Bacterial precursors and unsaturated long-chain fatty acids are biomarkers of North-Atlantic demosponges

Short title: Fatty acid profiles of deep-sea demosponges

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Abstract

Sponges produce distinct fatty acids (FAs) that (potentially) can be used as chemotaxonomic and ecological biomarkers to study endosymbiont-host interactions and the functional ecology of sponges. Here, we present FA profiles of five common habitat-building deep-sea sponges (class Demospongiae, order Tetractinellida), which are classified as high microbial abundance (HMA) species. *Geodia hentscheli*, *G. parva*, *G. atlantica*, *G. barretti*, and *Stelletta rhaphidiophora* were collected from boreal and Arctic sponge grounds in the North-Atlantic Ocean. Bacterial FAs dominated in all five species and particularly isomeric mixtures of mid-chain branched FAs (MBFAs, 8- and 9-Me-C\textsubscript{16:0} and 10 and 11-Me-C\textsubscript{18:0}) were found in high abundance (together ≥ 20% of total FAs) aside more common bacterial markers. In addition, the sponges produced long-chain linear, mid- and \(a(i)\)-branched unsaturated FAs (LCFAs) with a chain length of 24–28 C atoms and had predominantly the typical \(\Delta^{5,9}\) unsaturation, although also \(\Delta^{9,19}\) and (yet undescribed) \(\Delta^{11,21}\) unsaturations were identified. *G. parva* and *S. rhaphidiophora* each produced distinct LCFAs, while *G. atlantica*, *G. barretti*, and *G. hentscheli* produced similar LCFAs, but in different ratios. The different bacterial precursors varied in carbon isotopic composition (\(\delta^{13}C\)), with MBFAs being more enriched compared to other bacterial (linear and \(a(i)\)-branched) FAs. We propose biosynthetic pathways for different LCFAs from their bacterial precursors, that are consistent with small isotopic differences found in LCFAs. Indeed, FA profiles of deep-sea sponges can serve as chemotaxonomic markers and support the conception that sponges acquire building blocks from their endosymbiotic bacteria.
Introduction

Sponges are abundant inhabitants of nearly all aquatic ecosystems including the deep-sea (1). They are sessile filter feeders with unique features, such as their enormous filtration capacity and their symbiosis with dense and diverse communities of (sponge-specific) microbes (algae, bacteria, archaea) (2,3) that contribute to their ability to thrive at nearly all depths and latitudes. The endosymbionts, which can occupy >50% of sponge volume (4), serve as energy source for sponges and provide a diverse pallet of metabolites and metabolic pathways that are beneficial to the sponge (reviewed in (2)). A prominent class of metabolites produced by the sponge and its endosymbionts are lipids. Lipid analysis of sponges started in the 1970s (5,6) and was sparked by the diversity and unique structures of fatty acids (FAs), of which extensive reviews exist (7–9). Characteristic of sponges is the presence of unusual poly-unsaturated, long chain (≥24 carbons(C)) FAs (LCFAs), with a typical Δ5,9 unsaturation (named “demospongic acids”, because of their first discovery in demosponges (5,10)). These LCFAs constitute a major part of sponge membrane phospholipids (PLs) and probably serve a structural and functional role (11). Sponges, because of their endosymbionts, are rich in bacterial FAs with high diversity, including not only the common iso (i) and anteiso (a)-branched FAs, but also more unusual ones. Typical of demosponges are a high abundance of mid-chain branched FAs (MBFAs), that are thought to be produced by sponge-specific eubacteria (12), and a presence of branched LCFAs (12,13). As branching is assumed to be introduced by microbes and not by the sponge host, the presence of branched LCFAs provides information on biosynthetic interactions between endosymbionts and host (12,14). Monoenic FA, e.g. C_{16:1}ω7, abundant in bacteria (15), have been identified as precursors for LCFAs with ω7 configuration (16). Accordingly, the position of unsaturation also provides insight in bacteria-host biosynthetic interactions.
In addition, sponge FA composition may have taxonomic value, at least on a higher
classification level (e.g. class level), since Demospongiae, Hexactinellida (‘glass’ sponges),
Calcarea, and Homoscleromorpha have distinct FA profiles (17). However, the
chemotaxonomic value on a lower classification level is disputable, since composition may
alter with environmental conditions (18). The FA composition of sponges, especially
combined with (natural abundance) stable isotope analysis, has been shown a valuable tool to
infer dietary information on sponges, such as feeding on coral mucus (19), phytoplankton
(20) and methane-fixing endosymbionts (21).

The North-Atlantic Ocean is home to extensive sponge grounds, that are widespread
along the continental shelves, seamounts, and on the abyssal plains (22,23). Geodiidae and
other sponge species of order Tetractinellida (class Demospongiae) are major constituents of
these sponge grounds, representing >99 % of sponge ground benthic biomass (23–25).
Geodiidae spp. are high microbial abundance (HMA) sponges that harbor rich, diverse and
specific microbial communities (bacteria and archaea) involved in several biogeochemical
processes, as observed in G. barretti (26). This is reflected in the FA composition of G.
barretti that is dominated by bacterial FAs (12), including the distinct MBFAs that represent
28% of total FAs (12). However, the FA profiles of other Geodiidae are not described in the
literature yet.

In this study we analyzed the FA profiles of five common deep-sea Tetractinellids,
from different assemblages distinguished by temperature in the North Atlantic: the Arctic
sponge ground assemblages accommodate G. parva, G. hentscheli, and Stelletta spp. (e.g. S.
rhaphidiophora) dwelling at temperatures below 3–4 °C, and the boreal assemblages
accommodate G. barretti and G. atlantica amongst others, which are typically found at
temperatures above 3 °C (23,27). Based on the chemical configuration and the presence of
branching in LCFAs, we propose biosynthetic pathways and show that these are consistent
with the C isotope ($\delta^{13}C$) signatures of LCFAs and bacterial precursors. The high abundance of endosymbiont markers that are precursors of LCFAs, indicate that these deep-sea sponges use their endosymbionts as metabolic source.

**Methods**

**Sponge collection**

Common habitat-building sponges of class Demospongiae, order Tetractinellida, were collected in the North-Atlantic Ocean by remotely operated vehicle (ROV) and box cores during different scientific expeditions. *G. atlantica* ($n = 2$) specimens were collected on the Sula Reef between 266–295 m depth during an expedition in August 2017 with the Norwegian research vessel G.O. Sars ($64^\circ42'N 7^\circ59'E$). *G. barretti* ($n = 6$) individuals were obtained from the Barents Sea ($70^\circ47N 18^\circ03'E$) around 300 m water depth on a subsequent G.O. Sars expedition in August 2018 (28). During the same expedition, *G. hentscheli*, *G. parva*, *Stelletta rhaphidiophora* (all $n = 1$) were collected at 550–600 m depth on the summit of Schulz Bank ($73^\circ50' N, 7^\circ34' E$) (29). *G. hentscheli* ($n = 3$), *G. parva* ($n = 3$), and *S. rhaphidiophora* ($n = 2$) specimens were retrieved on an Arctic expedition with the German research vessel Polarstern (AWI Expedition PS101) in September–October 2016 at 690–1000 m depth from Langseth Ridge, located in the permanently ice-covered Central Arctic ($86^\circ N, 61^\circ E$). Sponges collected during the G.O. Sars expeditions were immediately dissected on board and sponges collected from Langseth Ridge were frozen at -20°C and dissected (frozen) in the lab. Subsamples ($n = 3$) from random parts of individual sponges were freeze-dried, grinded to obtain a fine powder. The powdered subsamples of sponges from Schulz Bank and Barents Sea were mixed to obtain a species representative sample, while a subsample of the interior of sponges was analyzed in case of Langseth Ridge specimens.
**Lipid extraction and FAME preparation**

Approximately 100 mg of sponge powder of each individual sponge was used per extraction. Sponge lipids were extracted with a modified Bligh and Dyer protocol (30), which was developed at NIOZ Yerseke (31–33). We adjusted this protocol by replacing chloroform with dichloromethane (DCM), because of lower toxicity. The whole protocol is available online: dx.doi.org/10.17504/protocols.io.bhnpj5dn. In short, sponge tissue samples were extracted in a solvent mixture (15 mL methanol, 7.5 mL DCM and 6 mL phosphate (P)-buffer (pH 7-8)) on a roller table for at least 3 hours. Layer separation was achieved by adding 7.5 mL DCM and 7.5 mL P-buffer. The DCM layer was collected, and the remaining solution was washed a second time with DCM. The combined DCM fraction was evaporated to obtain the total lipid extract (TLE), which was subsequently weighed. An aliquot of the TLE was separated into different polarity classes over an activated silica column. The TLE was first eluted with 7 mL DCM (neutral lipids), followed by 7 mL acetone (glycolipids) and 15 mL methanol (phospholipids). The phospholipid (PL) fraction, which was used for further analysis, was converted into fatty acid methyl esters (FAMEs) using alkaline methylation (using sodium methoxide in methanol with known δ^{13}C). Alkaline methylation is recommended for complex lipid mixtures (34). After methylation, FAMEs were collected in hexane and concentrated to ~100 µL hexane for gas chromatography (GC) analysis.

For this study, two individual sponge samples per species were selected for detailed analysis. Aliquots of the FAME samples were used for double bond identification using dimethyl disulfide (DMDS) derivatization (35). Samples reacted overnight at 40°C in 50 µL hexane, 50 µL DMDS and 10 µL 60 mg/mL I₂. The reaction was stopped by adding 200 µL hexane and 200 µL Na₂S₂O₃. The hexane layer was collected, and the aqueous phase was washed twice with hexane. The combined hexane fraction was dried, subsequently eluted...
over a small Na$_2$SO$_4$ column using DCM: methanol (9:1) to remove any water and re-
dissolved in hexane in a GC-vial for GC-analysis. Another aliquot of FAME sample was used
for methyl-branching identification using catalytic hydrogenation with Adams catalyst (PtO$_2$
and hydrogen. Each FAME sample, dissolved in ~3 mL ethyl acetate with 10-30 mg PtO$_2$ and
a drop of acetic acid, was bubbled with hydrogen gas for at least 1 h, after which the reaction
vial was closed, and stirred overnight at room temperature. Subsequently, each sample was
purified over a small column consisting of MgSO$_4$ (bottom) and Na$_2$CO$_3$ (top) using DCM
and analyzed after re-dissolving it in ethyl acetate.

FAME analysis

FAMEs were analyzed on a gas chromatograph (GC) with flame ionization detector
(FID) (HP 6890 series) for concentrations and GC-mass spectrometry (MS) (Finnigan Trace
GC Ultra) for identification on a non-polar analytical column (Agilent, CP-Sil5 CB; 25 m x
0.32 mm x 0.12 µm). The GC oven was programmed from 70–130 °C at 20 °C/min and
subsequently at 4 °C/min to 320 °C, at which it was hold for 20 min. The GC–FID was
operated at a constant pressure of 100 kPa, whereas the GC–MS was operated at a constant
flow of 2.0 mL min$^{-1}$. The MS was operated in Full Data Acquisition mode, scanning ions
from m/z 50–800. The $^{13}$C/$^{12}$C isotope ratios of individual FAMEs were determined by
analyzing samples in duplicate on a GC-combustion-isotope ratio mass spectrometer (IRMS)
consisting of a HP 6890N GC (Hewlett-Packard) connected to a Delta-Plus XP IRMS via a
type-III combustion interface (Thermo Finnigan), using identical GC column and settings as
for GC-MS.

Retention times were converted to equivalent chain length (ecl) based on the retention
times of C$_{12:0}$, C$_{16:0}$, and C$_{19:0}$ FAMEs. The $\delta^{13}$C values obtained by GC-C-IRMS were
corrected for the added C atom of the methylation agent. The data were analyzed and plotted in R (36) with R-package RLims (37).

**Results**

The lipid yield of *G. barretti, G. hentscheli, G parva,* and *S. rhaphidiophora* was similar, around 2–3 % of dry weight (DW). Only *G. atlantica* had a lower lipid yield, about 1.6 % of DW. The FA profiles of PL resembled those of TLE (Table S1). However, identification was more difficult using TLEs, because LCFAs co-eluted with sterols, hence PL chromatography was used for identification and composition analysis.

**Identification**

Chemical structures of individual FAs were identified by retention times (ecl), interpretation of their mass spectra and/or by identification using a NIST library. The assignments were verified with reference mixtures (bacterial and general FA mixtures from Sigma Aldrich) and by literature comparison (e.g. the reference ecl lists from NIOZ Yerseke (31)).

FAs are presented in both ω and Δ (IUPAC) annotation to avoid unambiguity and in a hybrid form, which is typical of sponge LCFA annotation (17,38) (Table 1). Unsaturation is described as C\textsubscript{x:y}, where x is the number of C atoms and y is the number of double bonds, which is followed by Δ and all double bond positions from the carboxylic acid end in Δ notation, and the position of the first double bond from the methyl (terminal) end in ω notation (Table 1, Fig. 1). Methyl branching according to IUPAC notation is described as y-Me-C\textsubscript{x}, where y is the position of the branching from the carboxylic acid end and x is the number of C atoms at the backbone, excluding the branching (Fig. 1). The ω notation follows
the terminology of IUPAC for MBFAs, but deviates for terminally branched FAs. The penultimate (ω2) and pen-penultimate methyl branching (ω3) are described with ω notation as iso (i-C_x) and anteiso (a-C_x) where x is the total number of C atoms, including the branching (Table 1, Fig. 1).

Table 1: Fatty acid (FA) composition in % of total PLFA of deep-sea demosponge species (order Tetractinellida): Geodia atlantica (Ga), G. barretti (Gb), G. hentscheli (Gh), G. parva (Gp) and Stelletta rhaphidiophora (Sr). FA names are given in ω and Δ notation and a hybrid form, with corresponding total C atoms (C) and equivalent chain length (ecl). FA categories match with those of Fig. 2. Only FAs with abundance ≥1 % (in at least one species) are shown. Numbers in bold are ≥ 10 % of total FAs.

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<th>Gp</th>
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Fig. 1: Illustration of $\omega$ and $\Delta$ annotation for the chemical structure of $i$-$C_{17:1}\omega7$ / $15-$Me-$C_{16:1}\Delta9$. The $a(i)$-notation for methyl branching describes the total number of C, while the Me-notation describes the number of C in the backbone. For sponge LCFAs, a mixture of both nomenclatures is, however, commonly used.

The elution order on an apolar column consists of FAMEs with methyl-branching close to the functional group to elute first, followed by the terminally (penultimate) branched iso ($i$, $\omega1$) and pen-penultimate anteiso ($a$, $\omega2$) FAMEs, and finally the unsaturated FAMEs, for which unsaturation closest to the functional group elutes first. Branched unsaturated FAMEs elute before branched straight FAMEs and straight FAMEs with the same C number elute last (Fig. 2, Table 1).

Figure 2: GC trace of the FAME fraction extracted from demosponge *G. hentscheli* from Langseth Ridge (Central Arctic). LCFA isomers often co-eluted or were at least not well separated as shown in this PLFA profile $C_{26:2}\Delta5,9$ and $C_{26:2}\Delta9,19$.

The position of branching was also verified with MS spectra, as $i$-branching was characterized by a more intense $[M^+ - 43]$ fragment ion and $a$-branching was characterized by an elevated fragment ion at $[M^+ - 57]$. The position of methyl branching in saturated MBFAs
was identified via diagnostic mass fragments similar to (12). The relative intensity of m/z 171, 185, 199 and [185+213] was used to identify the relative contributions of 8, 9, 10, and 11-Me branching, respectively (Table S1). Because 11-Me-branching produces equal fragments of m/z 185 and 213, the excess of m/z 185 (213 – 185) was produced by 9-Me branching (Table S1) (12). The branching within unsaturated MBFAs was performed in hydrogenated samples, using similar diagnostic fragments and ecl of saturated FAMEs (Table S1).

Identification of unsaturation positions was conducted after treatment with DMDS, which is straight-forward with mono-unsaturated FAMEs. However, for poly-unsaturated FAMEs, identification with DMDS becomes complicated, because of multiple possibilities for S(-Me) adducts. The $\Delta^{5,9}$ unsaturation, typical of sponge LCFAs, forms a cyclic thioether at the C$_6$ and C$_9$ position along with methylthio groups at C$_5$ and C$_{10}$ positions upon derivatization with DMDS. In addition, products are formed with either methylthio groups at C$_5$ and C$_6$ and a (unreacted) double bond at C$_9$ and C$_{10}$, and vice versa (39). This has been useful for identifying the typical $\Delta^{5,9}$ configuration in sponges (40). When unsaturation is far apart, i.e. positions $\Delta^{9,19}$ and $\Delta^{11,21}$, both double bonds are converted to dimethyl disulfide adducts (S1 Figure for their mass spectra). Based on ecl and a combination of DMDS and hydrogenation, we identified branched-monoenic and dienic FAs.

**Fatty acid composition**

The Arctic species (*G. hentscheli, G. parva, S. rhaphidiophora*) from Schulz Bank and Langseth Ridge had a similar FA profile (Table S1), so we pooled the compositional data from the two locations (Table 1). The data are standardized to % of total FAs to facilitate comparison, but actual concentrations (µg g$^{-1}$ DW) are available in Table S1.
**Bacterial fatty acids**

Bacterial FAs, comprising branched and monoenic FAs with chain length < C$_{20}$, constituted the majority of total FAs in all five deep-sea demosponge species (67 ± 6 % mean ± SD of total FAs, used throughout text, $n = 19$, across all species) (Table 1, Fig. 3) and can represent up to 79 ± 2 % (in *S. rhaphidiophora*).

**Fig. 3:** Average contribution of bacterial FAs (blue), sponge LCFAs (orange) and other FAs (green) to the total PLFAs of each species (abbreviated as in Table 1).

MBFAs dominated the FA profiles of all deep-sea demosponge species (Table 1, Fig. 2), among them the most abundant were Me-C$_{18:0}$ (12–23 %, Table 1), with branching at 9, 10, 11 with a predominance at position 11 ($m/z$ [213+185]; 49 % on average), followed by position 10 ($m/z$ 199; 37 % on average). The second most abundant FAs were Me-C$_{16:0}$ (8–14 % of total FAs, Table 1), with branching at 8, 9, 10, 11 and a predominance of position 9 ($m/z$ 185; 36 % on average) and 10 ($m/z$ 199; 33 % on average). Also, Me-C$_{14:0}$, Me-C$_{15:0}$, and Me-C$_{17:0}$ were present, but in much lower abundance ($\leq$ 3 % of total FAs for each, Table 1). Other branched (saturated) FAs found in all demosponges but less abundant, included $i$-C$_{15:0}$ (13-Me-C$_{14:0}$) and $a$-C$_{15:0}$ (12-Me-C$_{14:0}$) comprising 2–5 % of total FAs for each, and $i$-C$_{17:0}$ (15-Me-C$_{16:0}$) and $a$-C$_{17:0}$ (14-Me-C$_{16:0}$) ranging from 1 to 2 % of total FAs for each (Table 1).

Multiple monoenic FAs were found in the deep-sea demosponges. The most abundant were C$_{16:1}$ (ranging from 9 % in *G. atlantica* to 14 % in *G. parva* and *S. rhaphidiophora*), consisting of different isomers with the double bond at $\omega 5$, $\omega 7$, $\omega 9$, and $\omega 11$ positions.

Isomers of C$_{18:1}$ with double bonds at $\omega 7$, $\omega 8$, $\omega 9$, $\omega 11$, $\omega 12$, $\omega 13$, $\omega 14$, and $\omega 15$ positions constituted 4–7 % of total FAs. The $\omega 7$ unsaturation dominated in both C$_{16:1}$ and C$_{18:1}$ FAs. In addition, rare C$_{16:1}$ and C$_{18:1}$ FAs with methyl-branching were found in demosponges. The
dominating unsaturation in C\textsubscript{16:1} was \(\omega 7\) and the hydrogenated FAME sample indicated \(\textit{i}\)-branching; \(\textit{i}-\text{C}_{17:1}\omega 7\) (15-Me-\(\text{C}_{16:1}\Delta 9\)) represented 4–5 \% in boreal species (\textit{G. barretti}, \textit{G. atlantica}) and \(< 2\ %\) in Arctic species (\textit{G. hentscheli}, \textit{G. parva}, \textit{S. rhaphidiophora}) (Fig. 1, Table 1). The most abundant unsaturation in \(\text{C}_{18:1}\) FAs was \(\omega 12\) (\(\Delta 6\)) for \textit{G. parva}, \textit{G. hentscheli} and \textit{S. rhaphidiophora} and \(\omega 14\) (\(\Delta 4\)) for \textit{G. barretti} and \textit{G. atlantica}, and the Me group was in the middle of the chain, since no increased peaks for \(\textit{i}\)- and \(\alpha\)-\(\text{C}_{19:0}\) were found in the corresponding hydrogenated fractions. The mid-Me branched \(\text{C}_{18:1}\) isomers (Me-\(\text{C}_{18:1}\omega 4\) and Me-\(\text{C}_{18:1}\omega 12\)) were found in all demosponge species and ranged between 2–5 \% (Table 1). Also, low amounts (<1 \%) of \(\text{C}_{15:1}\) and non-branched \(\text{C}_{17:1}\) were found.

**Other fatty acids**

Linear FAs were predominantly \(\text{C}_{14:0}\), \(\text{C}_{16:0}\) and \(\text{C}_{18:0}\) in all species (Table 1). Sponges contained only low amounts of FAs with a chain length between \(\text{C}_{20}\) and \(\text{C}_{24}\), such as \(\text{C}_{20:5}\omega 3\) (<1 \% in all species) and \(\text{C}_{22:6}\omega 3\) (1.4 ± 0.9 \%, \(n = 8\)) in boreal species \textit{G. atlantica} and \textit{G. barretti} and <1 \% in Arctic species \textit{G. hentscheli}, \textit{G. parva} and \textit{S. rhaphidiophora}.

**Sponge fatty acids**

LCFAs (\(\geq \text{C}_{24}\)), typical of sponges, differed per species and consisted of 24–29 C atoms (Table 1). LCFAs represented 21 ± 6 \% (\(n = 19\), across all species), with the highest contribution (29 \%) in \textit{G. atlantica} (Fig. 3). The most common unsaturation in demosponges was \(\Delta 5,9\), but also unsaturation at \(\Delta 9,19\) and \(\Delta 11,21\) was observed.

- \(\text{C}_{25}\): The dominant LCFA in \textit{G. parva} was 23-Me-\(\text{C}_{24:2}\Delta 5,9\) (\(\text{i}-\text{C}_{25:2}\Delta 5,9\)), followed by 22-Me-\(\text{C}_{24:2}\Delta 5,9\) (\(\alpha\)-\(\text{C}_{25:2}\Delta 5,9\)), making up 15 ± 1 \% of total FAs. Isomers 23-Me-\(\text{C}_{24:1}\Delta 17\) (\(\text{i}-\text{C}_{25:1}\omega 7\)) and (mid-)Me-\(\text{C}_{24:1}\Delta 17\) (Me-\(\text{C}_{24:1}\omega 7\)) were present in boreal species (\textit{G. atlantica} and \textit{G. barretti}) representing together 2 ± 0.6 \% (Table 1).
The dominant LCFA in G. hentscheli was C_{26:2}\Delta^{8,19}, followed by C_{26:2}\Delta^{5,9}, together they represented 14 ± 6 % of total FAs in that species. G. barretti and G. atlantica also synthesized C_{26:2}\Delta^{5,9} and C_{26:2}\Delta^{9,19} in comparable amounts, representing together 11 ± 1% in G. barretti and 7 % in G. atlantica. Trace amounts (<1 %) of C_{26:2}\Delta^{5,9} were present in G. parva and S. rhaphidiophora. Similarly, trace amount of C_{26:2}\Delta^{11,21} (<1 %) was found in G. parva.

- C_{27}: (mid-)Me-C_{26:2}\Delta^{5,9} were abundant in boreal species (G. barretti: 4 ± 3 %; G. atlantica: 9 %). Also 25-Me-C_{26:2}Δ^{5,9} (i-C_{27:2}Δ^{5,9}), and 25-Me-C_{26:2}Δ^{9,19} (i-C_{27:2}Δ^{9,19}) were produced by boreal species, representing together 3 ± 2 % of total FAs in G. barretti and 5 % in G. atlantica. Because these peaks co-eluted, the individual concentrations might represent isomeric mixtures. G. hentscheli possessed low amounts of (mid-)Me-C_{26:2}Δ^{5,9} and 25-Me-C_{26:2}Δ^{9,19} (i-C_{27:2}Δ^{9,19}) (<2 %). Similarly, S. rhaphidiophora had low amounts of (mid-)Me- C_{26:2}Δ^{5,9} and (a)i-C_{27:2}Δ^{5,9} (together 2 %, Table 1).

- C_{28}: G. atlantica, G. barretti and G. parva contained C_{28:2} with Δ^{11,21} configuration, comprising 1.8 ± 1 % of total FAs in G. barretti, 1.9 % in G. atlantica and 3.6 ± 1.7 % in G. parva. S. rhaphidiophora contained a low amount of C_{28:2}Δ^{5,9} (1.5 ± 0.4 %) (Table 1).

- C_{29}: The dominant LCFA in S. rhaphidiophora was (mid)-Me-C_{28:2}Δ^{5,9} with a contribution of 5.5 ± 0.6 % to total FAs (Table 1).

**Stable C isotope values (δ^{13}C)**

Stable C isotope values (δ^{13}C) of dominant FAs ranged between -18‰ (95 percentile) and -26‰ (5 percentile) and showed similar patterns across all demosponges (Fig. 4, Table 2). The δ^{13}C values of the dominant MBFAs, Me-C_{16:0}, Me-C_{18:0}, and also Me-C_{18:1}\omega12 (and
ω14) were enriched in $^{13}$C compared to other bacterial fatty acids, $(a(i))-C_{15:0}$, $C_{16:1}\omega 7$, $C_{18:1}\omega 7$ (Fig. 4, Table 2). The most depleted FA was $i-C_{17:1}\omega 7$ ($-25.7 \pm 1.3 \% \delta^{13}C$). The different LCFA isomers were analyzed as one, because isomers co-eluted or were at least not well separated on GC (Fig. 2). However, we could assign separate isotope values for $(a)i-C_{27:2}$ and Me-C$_{26:2}$ (Fig. 4). The LCFA showed less isotopic variation compared to bacterial FAs, but still ranged between -25 and -19 % (5–95 percentile) (Fig. 4, Table 2). Me-C$_{26:2}$ and $(a)i-C_{27:2}$ had relatively similar $\delta^{13}C$ values, -20 and -21 %, in $G$. barretti and $G$. atlantica, but a more prominent difference of -21 and -24 % was observed in the hydrogenated samples ($n = 1$ per species), indicating that peak overlap blurred the isotopic values.

Table 2: $\delta^{13}C$ values (mean ± SD) of (bacterial) FA precursors and dominant LCFA of all species combined.

<table>
<thead>
<tr>
<th>Category</th>
<th>Fatty acid biomarker</th>
<th>Average $\delta^{13}C$ (‰) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(a)i$-branched FA</td>
<td>$(a)i-C_{15:0}$</td>
<td>-22.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>$(a)i-C_{25:2}$</td>
<td>-22.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>$i-C_{17:1}\omega 7$</td>
<td>-25.7 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>$i-C_{25:1}\omega 7$</td>
<td>-23.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>$(a)i-C_{27:2}$</td>
<td>-21.0 ± 1.2</td>
</tr>
<tr>
<td>Linear FA</td>
<td>$C_{16:0}$</td>
<td>-21.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>$C_{18:0}$</td>
<td>-20.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>$C_{16:1}\omega 7$</td>
<td>-23.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>$C_{18:1}\omega 7$</td>
<td>-23.6 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>$C_{26:2}$</td>
<td>-21.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>$C_{28:2}$</td>
<td>-23.2 ± 1.4</td>
</tr>
<tr>
<td>Mid-branched FA</td>
<td>Me-C$_{16:0}$</td>
<td>-19.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Me-C$_{18:0}$</td>
<td>-18.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Me-C$_{18:1}$</td>
<td>-17.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Me-C$_{26:2}$</td>
<td>-20.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Me-C$_{28:2}$</td>
<td>-19.4 ± 0.2</td>
</tr>
</tbody>
</table>

Fig. 4: $\delta^{13}C$ composition of precursors and dominant LCFA in analyzed demosponges.

Sponge species were pooled together, and the median is indicated in the box plot as black
line. The numbers depict the sample size (individual FAME samples). The colors are used to
match bacterial precursor FAs with sponge-produced LCFAs. Pink is used for (mid-)Me-
branched FAs, grey is used for linear FAs, blue and yellow indicate \((a)i\)-branched FAs with
distinct $\delta^{13}C$ that may end up in an isometric mixture, indicated by green (see Fig. 5 for
biosynthetic pathways).

**Discussion**

**Bacterial FAs**

High concentrations of isometric mixtures of MBFAs were found in all five sponge
species analyzed, independent of species and location (Table 1). A predominance of MBFAs
is considered to be a typical feature of Demospongiae, because it is not observed in any other
organism, sediment or water (12,17,41). Typical position of branching is between $\omega_5$ and $\omega_9$
(12), resulting in predominance of 8- and 9-Me-C$_{16:0}$ and 10- and 11-Me-C$_{18:0}$ in this study, in
agreement with previously reported MBFAs (42,43). MBFAs are typically produced by
bacteria, so they are presumably made by distinctive and sponge-specific eubacterial
symbionts. It has been hypothesized that these bacteria were widespread in the geological
past and were inherited in the protective environment of distinctive sponge hosts in modern
marine environments (8,12). This hypothesis has been further supported by genomic analysis
on *Geodia* sp. revealing similar microbial communities between species with little
geographical variation (44).

A proposed candidate phylum for MBFAs is Poribacteria, a unique and abundant
phylum in HMA sponges (45), since a positive relation between MBFA concentration and
Poribacteria abundance was found across several sponge species (43). Metagenome analyses
showed that Poribacteria are a prominent phylum in *G. barretti* (46,47), but are rare or even
absent in *G. hentscheli* (48), which shows a dominance of Acidobacteria, Chloroflexi, and
Proteobacteria, phyla that are also abundant in *G. barretti* (46,47). This suggests that either
the MBFAs belong to one of the above-mentioned phyla, or that the MBFAs are shared
among microbial phyla, as their chemotaxonomic resolution is lower compared to genomic
analysis. In the environment, MBFAs are primarily found in nitrogen and sulfur reducers
(chemoheterotrophs) and oxidizers (chemoautotrophs) that are mostly members of the (large)
proteobacteria family (49–52). Nitrogen and sulfur reduction and oxidation processes are
conducted in deep-sea sponges such as *G. barretti* (26,53,54), and oxidation processes are
coupled to CO$_2$ fixation, although associated CO$_2$ fixation is likely to contribute < 10 % of
the carbon demand of deep-sea sponges (55). The poribacteria in sponges were also
characterized as mixotrophic bacteria, able to fix CO$_2$ using the ancient Wood–Ljungdahl
(reversed acetyl-CoA) pathway (56). The isotopic enrichment in MBFAs (Fig. 4, Table 2),
agrees with earlier observations for *G. barretti* (57), and might thus be linked to nitrogen and
sulfur transforming processes and potentially CO$_2$ fixation. It will be interesting to perform
an isotope-tracer study (55) with $^{13}$C-CO$_2$ to assess CO$_2$ incorporation in the abundant
MBFAs, perhaps combined with nitrification (or sulfur oxidation) inhibitors, similar to
Veuger et al. (58).

The most depleted FA ($i$-C$_{17:1}$ω7, Fig. 4, Table 2) is considered a chemotaxonomic
marker for the sulfur reducing bacteria *Desulfovibrio* sp. (59). The isotopic difference
between $i$-C$_{17:1}$ω7 and MBFAs suggest that these markers are not from the same microbial
consortium. The more general bacterial markers (e.g. $(a)$-C$_{15:0}$, typical of gram-positive
bacteria and C$_{16:1}$ω7 and C$_{18:1}$ω7, typical of general gram-negative bacteria (15)) had
intermediate $\delta^{13}$C values (Fig. 4, Table 2). Such values can be the result of isotopic averages
from different pathways, since they are more general bacterial markers, or they might
represent general heterotrophy on organic matter with δ\(^{13}\)C value from -24 to -22 ‰ in the western Arctic (60).

The low contribution of FAs with a chain length of C\(_{20}\) to C\(_{24}\) typical of phytoplankton and zooplankton (e.g. C\(_{20:5}\)ω3 and C\(_{22:6}\)ω3) indicates that sinking zoo- and phytoplankton are not contributing much to sponge diet, at least not directly. These findings support increasing evidence that G. barretti (and other North-Atlantic deep-sea sponges) primarily feed on dissolved organic matter and pelagic and associated bacteria (61,62). A higher contribution of phytoplankton markers in boreal Geodia spp. (G. atlantica and G. barretti) compared to Arctic species (Table 1) might be linked to water depth, as boreal species were sampled from ~300 m and Arctic species from ~600 m, while also environmental factors, such as permanent ice coverage (Langseth Ridge) and a generally lower primary production in the Arctic compared to the boreal North-Atlantic ocean (63) might play a major role.

The overall high abundance of bacterial FAs (56–79 % of total FAs across all five analyzed deep-sea demosponge species, Fig. 3) fits with their classification as HMA sponges and supports the idea that microbial endosymbionts play a pivotal role in sponge metabolism (2,3). It is important to notice that the contribution of endosymbionts is likely even higher, since archaea are not detected with (PL)FA analysis (64), while they were also found to be abundant in G. barretti (46,47).

**Sponge LCFAs**

Although bacterial FA profiles were very similar among the studied Tetractinellid species, the sponge-specific LCFA composition was more species-specific. The dominant unsaturation in LCFAs, was double unsaturation at Δ\(^{5,9}\) position in all species analyzed, which is typical feature of demosponges (5,10). Similarly, the linear C\(_{26:2}\)Δ\(^{5,9}\), (a)i-C\(_{25:2}\)Δ\(^{5,9}\)
and/or i-C\textsubscript{27:2}Δ\textsuperscript{5,9}, present in all species analyzed (Fig. 5, Table 1), are common LCFAs of demosponges, (e.g. (17,38,65), for an overview see (8)). We found (mid-)Me-branched Δ\textsuperscript{5,9} LCFAs in all species, except G. parva (Fig. 5, Table 1). Also, Thiel et al. (17) found them in G. barretti and some other Demospongiae (Haliclona sp., Petrosia sp.) but not in all analyzed Demospongiae. We also identified novel LCFAs: i-C\textsubscript{27:2}Δ\textsuperscript{9,19} (in G. atlantica, G. barretti, and G. hentscheli) and Δ\textsuperscript{11,21} in C\textsubscript{26:2} and C\textsubscript{28:2} (G. barretti and G. parva). The presence of Δ\textsuperscript{11} unsaturation (C\textsubscript{26:2}Δ\textsuperscript{11,21} and C\textsubscript{28:2}Δ\textsuperscript{11,21}), identified via DMDS derivatization, is uncommon in sponge LCFAs. Barnathan et al. (66) found Δ\textsuperscript{11} unsaturation in a series of monoenic FAs, including C\textsubscript{28:1}, in a tropical demosponge species (order Axinellida), but no dienic LCFAs with Δ\textsuperscript{11} unsaturation have been described so far. The configuration indicates that Δ\textsuperscript{11} desaturase might be active in these species; however, the activity of this enzyme in sponges has not been reported.

G. atlantica and G. barretti had almost identical FA profiles (Table 1), suggesting that these species might be closely related, as was earlier suggested based on their sterol and amino acid composition (67), but deviates from molecular phylogeny that places them further apart (27). The FA profile of G. hentscheli resembled those of G. barretti and G. atlantica and based on molecular phenology, G. hentscheli is a sister species of G. barretti. On the other hand, G. parva produced distinct LCFAs compared to the other Geodia spp., the i- and a-C\textsubscript{25:2}Δ\textsuperscript{5,9} and this species is also phylogenetically apart from the other Geodia spp. (27). A dominance of (a)i-branched C\textsubscript{25:2} has been found in another Geodiidae family (Geodinella) (68). Finally, also S. rhaphidiophora produced a distinct LCFA, Me-C\textsubscript{28:2}Δ\textsuperscript{5,9}, a LCFA that has been described for demosponges of the family Aplysinidae (13,42).

Each of the three dominant Tetractinellids of Artic sponge grounds (G. hentscheli, G. parva and S. rhaphidiophora) produced distinct LCFAs (Table 1) that can serve as chemotaxonomic markers. The morphology of these sponges is very similar, so LCFA
analysis provides an additional method to identify each species. Furthermore, the distinct LCFA composition could be useful as trophic markers to study the ecological role of deep-sea sponges in the environment. No geographical differences in LCFA composition of Arctic Tetractinellids were found (Table S1) suggesting that the environment has a limited influence on the LCFA composition, which is a prerequisite for using LCFA as chemotaxonomic markers.

**Biosynthetic pathways of prominent sponge fatty acids**

The identification of branching in LCFA allows identification of its short chain precursors and biosynthetic pathways. As demonstrated by various in vivo incorporation studies with radioactive substrates (16,38,65,69), sponges elongate FA precursors by adding 2 C atoms at the carboxylic acid end and desaturate at $\Delta^5$ and $\Delta^9$ (visualized in (10)), revealing $\text{C}_{16:0}$ as precursor for the common $\text{C}_{26:2}\Delta^5,9$, while $\text{C}_{16:1}\omega7$ was identified as precursors for $\text{C}_{26:2}\Delta^9,19$ (Fig. 5). There is no evidence for branching to be introduced by sponges, so $i$- and $a$-$\text{C}_{15:0}$ were identified as precursors of $i$- and $a$-$\text{C}_{27:2}\Delta^5,9$ (Fig. 5) (38), while Me-$\text{C}_{16:0}$ has been identified as precursor for Me-$\text{C}_{26:2}\Delta^5,9$ and Me-$\text{C}_{28:2}\Delta^5,9$ (Fig. 5) (13,16,70). Finally, we hypothesize that $i$-$\text{C}_{17:1}\omega7$ is the precursor for $i$-$\text{C}_{25:1}\omega7$ and $i$-$\text{C}_{27:2}\Delta^9,19$ found in *G. atlantica*, *G. barretti*, and *G. hentscheli* (Fig. 5).

Application to the present study showed that most LCFA could be linked to precursors via established pathways, with hypothetical intermediates since hardly any were found in detectable abundance (Fig. 5). The C isotopic differences in bacterial precursors were (partially) reflected in C isotopic composition of LCFA (Fig. 4, Table 2), although the differences were not as prominent in LCFA compared to their precursors. One explanation is that a mixture of C sources is used by the host to elongate precursors to LCFA, while also methodological aspects might contribute. A (much) longer analytical column might help improving separation of LCFA.
Fig. 5: Proposed biosynthetic pathways of (microbial) precursors to LCFA in examined Tetractinellid species. FAs detected in the studied sponges are shown in a black rectangle, solid arrows indicate elongation, dashed arrows indicate $\Delta^{5,9}$ desaturation. The species encompassing each LCFA are indicated with abbreviated names as in Table 1 and names in bold mean that the LCFA is dominant in that specific species.

The schematization of Fig. 5 shows the benefit of using both $\omega$ and $\Delta$ (and mixed) nomenclatures in sponge lipid research. Annotations from the terminal end ($\omega$ and $(a)i$ notation) (Fig. 1) are convenient to show biosynthetic pathways as these positions do not change with elongation (Fig. 5). However, the typical $\Delta^{5,9}$ unsaturation is more convenient to show with $\Delta$ annotation, as an $\omega$ notation would alter with varying C chain length (Fig. 5). Ambiguity arises in $\omega$ notation of methyl-branching, because $a(i)$ notation is used for terminally branched FAs and describes total C atoms (including the methyl group(s)), while Me notation is used for MBFAs and describes the C number of the backbone (excluding methyl group(s)) and counts the position of the branching from the carboxylic acid end (and not the terminal $(\omega)$ end, Fig. 1). This might lead to confusion about the total C number, which is needed to correct measured isotope values for the extra methyl group, and about the $\omega$ position of unsaturation (start counting from the end of the backbone, excluding the methyl-group) and the conversion from $\omega$ to $\Delta$ notation (Fig. 1). We added this discussion to create awareness and would like to recommend including a description of the notation in the methods and presenting both nomenclature when a mixture of notation styles is used.
Conclusions

In this study we identified FAs of prominent habitat-building demosponges (order Tetractinellida) from the boreal-Arctic Atlantic Ocean. All five species investigated contained predominantly bacterial FAs, in particular isomeric mixtures of MBFAs (Me-C\textsubscript{16:0} and Me-C\textsubscript{18:0}) (together >20% of total FAs). The MBFAs were isotopically enriched compared to linear and (ante)iso-branched FAs. The sponge-produced LCFAs with chain lengths of C\textsubscript{24}-C\textsubscript{28} were linear, mid- and (a(i)-branched and had predominantly the typical Δ\textsuperscript{5,9} saturation. They also produced (yet undescribed) branched and linear LCFAs with Δ\textsuperscript{9,19} and Δ\textsuperscript{11,21} unsaturation, namely i-C\textsubscript{27:2}, C\textsubscript{26:2}, and C\textsubscript{28:2}. G. parva and S. rhaphidiophora each produced distinct LCFAs, while G. atlantica, G. barretti, and G. hentscheli had a similar LCFA profile, although each species had different predominant ones. The typical FA profiles of North-Atlantic deep-sea demosponges can be used as chemotaxonomic and trophic markers. We proposed biosynthetic pathways for dominant LCFAs from their bacterial precursors, which were supported by small isotopic differences in LCFAs that support the idea that sponges acquire building blocks from their endosymbiotic bacteria.

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**Supplementary information**

S1 Fig. Mass spectra of DMDS conducts of C$_{26}$ (a,b) and C$_{28}$ (c,d) LCFA with $\Delta^{9,19}$ (a,c) and $\Delta^{11,21}$ (b,d) unsaturation

S1 Table. All fatty acid compositional data. This excel file contains fatty acid data (µg g DW$^{-1}$ and relative abundance (%), in PL and TL) of individual specimens. The excel file also contains the fragments of Me-branched C$_{16}$ and C$_{18}$, the relative positions of saturated (branched and linear) FAMEs in hydrogenated samples and the isotope data.
$i-C_{17:1}\omega 7/15-Me-C_{16:1}\Delta^9$

Figure 1
Figure 2
Figure 4
Figure 5