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EDITED BY
Paco Bustamante,
Université de la Rochelle, France

REVIEWED BY
Andrey Tikunov,
University of North Carolina at Chapel Hill, United States
Valerie Thiery,
Université de la Rochelle, France

*CORRESPONDENCE
Christian Bock
Christian.Bock@awi.de

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^{13}C-enrichment NMR spectroscopy: a tool to identify trophic markers and linkages

Christian Bock*, Tianyi Zhao, Sandra Götze, Felizitas C. Wermter and Gisela Lannig

Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany

Current climate change, particularly ocean warming, will induce shifts in marine species distribution and composition, affecting the marine food web and, thus, trophic interactions. Analyses of the stable isotopes ^{13}C and ^{15}N are commonly used to detect trophic markers for food web analyses. With the current standard methods used in food web ecology, it is still challenging to identify potential changes in the uptake and utilization of trophic markers. In this work, we present a ^{13}C-enrichment analysis by NMR spectroscopy to track the uptake and utilization of dietary carbon in a simple laboratory experiment of a primary producer and its consumer (algae and bivalve). In particular, we tested the hypothesis of a temperature-dependent use of dietary carbon by tracing the incorporation of ^{13}C-atoms. Unicellular phytoplankton, *Phaeodactilum tricornutum*, was reared in a medium containing ^{13}C-labeled bicarbonate. The accompanying ^{13}C-NMR spectra of labeled *P. tricornutum* showed a specific profile of ^{13}C-labeled compounds, including typical trophic markers such as the polyunsaturated omega-3 fatty acid eicosapentaenoic acid (EPA). Afterwards, ^{13}C-labeled *P. tricornutum* was fed to King scallops, *Pecten maximus*, kept at two different temperatures (15°C and 20°C). Tissue-specific NMR spectra of *P. maximus* revealed elevated ^{13}C-NMR signals, particularly of the fatty acid EPA in the digestive gland, which was not evident in muscle tissue. The comparison between the two temperatures indicated a change in trophic markers. At the higher temperature, less unsaturated fatty acids were detected in the digested gland, but increased ^{13}C-labels in sugars were detected in the adductor muscle. This might indicate a change in the uptake and utilization of the trophic marker EPA in *P. maximus* due to a shift in energy conversion from favored beta-oxidation at colder temperatures to conversion from carbohydrates in the warmth. Our approach indicates that besides the accumulation of trophic markers, their incorporation and conversion are additional important factors for the reliable interpretation of trophic linkages under climate change.

KEYWORDS

nuclear magnetic resonance, stable isotopes, ^{13}C-labeling, food web, climate change

Introduction

Current and ongoing climate change is accompanied by warming, ocean acidification, and deoxygenation, leading to oxygen minimum zones of the oceans (Pörtner et al., 2022). This directly impacts ectothermic organisms through increased energy demand and supply (Pörtner et al., 2017). In this context, the importance of the interactions between energy supply, energy storage, and energy allocation and their trade-offs in climate change has only recently been emphasized (Grunst et al., 2023). However, climate change will not only affect the organism`s energy budget. Still, it will also lead to shifts in the species diversity in the ecosystems, with species migrating out of an ecosystem and new species migrating in (Pinsky et al., 2020). In particular, phytoplankton communities will change their abundance and distribution (Hays et al., 2005). This results in earlier plankton blooms and a shift in distribution towards more thermophilic species, such as cyanobacteria and dinoflagellates (Paerl and Scott, 2010). This will have consequences for the quantity and quality of the phytoplankton as a food resource. Indeed, it is long known that the lipid composition of phytoplankton shifts to less unsaturated fatty acids at higher temperatures (Thompson et al., 1992). The potential impact of climate change changing diets is currently being extensively discussed for the availability of essential fatty acids, like omega-3 long-chain polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are important for almost all organisms' survival, development and growth (for a recent review, see Tan et al., 2023).

Last but not least, there will be alterations in behavior due to climate change, which will, for example, lead to changes in the feeding behavior of organisms. This factor has been given limited attention in the literature on the effects of climate change. Only recently, Gauzens et al., 2024 reported on potential changes in the foraging behavior of predators under warmer conditions, which can lead to unexpected effects, such as a lower consumption efficiency despite increased energy demand in the warmth (Sokolova et al., 2012). However, the underlying mechanisms for this are little or not understood. This might restructure marine food webs with potentially severe consequences, as already apparent in the Arctic (Kortsch et al., 2015). There is, therefore, an urgent need to better understand the potential changes in marine food webs and their trophic linkages to make reliable predictions about consequences for the biodiversity in an ecosystem. Food web ecology to describe trophic linkages and relationships is based on three basic techniques: stomach content analysis, stable isotope analysis, and fatty acid or sterol analysis. All three methods have advantages and disadvantages, extensively discussed in the relevant literature (for an overview, see Pasquaud et al., 2007).

Analyzing stable isotopes and determining fatty acids allows for insights into the energy transfer and energy flow in a food web and across predator-prey interactions. Both techniques can identify trophic markers and their linkages and relationships. In the following, we will present a method that integrates both analyses and is particularly suitable for detecting trophic markers. Stable isotope analysis has a long-standing tradition for tracing food- and trophic biomarkers to follow trophic linkages and energy flow (Peterson and Fry, 1987). Such investigations are extended to study the impact of environmental changes in ecosystems (for recent reviews, see Cresswell et al., 2020; Hobson, 2023). Nowadays, such studies on trophic biomarkers and linkages can be easily performed with the combined analysis of stable isotopes such as ^{13}C and ^{15}N, where carbon elucidates the energy flow, while nitrogen gives the position at the trophic level (Pasquaud et al., 2007). Stable isotope analysis recently gained a new renaissance due to the development of compound-specific isotope analysis (CSIA) tools, particularly for bulk stable isotope and fatty acid composition analysis (Twining et al., 2020).

^{13}C-enrichment NMR spectroscopy is a unique tool to follow the uptake and incorporation of carbon in an organism due to the magnetic properties of the ^{13}C-isotope in contrast to the ^{12}C-isotope with a missing nuclear spin. ^{13}C-enrichment NMR spectroscopy has been extensively used in studies (*in vitro* and *in vivo*) and has a long tradition in physiology and medical research. In particular, it allows the detection of ^{13}C, which was incorporated in carbohydrates, such as glucose (Shulman and Rothman, 2001), amino acids (Morris and Bachelard, 2003), and fatty acids (Hwang et al., 2003). Since the ^{13}C-isotope can be used to trace food sources and help to identify trophic markers in aquatic organisms, it is obvious that applications from biomedical research should be transferred to the challenges in trophic ecology. Therefore, the present study aimed to demonstrate the applicability of ^{13}C-enrichment NMR spectroscopy to follow the energy flow in combination with the detection and analysis of incorporated carbohydrates, amino acids and fatty acids in a simple food chain model under global warming conditions. In the first step, ^{13}C-enrichment NMR spectroscopy was used to follow the incorporation of ^{13}C-atoms into energy-rich substances in the unicellular diatom, *Phaeodactilum tricornutum*, after cultivation in a medium containing ^{13}C-labeled bicarbonate. In a second step, the algae were subsequently fed to our animal model consumer, the King scallop, *Pecten maximus*, at two different temperatures (temperate 15°C as the control temperature versus a moderate-warm temperature of 20°C). Finally, the incorporation of carbon sources into the scallop`s digestive gland and adductor muscle was screened using ^{13}C-enrichment NMR spectroscopy.

Two objectives should be demonstrated in this work using ^{13}C-enrichment NMR spectroscopy. Firstly, identifying trophic markers via ^{13}C-tracing and their fate after absorption into another organism. Secondly, the potential for detecting changes in the use of metabolic substrates and energy conversion caused by climate warming.

Material and methods

Algae cultivation and incubation

Phaeodactylum tricornutum cultures were obtained from BlueBioTech GmbH (Büsum, Germany). Four batch cultures with $1*10^5$ cells/mL starting concentrations were set up and incubated in

f/2+Si medium (Guillard, 1975) with 0.2µm filtered seawater. In a trial experiment, unlabeled bicarbonate was replaced by ^{13}C-labeled bicarbonate (Sigma/Aldrich, Germany) and added to the batch cultures in 1 mM and 3 mM (duplicate), respectively. Algae cultures were incubated inside a temperature control room with a constant temperature of 15°C. The light was constantly provided by a white fluorescent tube with a light intensity of 64.25 µEinstein m^{-2} s^{-1}. Algae were left growing undisturbed for 7 days. Cell concentrations of each incubation bottle were determined using Utermoehl counting chambers and were manually counted under an Axio Observer (Zeiss, Germany). After incubation, the algae were transferred to falcon tubes and centrifuged for 10 minutes at 800rcf at 4°C. This procedure was repeated twice. The supernatant was discarded, and the algae pellet was stored at -80°C until further processing.

For the feeding experiments, *P. tricornutum* was incubated under the same conditions again, but only with a final ^{13}C-labeled bicarbonate concentration of 3 mM and a starting concentration of $1*10^6$ cells/mL. After harvesting the ^{13}C-labeled cultivated algae, the algae were fed directly to the scallops.

Algae extraction

Algae pellets were prepared for NMR spectroscopy using a methanol-chloroform extraction protocol (adapted from Bligh and Dyer, 1959). $2*10^8$ cells were transferred to an ice-chilled precellys tube containing 400µL methanol and 125µL milli-Q water. The samples were homogenized using a precellys with 1 cycle of 20s at 6000 rpm (Precellys 24, Bertin technologies, France). Finally, 400µL chloroform and 400µL milli-Q water were added to the tubes and vortexed for 15s. After settling on ice for 10 min, the samples were centrifuged for 10 min with 3000 rpm at 4°C. The upper layer, containing the methanol soluble metabolites, was transferred to 1.5mL Eppendorf tubes and was dried in a vacuum concentrator (Speed-Vac, RVC 2-18 CD plus, Christ freeze dryers GmbH, Germany) for 12h. The lower part containing chloroform with the lipid phase was dried overnight in open brown glass bottles under a fume hood.

Tissue extraction

Extracts of muscle and digestive gland tissues of *P. maximus* were prepared for NMR spectroscopy using a similar extraction protocol as described by (Tripp-Valdez et al., 2017; Götze et al., 2020). Around 60 mg of grounded frozen tissue was taken and homogenized in methanol/water using a cell homogenizer (Precellys 24, Bertin Technology, France). In the second step, chloroform/water was added, vortexed, and settled on ice for 10 minutes. After centrifugation for 10 min at 3000 rpm and 4°C, the upper and lower layers were separated and dried as described for the algae extraction. The dried samples were stored in a fridge at -80°C until further processing.

Feeding experiment incubation and experimental protocol

A simple feeding experiment was designed for the giant scallop, *Pecten maximus*, under laboratory conditions (indoor simulated moderate-cold (15°C) and moderate-warm temperatures (20°C), in accordance with Bock et al., 2019. Both temperatures are typical temperatures, which these species experience during seasonal changes on the Atlantic coast.

For the feeding experiments, three individuals were placed in three separate water containers with a defined volume of 10 liters. The containers were continuously aerated with air and kept at their respective temperatures (15°C and 20°C, respectively). After one day of acclimation under starvation, a defined amount ($4*10^9$ cells) of ^{13}C-labeled algae was added to each single container. Scallops were fed daily for 3 days. It was ensured that the animals were open during feeding and thus had the opportunity to filter feed. After the feeding experiments, the animals were sacrificed, and samples from the digestive gland and striated muscle tissue were snap-frozen in liquid nitrogen and stored at -80°C for later analysis.

NMR spectroscopy

The prepared extracts were analyzed to incorporated ^{13}C-labeled compounds using ^{1}H- and ^{13}C-NMR spectroscopy. Using a methanol/chloroform protocol allowed us to analyze the cytosolic and lipid fractions within one sample, as described in (Amiel et al., 2020). In this way, we could detect carbohydrate and amino acid energy sources located in the cytosol and, in addition, the main and essential fatty acids located in the lipid fraction. The dried methanol phase, containing the cytosolic fraction of algae and tissue extracts, was dissolved in deuterated water (D_2O) containing 0.05% trimethylsilylpropionic acid (TSP, Sigma-Aldrich, USA) as chemical shift standard. The dried lipid fractions were dissolved in deuterated chloroform ($CDCL_3$) containing 0.03% tetramethylsilane (TMS) as the chemical shift standard for the lipid phase. All dried tissue samples were dissolved with a 1:1 ratio (initial tissue weight to volume) and transferred into 1.7 mm NMR tubes. Algae were either measured as a crude extract in the NMR spectrometer similar to Bock et al., 2024, or $2*10^8$ cells/mL were extracted (see above) and re-suspended in 60µL D_2O.

All NMR measurements were conducted on a wide bore vertical 9.4T NMR spectrometer (400 MHz) with Avance III HD electronics (Bruker Biospin, Germany). All samples were measured with a 1.7 mm diameter triple-tuned (^{1}H-^{13}C-^{15}N) probe at room temperature (21°C).

For ^{1}H-NMR measurements of cytosol samples, as well as for the measurements of the lipid phase, a classical Carr-Purcell-Meiboom-Gill, including f1 presaturation pulse sequence, was used. Subsequently, after each ^{1}H-NMR measurement, ^{13}C-enrichment NMR studies were performed using a 1D pulse and acquired sequence with gradient enhancement.

The acquisition parameters for the NMR measurements were as follows:

^{1}H-NMR spectroscopy: pulse program: Bruker protocol cpmgpr1d: Acquisition time (AQ) 4.01s, time domain (TD) 70656, sweep width (SW) 8802 Hz (22 ppm), delay (D1) 4s, dummy scan (DS) 4, number of scans (ns) 256.

^{13}C-enrichment NMR spectroscopy: pulse program: Bruker protocol zgig: TD 65536, SW 22055 Hz (220 ppm), AQ 1.485 s, D1 4s, DS 2, ns 8192.

^{1}H- and ^{13}C-NMR spectra were acquired from algae cultures raised under 1 mM and 3 mM concentrations of ^{13}C-labeled bicarbonate to find the optimal incorporation of the ^{13}C-label into the chemical compounds of *P. tricornutum* (n=3, respectively). NMR spectra of the cytosolic and lipid phases from tissue extracts from the muscle and digestive gland of *P. maximus* after feeding at the two temperatures (n=3, respectively) were acquired using ^{1}H- and C^{13}-NMR spectroscopy.

NMR processing

All NMR spectra were processed and analyzed using Bruker's NMR spectrometer software TopSpin 3.1 pl. ^{1}H-NMR spectra were zero-filled to 8k and processed using an exponential multiplication with a lb factor 0.5. Spectra were automatically phased, and the baseline was corrected using the TopSpin routine. ^{13}C-NMR spectra were zero-filled to 32k, and the first 350 points of the free induction decay (FID) were discarded before Fourier transformation. Afterwards, an exponential multiplication on the FID with a line broadening (lb) factor of 5 was applied, the spectrum was automatically phased, and the baseline was corrected. All spectra were calibrated to TSP or TMS to 0.0 ppm, respectively. Integrals from selected ^{13}C-labeled signals from ^{13}C-NMR spectra were calculated using the integral routine within TopSpin to determine relative concentration changes. The noise levels for the ^{13}C-NMR spectra of cytosol were set to a constant level; for the spectra of the lipid phase, the chloroform signal was set to a constant integral to assure a maximum comparability of integrals between spectra from different individuals. As a note, metabolite concentrations from ^{13}C-NMR signals for comparisons between spectra can be achieved via the natural abundance of the ^{13}C-NMR signal of the solvent as an internal calibration standard. In the case of chloroform, however, possible evaporation must be considered. Therefore, measuring the samples directly and consecutively is advisable to minimize the relative error. Integrals are given in arbitrary units of concentration and were saved in a csv file for comparison and further statistical analysis. The NMR spectra for the figures were prepared using MestReNova 14.2.0 (Mestrelab Research S. L., Santiago de Compostela, Spain).

^{13}C-NMR signals were assigned to specific metabolites and fatty acids according to NMR libraries from the literature (Spectral Database for Organic Compounds (SDBS), https://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi; Human Metabolome Database (HMDB), https://hmdb.ca/). In addition, ^{1}H-^{13}C-HSQC spectra were performed on some samples to confirm the identification of metabolites and lipid signals within the ^{1}H- and ^{13}C-NMR spectra (see Supplementary Figure S1).

Statistics

Statistically significant differences between the integrals of ^{13}C-labeled NMR signals of metabolites in the two temperature groups were determined using linear models. The linear models (function lm) were applied to the temperature groups using the statistical programming environment R (R Core Team, 2022), according to the tutorial by Winter (2013).

Results and discussion

The main aim of this study was to implement ^{13}C-enrichment NMR spectroscopy to detect trophic markers and track trophic linkages. To this end, under laboratory conditions, a simple feeding experiment was designed for the King scallop, *Pecten maximus*. Figure 1 compares ^{13}C-NMR spectra of algal extracts grown in media containing 1 mM and 3 mM ^{13}C-labeled bicarbonate, respectively. All ^{13}C-NMR spectra show a variety of ^{13}C-labeled NMR signals. The direct comparison of the spectra reveals an elevation of the most prominent signals in the ^{13}C-NMR spectra from the algae extracts incubated in 3 mM ^{13}C-labeled bicarbonate media. This is clear evidence for higher incorporation of ^{13}C-atoms into metabolites and lipids with increasing concentration of ^{13}C-labeled bicarbonate in the growth medium. All spectra were acquired with the same acquisition parameters and processed identically for a direct comparison. Despite the differences in concentration, no additional signals could be observed in the individual spectra, indicating that the incorporation of ^{13}C depends on the metabolization and relies on the concentration of ^{13}C-labeled bicarbonate. The contribution of ^{13}C-signals from compounds with natural abundance can be neglected with the selected measurement parameters. This can be concluded from the relatively low signal-to-noise ratio of the algae extracts obtained from *P. tricornutum* incubated in the growth medium with 1 mM ^{13}C-labeled bicarbonate, which is close to the detection limit (Figures 1A, C). Following the literature and to achieve the best possible incorporation of ^{13}C into the algae, a concentration of 3 mM ^{13}C-labeled bicarbonate was chosen for the upcoming feeding experiments.

In total, ^{13}C-labeled signals of seven main metabolites could be unambiguously assigned in the ^{13}C-NMR spectrum of the cytosolic phase of *P. tricornutum*, which belongs to glucose and amino acids (see Table 1). The metabolites identified are organic osmolytes, typically appearing in considerable concentrations in ^{13}C-NMR spectra of algae (e.g. Karsten et al., 1995). The signal pattern of the ^{13}C-enrichment NMR spectrum of the lipid phase from the algae compares very well with typical ^{13}C-spectra from classical oils, which are standardly used for the evaluation of ^{13}C-NMR lipid measurements (e.g. Hwang et al., 2003; Amiel et al., 2020).

^{13}C-NMR spectra of fatty acids can be very similar, which makes it difficult to assign the signals to specific individual fatty acids. Therefore, in lipid analyses using NMR spectroscopy, only the ratios of the functional carbon groups are often given. In the present study, the ^{13}C-NMR spectra of the most abundant fatty acids in *P. tricornutum* were taken from the literature (see Qiao et al., 2016) and compared with the labeled signals from the ^{13}C-

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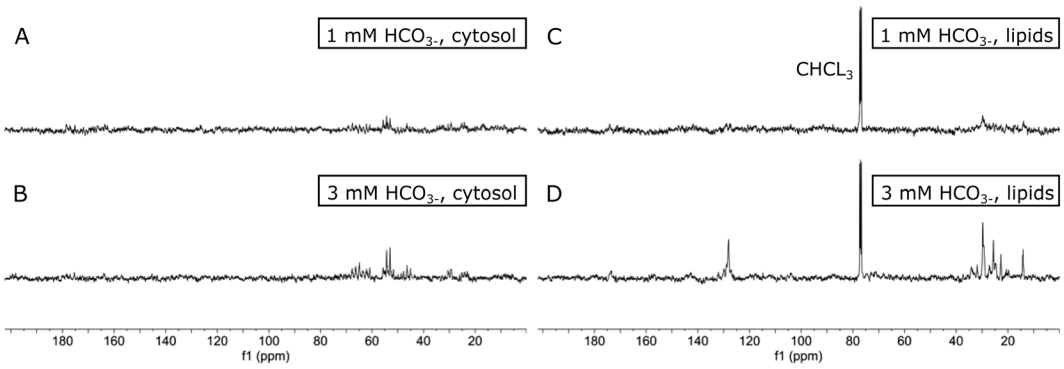

FIGURE 1
Comparison of ^{13}C-NMR spectra from *Phaeodactilum tricornutum* extracts grown in media with 1 mM (upper spectra) and 3 mM ^{13}C-labeled bicarbonate (lower spectra). The cytosolic fraction is displayed in **(A+B)**, and the lipid phase is in **(C+D)**. The natural abundance of ^{13}C-NMR signals from the solvent chloroform ($CHCL_3$) were used to calibrate and scale the NMR spectra.

NMR spectra of the lipid phase. The five fatty acids taken from the literature with a proportion of more than 5% in the late linear growth phase of *P. tricornutum* could also be assigned from the ^{13}C-NMR spectra (Qiao et al., 2016, see Table 2). In particular, the signals of the polyunsaturated omega-3 fatty acid eicosapentaenoic acid (EPA) in *P. tricornutum* could be identified in the ^{13}C-NMR spectrum of the lipid phase of *P. tricornutum* (see bold numbers in Table 2), confirming the exceptionally high content of EPA of more than 30% in *P. tricornutum* during the late linear growth phase (Qiao et al., 2016; Ding et al., 2023).

The uptake and incorporation of ^{13}C-labeled atoms from *P. tricornutum* into the digestive gland and muscle tissue of the King scallop *P. maximus* could be tracked clearly by ^{13}C-enrichment NMR spectroscopy. Figure 2 shows the ^{13}C-NMR spectra of the cytosolic and lipid phases obtained from the digestive gland (DG) tissue of *P. maximus* after feeding with unlabeled and ^{13}C-labeled *P. tricornutum*. In the scallops spectra of the digestive gland fed with unlabeled *P. tricornutum*, there was a visible contribution of naturally abundant ^{13}C-NMR signals from metabolites in the cytosolic and lipid phases (Figures 2A, C). However, the comparison with the spectra of the digestive gland of scallops fed with ^{13}C-labeled algae shows a substantial enrichment in these naturally abundant ^{13}C-NMR signals (Figures 2B, D), suggesting that these metabolites are preferentially synthesized. Interestingly, irrespective of the extract phase, only a few additional signals appeared in the ^{13}C-NMR spectra after feeding with labeled algae. These extra signals belong most likely to the digestive gland's specific metabolite pool, which was previously masked by noise and now became visible due to the increased incorporation of the ^{13}C-label.

A comparison of the ^{13}C-enrichment NMR spectra from the digestive gland of scallops with the spectra of ^{13}C-labeled algae indicates some substantial differences. The signals match between 45-80 ppm, while in the range between 170-180 ppm, the digestive gland shows additional signals compared to the algae spectra. In addition to the metabolites identified in *P. tricornutum*, the amino acids taurine, threonine and succinate could be assigned in the spectrum of the digestive gland of scallops fed with ^{13}C-labeled algae. This is particularly visible in the NMR signals of the C1 atoms of amino acids around 170-180 ppm (Figure 2B, Table 1), which are not detected in *P. tricornutum* spectra. This is evidence of incorporating the ^{13}C-label into *de novo* synthesized amino acids. The ^{13}C-NMR spectrum from the cytosol of the muscle was almost identical to the digestive gland spectrum except for additional signals from the amino acid l-arginine (at 155 ppm; see spectra in the supplement). This result is not surprising, as phosphor-l-arginine is provided in the phasic muscle for rapid energy supply, for example, during swimming (e.g. Bock et al., 2019) and is

TABLE 1 Identified ^{13}C-labeled NMR signals of the most prominent metabolites in the cytosolic extract phase of *Phaeodactilum tricornutum*.

C-atoms	Alanine	Betaine	Glucose	Glutamate	Glutamine	Glycine	Leucine
C1	178.3	172.01	93.99	177.3		175.2	178.3
C2	55.5	71.6	68.0	63.2	55.35	44.2	56.2
C3	18.9	69.0	66.7	31.8			42.6
C4		56.0	65.2	20.6			26.8
C5			62.5	19.3			24.7
C6			61.1				23.6
C7							

The numbers reflect the ^{13}C-labeled NMR signals of C-atoms identified in the ^{13}C-NMR spectra. Values are given in ppm relative to the internal standard TSP.

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TABLE 2 Chemical shift values of the functional groups of ^{13}C-labeled fatty acids identified in the lipid extract phase of *Phaeodactilum tricornutum*.

Carbon signal	C14:0	C16:0	C16:1n-7	C18:1n-9	C20:5n-3 (EPA)
-COOH	180.62	182.62	180.21	180.50	179.87
=CH-			**129.73/130.02**	**130**	**127.5-129.64**
-CH2COOH	34.2	36.68	34.05	33.96	34.02
-CH2	29-32	32-34	29-28.74	29-31	25.5-26.5
=CH-CH2			**27.22**	**27.12**	**129.10**
=CH-CH2-CH=					**132.1**
-CH2CH2COOH	24.74	27.33	24.65	24.59	24.54
-CH2CH3	22.74	25.36	22.67	22.52	20.61
-CH3	14.12	16.78	14.13	14.07	14.12

Note that the chemical shift values in bold reflect the double bonds of unsaturated fatty acids. All values are given in ppm relative to the internal standard TMS.

therefore found in high concentrations in the phasic muscle of *P. maximus* compared to other tissues (e.g. Bock et al., 2024). All identified metabolites correspond very well with the literature data from metabolomics studies of digestive gland and muscle tissue from bivalves, including scallops (Cappello et al., 2018; Bock et al., 2024). Contrasting to the cytosol phase, the comparison of the ^{13}C-NMR lipid spectra revealed no additional signals between the spectra obtained from algae and scallops. Indeed, the ^{13}C-enriched NMR spectrum from the lipid phase of the digestive gland is very comparable with the spectrum of the lipid phase of *P. tricornutum*. In particular, the signals of the essential unsaturated fatty acids now appear prominently in the ^{13}C-spectrum of the digestive gland (see region between 128-132 ppm in Figure 2D, Table 2). In contrast, the ^{13}C-enrichment spectra of the lipid phase of muscle spectra showed no incorporation of ^{13}C-labeled atoms (see Supplementary Figure S2).

The increased ^{13}C-NMR signals in the labeled ^{13}C-NMR spectra may arise from three contributors: 1. Signals from naturally abundant metabolites of the tissue. 2. Signals that belong to ingested ^{13}C-labeled algae that were not removed during the extraction process and contributed to the ^{13}C-NMR spectrum of the digestive gland; and 3. ^{13}C-NMR signals that arise from *de novo* synthetized metabolites. The contribution from naturally abundant metabolites is unlikely, as the signals between the labeled and unlabeled digestive glands are too different in intensity to be explained by variations between individuals only. A possible signal contribution of undigested algae remaining that are still present in the digestive gland, is rather unlikely. During the extraction process (see M&M), the remaining undigested algae will most likely be removed. In addition, potential contamination cannot explain the increase in ^{13}C-labels in amino acids that are not present in the cytosolic ^{13}C-NMR spectrum of *P. tricornutum*. Furthermore, increased ^{13}C-labeled signals were also observed in muscle tissue, where side effects due to contamination of undigested algae can be ruled out (see Supplementary Figure S2). Therefore, all indications support a *de novo* synthesis of metabolites in the

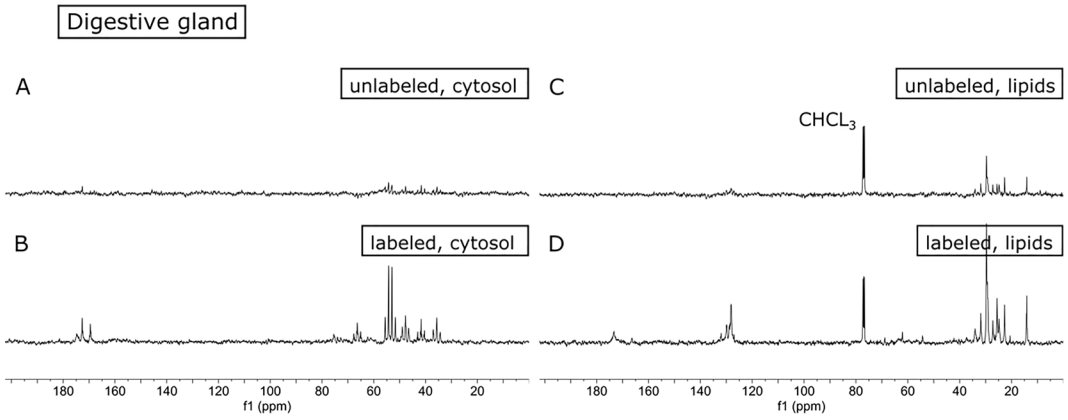

FIGURE 2
Comparison of ^{13}C-NMR spectra of the specific cytosolic and lipid phases from digestive gland tissue of the scallop *Pecten maximus* fed with unlabeled algae (**A**+**C**) and after feeding with ^{13}C-labeled *Phaeodactilum tricornutum* (**B**+**D**). Note the increase in ^{13}C-NMR signals in the digestive gland tissue spectra of the scallop fed with ^{13}C-labeled algae. Note the additional signals of the C1-atoms of the amino acids. The natural abundance of ^{13}C-NMR signals from the solvent chloroform ($CHCL_3$) and the noise level were used for scaling the NMR spectra.

scallops, especially of amino acids. However, a potential contribution by undigested algae in the spectra of the digestive gland can be regarded as insignificant.

The situation of the ^{13}C-enriched NMR spectrum of the lipid phase differs from that of the cytosol. Here, very distinct signals from essential fatty acids of the algae appear. These signals are particularly visible in the 128-132 ppm range when comparing Figures 1D and 2C, D. These ^{13}C-NMR signals can be attributed to the double-bonded carbon of unsaturated fatty acids (see above), in the case of *P. tricornutum* mainly to the polyunsaturated fatty acid EPA. Essential fatty acids such as EPA are necessary for the survival and growth of bivalves (da Costa et al., 2015). It is still unclear, however, whether scallops can directly synthesize essential fatty acids such as EPA (Knauer and Southgate, 1999), but it is well known that essential fatty acids can be selectively assimilated in scallops if the diet is unlimited (da Costa et al., 2015). EPA can, therefore, be identified as an unequivocal trophic marker for scallops, as shown in, e.g. *Daphnia magna* (Sperfeld and Wacker, 2009, 2011). In this way, the signals between 128-132 ppm in the ^{13}C-NMR spectra of the lipid phase can be used as indicators for trophic markers. Interestingly, in the lipid phase of the phasic muscle, neither naturally abundant ^{13}C-NMR signals nor labelled signals can be detected (see figure in supplement). This can be explained on the one hand by the fact that the lipid content in the phasic muscle of *P. maximus* is very low (Topić Popović et al., 2020), and on the other hand by the metabolism of the phasic muscle of *P. maximus*. The phasic muscle of scallops requires energy more immediately, e.g. for escape swimming. Since carbohydrate oxidation has a higher ATP synthesis rate than fatty acid oxidation (Hargreaves and Spriet, 2020), it is quite conceivable that fatty acids are only incorporated slowly or to a small extent. In addition, Guderley and co-workers who investigated isolated mitochondria from the phasic muscle of the tropic scallop *Euvola ziczac*, could show that carbohydrates rather than fatty acids are used for energy production (Guderley et al., 1995). This may explain the lack of incorporation of ^{13}C-labeled atoms in fatty acids and the generally low to non-detection of ^{13}C-atoms in the lipid phase of phasic muscle tissue under the present experimental conditions.

After discussing the applicability of ^{13}C-enrichment NMR spectroscopy in a simple feeding experiment of ^{13}C-labeled algae into bivalves, the next paragraph deals with our second approach to identify temperature-induced changes using metabolic substrates and energy conversion. Figure 3 presents ^{13}C-NMR spectra from the cytosolic fraction of the phasic muscle of *P. maximus* after feeding with ^{13}C-labeled algae at different temperatures, 15°C vs 20° C, respectively. At first glance, there are no major differences between the spectra from the cytosolic phase of the phasic muscle between the two temperatures. A closer inspection, however, showed a few but remarkable differences in the cytosolic fraction of the muscle tissue fed at 15°C compared with those fed at 20°C. In particular, there is an increase of a signal at 155 ppm (see bracket box in Figure 3) at 20°C in the cytosolic fraction of the phasic muscle, which can be attributed to the C6-atom of l-arginine and two more pronounced small signals in the region between 70 and 80 ppm (albeit not significant, see supplement for statistics). Carbon atoms of sugars, such as alpha- and beta-glucose, can be typically identified in this region of the ^{13}C-NMR spectrum (70-80 ppm, see Table 1).

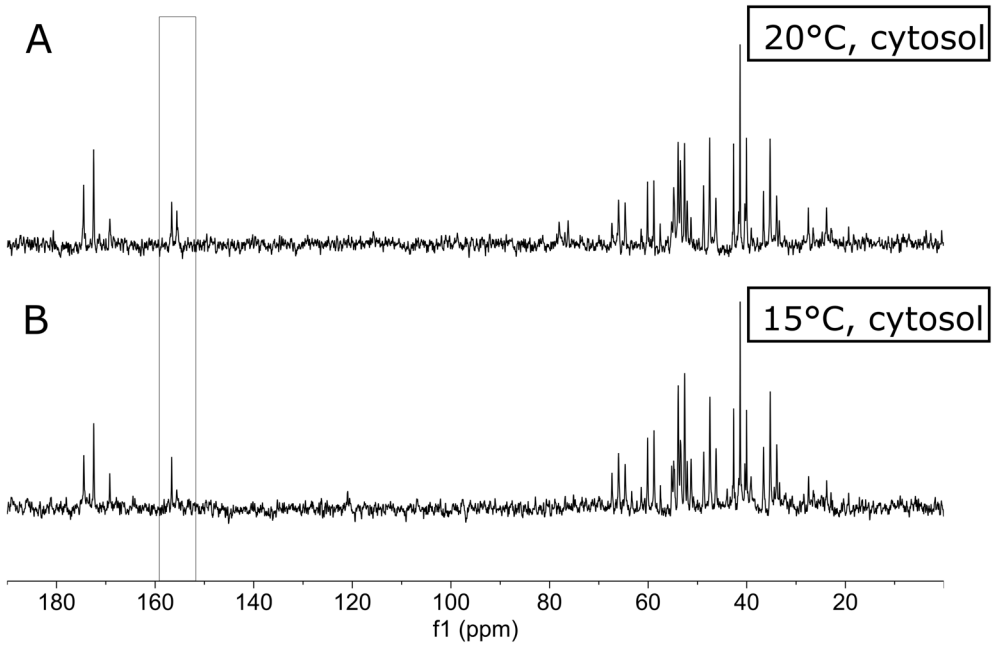

FIGURE 3
^{13}C-NMR spectra from the cytosolic fraction of scallop muscle tissue. Scallops were fed with ^{13}C-labeled algae at 20°C **(A)** and 15°C **(B)**. Spectra are the sum of spectra from three individual scallops from both groups. The bracket box points to the increased ^{13}C-labeled signal at 155 ppm in the muscle tissue from scallops fed at 20°C. That signal is significantly elevated and could be attributed to l-arginine.

It is well known from the literature that scallops such as *P. maximus* have higher metabolic costs at higher temperatures (e.g. Schalkhausser et al., 2014), reflected in higher filtration rates. In the adductor muscle, particularly the phasic muscle, higher metabolic costs, such as swimming, are compensated via increased use of phospho-l-arginine (PLA) to fuel ATP (Tremblay et al., 2012). The ^{13}C-label under sufficient food availability will be increasingly incorporated into l-arginine at the beginning of moderately higher temperatures to prepare for this under routine conditions. In the case of longer-term warming conditions and energy requirements, however, there is a reduction in PLA and l-arginine (Bock et al., 2019, 2024). Another compensation process for the increased energy requirement at warmer temperatures is the increased incorporation of carbohydrates such as glucose (signals at 76.2 and 78.0 ppm). These are then utilized for energy production in the mitochondria of the muscle (see above, Guderley et al., 1995). In addition, bivalves can show a seasonal-dependent preferred use of carbohydrates in summer at higher temperatures and fatty acids in autumn and winter at lower temperatures, as recently has been shown for *Pecten jacobeus* (Topić Popović et al., 2020). Furthermore, the triglyceride content in the muscle is an order of magnitude lower than in the digestive gland in *P. jacobeus* (Topić Popović et al., 2020). A scallop species that is closely related to *P. maximus*. The increased ^{13}C-content on the sugars of the muscle tissue indicates that these sugars are incorporated from ^{13}C-labeled algae. Therefore, the increased filtration rate at 20°C and higher energy demand at 20°C may explain the increased incorporation rate of the ^{13}C-label into sugars in the muscle tissue of scallops from the 20°C group.

Figure 4 compares ^{13}C-NMR spectra from the lipid fraction of the digestive gland from scallops fed at 15°C and 20°C. Here, the broad signal cluster at 127-129 ppm and the prominent signal cluster around 25 ppm are significantly higher in the 15°C spectrum than the sum spectrum at 20°C (see supplement for statistics). Together with the signals at 20 ppm and a signal at 14 ppm, these signals can be attributed to the polyunsaturated fatty acid EPA (see Table 2). EPA as a classical essential fatty acid was also identified in the ^{13}C-NMR spectra from *P. tricornutum* extracts (Figure 1). As described above, EPA is an unequivocal trophic marker (Sperfeld and Wacker, 2009, 2011).

The higher levels of unsaturated fatty acids found in the digestive gland of the 15°C group can be explained by selectively incorporating more unsaturated fatty acids from the algae into their digestive gland than the 20°C group (da Costa et al., 2015). This interpretation is supported by the fact that scallops use more fatty acids for energy production at colder temperatures. A phenomenon that can be observed across the animal kingdom. At warmer temperatures, carbohydrates are used for energy provision, as shown for the tropical scallop *E. ziczac* (Guderley et al., 1995) and supported by the increased ^{13}C-NMR glucose signals in the muscle tissue at 20°C (see above). Furthermore, unsaturated fatty acids are incorporated into cellular membranes at colder temperatures to keep the fluidity of cell membranes. A phenomenon frequently reported for polar organisms is called homeoviscious adaptation (Sinensky, 1974). However, it is most unlikely that it is necessary to modify the membrane fluidity in a moderate temperature difference range from 15° to 20°C. In addition, temperature-induced adaptations to membrane composition to keep membrane fluidity should affect all tissues.

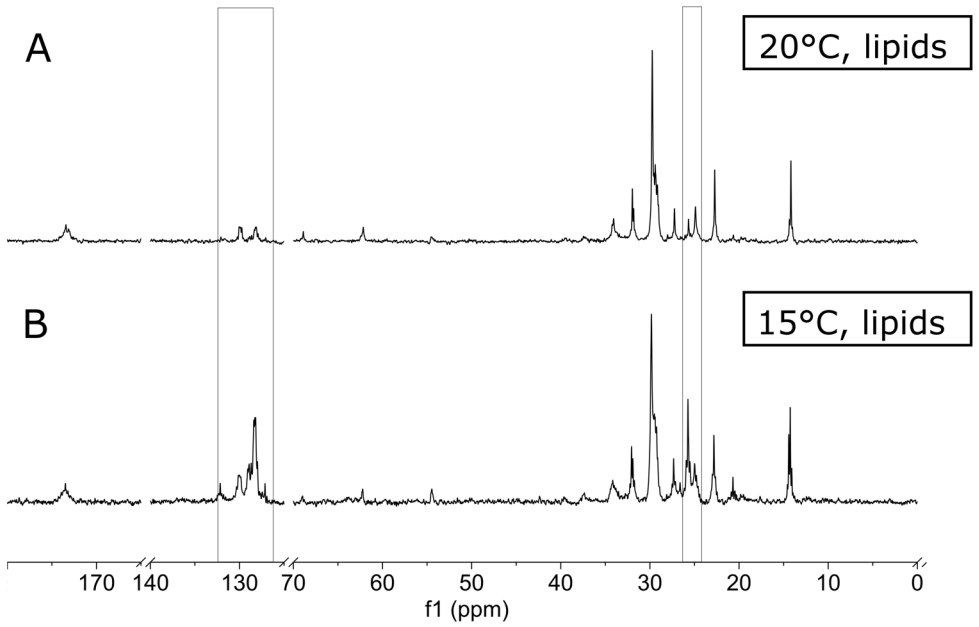

FIGURE 4
^{13}C-NMR spectra from the lipid fraction of scallop digestive gland tissue. Scallops were fed with ^{13}C-labeled algae at 20°C **(A)** and 15°C **(B)**. Spectra are the sum of three spectra from three individual scallops from both groups. The most notable changes of ^{13}C-labeled atoms in the digestive gland from scallops can be observed inside the bracket boxes. Note the prominent decrease of double-bound ^{13}C-atoms around 129 ppm, reflecting the double bonds of unsaturated fatty acids (in particular EPA, Table 2).

However, we did not observe such changes in scallop muscle tissue. Therefore, the observed increase in unsaturated fatty acids is more likely to be explained as a reserve for energy provision. This was also suggested in a recent metabolomic study on the impact of Ocean warming and acidification on the gills, mantle and adductor muscle of *P. maximus* (Bock et al., 2024). In summary, our simple experiment demonstrates the potential of NMR experiments with ^{13}C-enrichment to detect environmentally induced changes in metabolic pathways (e.g. Lannig et al., 2010; Tikunov et al., 2014) or the specific use of metabolic substrates (Guderley et al., 1995) in laboratory experiments.

Conclusions

We showed that ^{13}C-enrichment NMR spectroscopy can be used to monitor the uptake of ^{13}C-labeled algae in filtration feeders. We could identify trophic markers in a very simple experiment with high reproducibility. The experimental approach indicates that besides the accumulation of trophic markers, their use and conversion might be an additional important factor for the reliable interpretation of trophic linkages under climate change scenarios. The experimental approach should be easily transferable to other and more complex systems, such as predator-prey interactions under more complex climate change scenarios. It should also be practicable in the field.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.pangaea.de/.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because it is not required for bivalves according to german legislature.

Author contributions

CB: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Formal analysis. TZ: Investigation, Writing – original draft. SG: Investigation, Writing – review & editing. FW: Formal analysis, Visualization, Writing – review & editing. GL: Data curation, Formal Analysis, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1446998/full#supplementary-material

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