produced by aquatic organisms: (1) the δ2H value of the source water (Chikaraishi & Naraoka, 2003); and (2) the biosynthetic δ2H fractionation (δ2Hbio) (Sternberg et al., 1984; Yakir & DeNiro, 1990; Luo et al., 1991; Zhang & Sachs, 2007). The latter involves several biochemical pathways and is calculated as the δ2H fractionation between water and the synthesised organic material. Because of the strength of the C-H bond in lipids, δ2H values of lipids are relatively stable over time. Isotopic exchanges of C-bound H can nevertheless slowly occur over geological timescales. Such exchange can be recognised (e.g. via comparison of coeval n-alkyl and isoprenoid hydrocarbons) and does not preclude the valuable use of δ2H values in geological studies (Sessions, 2016). Most palaeostudies using δ2H derived from terrestrial plant biomarkers have considered δ2Hbio to be constant within a species (Sachse et al., 2006) simplifying the application of biomarker δ2H values as a (palaeo)hydrological proxy (Rach et al., 2014, 2017; Ladd, 2021). In plankton, however, salinity and growth rate are also known to influence 2H-enriching post-photosynthetic fractionation pathways (Englebrecht & Sachs, 2005; Schouen et al., 2006). It is mainly because of the influence of salinity on δ2Hbio that δ2H values can be used to derive palaeosalinity in aquatic basins (Ladd & Sachs, 2015). Evaporation in (semi-)closed aquatic basins also influences both salinity and source water δ2H values, which is correspondingly recorded in the δ2H values of the biomarkers produced in those basins (Nelson & Sachs, 2016).

Most models of δ2H values in organic compounds have endeavoured to minimise the impact of metabolism in order to explain the variability in δ2H values as a result of a specific environmental parameter. Roden et al. (2000) suggested that in plants, 2H-depleting photosynthetic fractionation pathways and 2H-enriching post-photosynthetic fractionation pathways determine a constant value for δ2Hbio. While this approach is very useful for interpreting hydrological conditions from cellulose in tree rings, it does not capture the effects of environmental change on the

There are indications that the relative abundance of hydrogen (H) isotopes in lipids (i.e. deuterium (2H) and protium (1H), expressed as δ2H values) is highly sensitive to the metabolism of bacteria (Zhang et al., 2009; Wijker et al., 2019), vascular plants (Luo & Sternberg, 1992; Cormier et al., 2018), and parasitic plants (Cormier et al., 2019).
balance between photosynthetic and post-photosynthetic processes and their impact on $\delta^{2}H$ values. Additionally, the approach has not yet been extended to compounds other than cellulose or to taxa other than angiosperms.

**A new conceptual view of H isotope ratios**

To fill these gaps, a conceptual biochemical model has been proposed to describe how post-photosynthetic processes imprint a strong metabolic signal in $\delta^{2}H$ values of plant-derived organic compounds (i.e. up to 60%o) in response to environmental changes (Cormier et al., 2018, 2019). These biochemical pathways leave a metabolic signal on the $\delta^{2}H$ values because they induce a different isotopic fractionation. The model expresses that, overall, photosynthetic pathways deplete organic compounds in $^{2}H$, while post-photosynthetic (or heterotrophic) pathways enrich compounds in $^{2}H$.

Specifically, in Cormier’s model, the photosynthetic carbohydrate supply rate affects $^{2}H$-bio for carbohydrates and lipids (Fig. 2). This pattern is mostly driven by the carbohydrate pool size, the cycling rates of individual organic molecules in their respective

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**Fig. 2** Schematic view of H flow during processes leading to lipids, polysaccharides and nonstructural carbohydrates (NSC) $^{2}H$-bio. The key enzymes and pathways responsible for H flow are indicated by their following abbreviations and are based on known biochemical pathways: 2-OGDH, 2-oxoglutarate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; ACP, acyl carrier protein; ALD, aldolase; ENO, enolase; FNR, ferredoxin-NADP$^{+}$ reductase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KA, ketoacyl; ME, malic enzyme; NADP, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; oxPPP, oxidative pentose phosphate pathway; PDH, pyruvate dehydrogenase; PGI, phosphoglucose isomerase; PK, pyruvate kinase; R, reductase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TE, trans-enoyl; TPI, triosephosphate isomerase; and TPT, triose phosphate translocator. Simplified from Cormier et al. (2018) and references therein.
pools, the associated exchange of C-bound H with \(^{2}H\)-enriched cellular water and preferential removal of light \(^{1}H\) via the oxidative pentose phosphate pathway (oxPPP) (Cormier et al., 2018). Consequently, a high photosynthetic (e.g. autotrophically dominated) carbon supply results in \(^{2}H\)-depleted organic molecules, reflecting the \(^{2}H\)-depleted signal of NADPH generated by the light reaction of photosynthesis (i.e. grey zone; Fig. 2). By contrast, a low photosynthetic (e.g. heterotrophically dominated) carbon supply leads to \(^{2}H\)-enriched lipids, where higher cycling rates of individual organic molecules are associated with an increasing exchange of C-bound H with \(^{2}H\)-enriched cellular water and removal of \(^{1}H\) from the C-skeletons via the oxPPP. While such a model highlights the likely considerable effect of fluxes through metabolic networks on H isotopic fractionation (Kruger & Ratcliffe, 2015), it also explains why a metabolic signal in H isotopic fractionation due to a shift in trophic behaviour is discernible in diverse biological organisms. As such, Gebauer et al. (2016) recently adopted this rationale successfully for analysis of environmental samples to study orchid ecology and the prevalence of heterotrophic and mycoheterotrophic orchid taxa in Europe. Similar to most marine protists, orchids behave auto-, mixo- and heterotrophically. The successful application of \(\delta^{2}H\) values to study orchid ecology underlines the potential of compound-specific \(\delta^{2}H\) values as a metabolic proxy in diverse environments and biological systems. In plants, studying such biochemical effects is greatly complicated by the spatial and temporal variation of the isotopic composition of water, the H source (i.e. within soil–water gradients and throughout plant stems and leaves). By contrast, sea water has an essentially constant \(\delta^{2}H\) value in comparison with the scale of the biological fractionations, allowing a primary focus on biochemical variations in plankton. In preliminary work (Supporting Information Notes S1), we have observed that hydrogen isotopic fractionation during fatty acid biosynthesis in the green algae Chlorella sorokiniana is also sensitive to heterotrophy (Fig. 3). This metabolic sensitivity represents a fundamentally different philosophical approach to most previous studies of \(^{2}H\) fractionation in photo(auto)trophs, which have assumed near-constant biochemistry and sought to reconstruct environmental variables.

The way towards the new tool

Laboratory-based calibrations are essential for in situ monitoring of protist trophic behaviours (including phago(hetero) trophic vs
photo(auto)trrophic growth) using isotopic measurements of lipid biomarkers from environmental samples. For instance, the magnitude of the photosynthetic $^{2}$H fractionation, which occurs during the light reaction of the photosynthesis (Fig. 2, grey zone), has only been estimated by Yakir & DeNiro (1990) who reported a fractionation of $-171\%$ for cellulose in the multicellular aquatic plant *Lemna gibba*. This value, although widely utilised, most likely varies appreciably between organisms and trophic behaviours. Moreover, even if some variables are known to influence post-photosynthetic $^{2}$H fractionation during lipid biosynthesis (e.g. temperature, salinity, light intensity, growth rate, biosynthetic pathway, metabolic network and metabolic source of NADPH), a comprehensive understanding of how these variables impact, individually and synergistically, on $^{2}$H fractionation in marine microbes is still lacking (Fig. 4).

With the appropriate investigations targeting these variables, compound-specific isoee analysis has the potential to provide a valuable research tool: a metabolic proxy for the quantitative assessment of the ratio between autotrophic and heterotrophic metabolism in diverse marine microorganisms and their contribution to the global carbon cycle in modern and, potentially, palaeoenvironmental contexts (i.e. assuming that the metabolic effects can be deconvoluted from the environmental and sedimentological effects using other proxies). Moreover, defining the metabolic influences on $^{2}$H isotope fractionation during biomarker synthesis will provide a much better understanding of their $\delta^{2}$H values in response to changes in environmental conditions and will improve their utility as versatile palaeoecological proxies for, *inter alia*, temperature (Feng & Epstein, 1994), hydrological conditions (Sachse *et al.*, 2012) and sea surface salinity (Kasper *et al.*, 2014).

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**Author contributions**

M-AC, REMR and NJK planned and designed the research. M-AC, J-BB and GB designed and performed the experiment and analysed the data. M-AC and J-BB performed chemical measurements. M-AC, KJF and REMR wrote the manuscript with contributions from CNT, DJM and RSL.

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**Data availability**

The data that support the findings of this study are available in Supporting Information Notes S1.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Notes S1** Cultures of *Chlorella sorokiniana* and compound-specific isotope analyses.

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