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Methodological comparison for the isolation of shell-bound organic matter for carbon, nitrogen and sulfur stable isotope analysis

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Abstract

Shell-bound organic matter (SBOM) is present in the shells of biomineralizing organisms and can act as an isotopic proxy for nutrition. Stable isotope analysis of SBOM generally requires its isolation from the mineral component of the shell, and this study shows that various shell removal techniques (cation exchange resin, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), and acetic acid) can influence the carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and sulfur ($\delta^{34}S$) stable isotope values of both total SBOM and intra-crystalline SBOM to varying extents. In addition, isotopic and compositional differences are reported here between the different SBOM pools in the shell: total SBOM and intra-crystalline SBOM. Total SBOM isolated from Mytilus edulis, Ruditapes decussatus and Cerastoderma edule show minor differences in $\delta^{15}N$ values between methods, but all treated samples have slightly higher $\delta^{15}N$ values when compared to untreated shell powder. Methodological differences for $\delta^{15}N$ values of intra-crystalline SBOM are also limited to ~1‰, with the exception of cation exchange resin (max. -4‰ compared to mean values). Use of the cation exchange technique is also discouraged for obtaining $\delta^{13}C$ and $\delta^{34}S$ values for total and intra-crystalline SBOM, due to large deviations from mean values (to a maximum of -2‰ and -10‰, respectively). The other tested methods produce data with a 2‰-range for $\delta^{13}C$ values for total SBOM, although for intra-crystalline SBOM $\delta^{13}C$ values the use of acetic acid produced negative outliers. For sulfur stable isotope analysis extraction by EDTA is recommended, as acidification methods produce 1-2‰ lower $\delta^{34}S$ values for total SBOM, and using HCl can result in extremely negative intra-crystalline SBOM $\delta^{34}S$ values.

Keywords: Shell-bound organic matter, stable isotopes, filter feeding bivalves

1. Introduction

Shell-bound organic matter (SBOM) is the organic component of mollusc and brachiopod shells, consisting of a proteinaceous framework surrounding the mineral crystals (inter-crystalline SBOM), and a minor fraction that is present within individual crystals (intra-crystalline SBOM) (Lowenstam and Weiner, 1989). Together these fractions make up the total SBOM, which regulates biomineralisation by controlling the growth, mineralogy and structural organisation of newly formed crystallites (Marin et al., 2012). Because SBOM is secreted by mantle epithelial cells it has potential as an isotopic proxy for nutrition, and could act as a decay-resistant alternative to the animal's soft tissues in both modern and fossil shelled invertebrates (e.g. O'Donnell et al., 2003; Mae et al., 2007; Dreier et al., 2012). In addition to bivalves, carbonate-associated organic matter can also be obtained from other taxonomic groups, including foraminifera (e.g. Ren et al., 2009), corals (e.g. Wang et al., 2014), and fish otoliths (e.g. Lueders-Dumont et al., 2018). Stable isotope analysis of these organics is also revealing a valuable archive of environmental and ecological information, and our study could potentially be of interest in these research areas.

Stable isotope analysis of SBOM requires its separation from the mineral component of the shell (calcium carbonate for all organisms in this study), because the mineral carries an environmental carbon and sulfur isotopic signal (Immenhauser et al., 2016), and is much more abundant than SBOM in the shell. The effects of chemical isolation on the carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and sulfur ($\delta^{34}S$) stable isotope signatures of SBOM are poorly understood and have mostly not been tested, even though small isotopic deviations can indicate different food sources or different trophic levels (Michener and Kaufman, 2007), and artifacts imposed by the isolation of SBOM could potentially confound interpretations about an animal's ecology and environment. To address this knowledge gap, we have directly compared the effects of the most commonly applied reagents for shell removal:

ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), acetic acid (AA), and cation exchange resin (RESIN). Tests were performed on both total SBOM and intra-crystalline SBOM. Because intra-crystalline SBOM is protected from the external environment, it has a greater preservation potential on longer timescales. (Sykes et al., 1995; Penkman et al., 2008). In this study bivalve intra-crystalline SBOM has also been analysed separately to investigate any isotopic and compositional differences between the two SBOM pools.

Previous studies have explored the limitations of various shell dissolution methods. Acidification is the most common method used in ecological studies to obtain biological organic matter from carbonate-rich samples, whereby inorganic carbon is expelled as carbon dioxide. A review study by Schlacher and Connolly (2014) summarised that acidification of calcified structures can result in both higher and lower $\delta^{13}C$ and $\delta^{15}N$ values of biological organics to varying extent, but in many cases the exact mechanisms for these changes have remained undetermined. Potential causes are loss or chemical transformation of organic matter, particularly due to the break-up of protein complexes and the solubilisation of proteins (Schlacher and Connolly, 2014). Darrow et al. (2017) conducted a method comparison using $\delta^{15}N$ values of oyster shells between untreated, acidified-filtered, and acidified-centrifuged methods to obtain total SBOM, and report no statistical difference between the methods. The study does warn that untreated samples could result in low total nitrogen content and subsequent reduced quality of $\delta^{15}N$ data, but Gillikin et al. (2017) have shown that even with very low nitrogen content, simple combustion of untreated shell powder is possible for $\delta^{15}N$ analysis. Similarly, Carmichael et al. (2008) report that increased concentration or prolonged exposure to acidification can also produce less reliable $\delta^{15}N$ values due to reduced nitrogen recovery. No published studies have be found on the potential effects of acidification for $\delta^{34}S$ analysis of SBOM.

An alternative method to acidification is the calcium-chelating agent EDTA (Albeck et al., 1996; Mae et al., 2007; Dreier et al., 2012) that isolates SBOM by binding calcium, and, compared to acidification, has the benefit of working at neutral pH (Meenakshi et al., 1971). However, EDTA can be very difficult to remove from the SBOM without specialized filtration systems, due to the formation of EDTA-calcium-protein complexes (Curry et al., 1991). Because EDTA molecules contain carbon and nitrogen, this technique could potentially influence SBOM $\delta^{13}C$ and $\delta^{15}N$ values by introducing exogenous carbon and nitrogen.

The cation exchange resin method of shell removal (Albeck et al., 1996; Gotliv et al., 2003) does not introduce any additives, and isolates SBOM by binding calcium ions, whilst releasing carbon dioxide. We designed a novel set-up of this method for performing large batches of these isolations. In addition, we tested the potential of this method for simultaneous isolation of carbonate associated sulfate (CAS) for $\delta^{34}S$ analysis. CAS is trace sulfate incorporated into the lattice of carbonate minerals, and in marine carbonates records the sulfur isotopic composition of ambient seawater sulfate (Kampschulte and Strauss, 2004).

2. Materials and Methods

2.1. Material

Analyses were carried out on homogenized shell samples from three marine bivalve taxa: Mytilus edulis (blue mussel), Ruditapes decussatus (grooved carpet shell) and Cerastoderma edule (common cockle). The shell of M. edulis consists of a calcite/aragonite mix, whilst the other two species have aragonite shells. These species are primary consumers that filter feed on suspended organic matter from the water column. M. edulis has an epifaunal lifestyle, whilst the other two species are infaunal sediment dwellers.

Material for this study was obtained from the local fish market in Leeds (UK) in October/November 2012, and comprised 1 kilogram of live specimens originating from Wales, UK (M. edulis), southern France (R. decussatus) and Dorset, UK (C. edule). The shells were washed in deionised water and soft tissue was excised, the shells were then cleaned of remaining organic material (internal soft tissues, periostracum and ligament) using a scalpel and a Dremel rotary tool, rewashed with DI water and air-dried. The dry valves were then ground using a ceramic mortar and pestle, sieved to <125 µm particle size in a stainless steel sieve, and homogenized for SBOM isolation. A minimum of 3 replicates was performed for each isolation method (see: Supplementary Material for details).

Soft tissues of several specimens from the three test species were also analysed, these results are reported in the Supplementary Material (Table A3).

2.2. Isolation of SBOM

2.2.1. Isolation of intra-crystalline SBOM

Intra-crystalline SBOM samples were obtained from shell powder from which the inter-crystalline SBOM pool was removed, following the procedures of Penkman et al. (2008) and Demarchi et al. (2012). Prepared shell powder was oxidized with 12% w/v NaOCl (VWR International, Carnot, France) for a 48 hour period within a glass beaker (50 µL per mg of shell powder). After completion the samples were rinsed a minimum of three times with excess DI water and air-dried on 20-25 µm filter paper. To isolate the intra-crystalline SBOM, the shell powder with inter-crystalline SBOM removed was processed using the same isolation methods as used for unbleached shell powder. Because of the grinding process it is possible that part of the intra-crystalline SBOM is also exposed to the NaOCl and removed, so potentially only a subset of the intra-crystalline SBOM is analysed.

2.2.2. SBOM isolation using cation exchange resin

SBOM was isolated using cation exchange resin (Dowex 50WX8 50-100 mesh, Acros Organics, New Jersey, USA) based on a modification of the methodologies from Albeck et al. (1996) and Gotliv et al. (2003). Approximately 2 g of shell powder suspended in DI water were placed inside a dialysis bag (3500D, Spectra Por 3, 18 mm width, SpectrumLab, Inc., Rancho Dominguez, USA). The dialysis bag was placed in a glass vial with 75 mL resin and 25 mL DI water. The dialysis tube was vented through the lid of the vial and the reaction vessel was placed in a horizontal shaker for two weeks. The pH of the solution stayed constant around 1.5-2. After shell dissolution was completed, the dialysis bag with SBOM was dialysed for five days in DI water, frozen and freeze-dried. Dried SBOM was weighed for calculation of recovery.

2.2.3. SBOM isolation using EDTA

For the EDTA technique, the methodology of Dreier et al. (2012) was followed (see also Mae et al., 2007). Approximately 2 g of shell powder were suspended in DI water within dialysis tubing, and placed in 100 mL 0.5M EDTA (VWR International, Leuven, Belgium) made up with MilliQ water (adjusted to pH 7.4 using potassium hydroxide) in glass beakers. Shell dissolution was complete after two weeks, and the dialysis bag with SBOM was dialysed for five days in MilliQ water changed daily. Subsequently, the SBOM was placed in centrifuge tubes, centrifuged and rinsed three times with MilliQ water, before freezing and freeze-drying.

2.2.4. SBOM isolation using acid

For HCl dissolution of the mineral component (following Mae et al., 2007) 10 mL 6 M HCl (Sigma-Aldrich, Steinheim, Germany) was slowly added to 2 g of shell powder in a 50 mL

centrifuge tube. For dissolution using 10% HCl (v/v) 40 mL was added to 2 g shell powder in a glass vial. The SBOM was subsequently centrifuged and rinsed three times with DI water to de-acidify the organics. A similar procedure was followed with acetic acid 10% v/v (Aldrich, Dorset, England), whereby 40 mL acid was slowly added to 2 g of shell powder in glass vials left overnight. The SBOM was centrifuged and rinsed three times with DI water. The SBOM samples obtained using HCl and acetic acid were then frozen and freeze-dried.

2.3. CAS isolation using resin or HCl

Carbonate-associated sulfate (CAS) was obtained from the DI water containing the cation exchange resin as described in section 2.2.2 (surrounding the dialysis bag). The water was filtered using 20-25 μm filter paper to remove residual resin, then placed in clean glass beakers, and the pH was adjusted to 2-3 using 10% HCl or 10% NH_4. To precipitate the $BaSO_4$ for isotopic analysis the solutions were heated to ~70 °C on a hot plate, 10% BaCl was added as 10% of the total volume, and the solution was kept at this temperature for an hour. After having cooled down overnight, the precipitated $BaSO_4$ was vacuum filtered out on 0.45μm cellulose/nitrate filter paper, that were left to dry in a drying cabinet (~50°C) and stored in glass vials. As a control for the cation exchange resin method, CAS was also isolated from C. edule bulk shell material using 10% HCl, and precipitated as described for the resin method.

2.4. Stable isotope and concentration analyses

Carbon, nitrogen and sulfur isotope analyses were performed on freeze-dried SBOM and the EDTA reagent and cation exchange resin. $\delta^{15}N$ SBOM values were also measured on untreated shell powder via direct combustion for comparison with extracted total SBOM. This is possible because SBOM is the only nitrogen pool in the shell. The amounts of intra-

crystalline SBOM pool present in bleached shell powder are however insufficient for $\delta^{15}N$ stable isotope analysis via direct combustion

The isotopic analyses were performed on an Isoprime continous flow mass spectrometer coupled to an Elementar Pyrocube elemental analyser. For all analyses the sample was weighed into 8 x 5 mm tin cups and combusted to N_2, CO_2 and SO_2 at 1150°C in the presence of pure oxygen (N5.0) injected into a stream of helium (CP grade). Quantitative conversion to N_2, CO_2 and SO_2 was achieved by passing the combustion product gas through tungstic oxide packed into the combustion column. Excess oxygen was removed by reaction with hot copper wires at 850°C and water was removed in a Sicapent trap. All solid reagents were sourced from Elemental Microanalysis, UK, and all gases were sourced from BOC, UK. One aliquot of each SBOM sample (0.05-0.1 mg) was analysed for its carbon composition, whilst duplicate larger aliquots (0.5-2.0 mg) were analysed for their nitrogen and sulfur composition in the same run, to limit the amount of SBOM necessary for the analyses. These sample weights resulted in approximately 20-40 μg of carbon, 50-200 μg of nitrogen, and 20-60 μg of sulfur analysed for total SBOM, and 15-40 μg of carbon, 15-45 μg of nitrogen and10-30 μg of sulfur for intra-crystalline SBOM. Samples for combined $\delta^{15}N/\delta^{34}S$ analysis were analysed in duplicate because of a small sulfur isotopic memory effect imparted during processing of SO_2 gas in the Pyrocube. In all analyses, N_2 produced by combustion continued through the system unchecked whilst CO_2 and SO_2 were removed from, and re-injected into, the gas stream using temperature controlled adsorption/desorption columns.

The blanks for carbon, nitrogen and sulfur isotope analyses were 0.2, 0.05 and 0.006 nA or better, respectively. No blank correction was performed but standards were run at approximately the same peak height as the average sample run. The range of sample peak heights for carbon, nitrogen and sulfur analyses was 3-9, 1-10, and 1-4.0 nA respectively. Average sample peak heights for C, N and S were normally around 6, 4.5 and 2.3 nA

225 respectively and standards were weighed to produce peak heights within ~0.5 nA of these
226 values.
227 The $\delta^{13}C$ value of the sample is derived from the integrated m/z 44, 45 and 46
228 fragment ions from the pulse of sample CO_2, compared to those in an independently
229 introduced pulse of CO_2 reference gas (CP grade). These ratios are then calibrated to the
230 international Vienna-Pee Dee Belemnite (V-PDB) scale using urea and C4 sucrose lab
231 standards with assigned values of -11.93‰ and -46.83‰ respectively. These values were
232 assigned by calibration using the international standards (assigned V-PDB value in brackets)
233 LSVEC (-46.479‰), CH7 (-31.83‰), CH6 (-10.45‰), and CO-1 (+2.48‰). The precision
234 obtained for repeat analysis of standard materials is generally 0.2‰ or less (1 standard
235 deviation). Repeat analyses of a lab C3 sucrose produced an average of -26.5‰ with a
236 standard deviation of 0.1‰.
237 The sample $\delta^{15}N$ value is derived using the integrated m/z 28 and 29 fragment ions
238 relative to those in a pulse of N_2 reference gas (N5.0). These ratios are calibrated to the
239 international AIR scale using USGS-25 and USGS-26 (both ammonium sulfate) which have
240 been assigned values of -30.4‰ and +53.7‰ respectively. The precision obtained for repeat
241 analyses of standard materials is generally 0.3‰ or less (1 standard deviation). Repeat
242 analyses of a yeast sample produced an average of -0.8‰ with a standard deviation of 0.1‰.
243 The sample $\delta^{34}S$ value is derived using the integrated m m/z 64 and 66 ions relative to
244 those in a pulse of SO_2 reference gas (N3.0). These ratios are calibrated to the international
245 V-CDT scale using an internal lab barium sulfate standard derived from seawater (SWS-3)
246 which has been analysed against the international standards NBS-127 (20.3‰), NBS-123
247 (17.01‰), IAEA S-1 (-0.30‰) and IAEA S-3 (-32.06‰) and assigned a value of 20.3‰, and
248 an inter-lab chalcopyrite standard CP-1 assigned a value of -4.56‰. The precision obtained
249 for repeat analyses of SBOM is generally 0.5‰ or smaller (1 standard deviation) and 0.2‰

or smaller (1 standard deviation) for CAS. Repeat analyses of a sulphanilamide sample produced an average of -0.2‰ with a standard deviation of 0.3‰.

Weight percent nitrogen (%N) and sulfur (%S) data were calculated by the Pyrocube software using a calibration based on multiple analyses of sulphanilamide samples with a range of weights. Relative standard deviations on analyses of %N and %S contents of the isotope calibration materials were 5% or better. The very small amount of material needed for $\delta^{13}C$ analysis meant that the sample size was below the calibration range for weight percent carbon (%C), and %C content was obtained from $\delta^{15}N/\delta^{34}S$ analyses of the same sample. The amount of %C data is therefore limited and given in Table A1 of the Supplementary Material.

2.5. Thermally assisted hydrolysis and methylation (THM) of SBOM in the presence of tetramethylammonium hydroxide (TMAH)

THM of total and intra-crystalline SBOM from *M. edulis* (obtained using cation exchange resin) in the presence of TMAH (also known as TMAH thermochemolysis) was performed on a CDS Pyroprobe 1000 via a CDS1500 valved interface (320°C), to a Hewlett-Packard 6890GC injector (320 °C) linked to a Hewlett-Packard 5973MSD (electron voltage 70eV, filament current 220 μA, source temperature 230°C, quadrupole temperature 150°C, multiplier voltage 2200V, interface temperature 320°C) (Robertson et al., 2008). The acquisition was controlled by a HP kayak xa chemstation computer, in full scan mode (50-650amu). Approximately 0.3 mg of the SBOM sample was weighed into a quartz tube with glass wool end plugs. The methylating agent tetramethylammonium hydroxide (TMAH) and the internal standard 5α-androstane were added to the samples. The tube was then placed into a pyroprobe platinum heating coil and then sealed into the valved interface. The sample was pyrolysed at 610 °C for 10 seconds (Abbott et al., 2013). At the same time the GC temperature programme and data acquisition commenced. Separation was performed on a

fused silica capillary column (60 m x 0.25 mm i.d) coated with 0.25 μm 5% phenyl methyl silicone (HP-5). Initially the GC was held at 50°C for 5 minutes and then temperature programmed from 50 – 320°C at 5° C per min and held at the final temperature for 5 minutes, giving a total run time of 65 minutes. The carrier gas was helium (constant flow 1 mL/min, initial pressure of 50 kPa). Peaks were identified and labelled after comparison of their mass spectra with the NIST05 library.

2.6. Data averaging and statistical methods

Duplicate samples were averaged to produce the final isotopic value. $\delta^{34}S$ values can be affected by a small memory effect, and only values 0.6‰ or less apart are considered to be identical and averaged, as normal reproducibility is ~ 0.3‰.

Different SBOM isolation methods were compared using unpaired t-tests (significance threshold: $p < 0.05$), excluding identified outliers.

Results are displayed in box-and-whisker plots. In these plots the median is represented by the horizontal line and the limits of the box and whiskers contain 50% and 100% of the data, respectively. If outliers are present, they fall outside 1.5*inter-quartile range (which is then indicated by the length of the whiskers) and are shown as asterisks.

3. Results

Total SBOM and intra-crystalline SBOM were successfully isolated using all shell removal methods. The weight percent (wt%) of total SBOM obtained from the shell powder varied between species (M. edulis = 0.7% to 1.0%; R. decussatus = 0.2% to 0.4% ; C. edule = 0.1% to 0.4% using ion-exchange resin) and the intra-crystalline fraction makes up a small percentage of the total (shell wt%: M. edulis = ~ 0.01%, R. decussatus and C. edule = ~ 0.005%, using ion-exchange resin). SBOM is voluminous, and has a uniform colour for each

species. The dark blue shelled M. edulis contains SBOM with a dark brown colour, whilst the other two species have lighter beige-brown shells and contain SBOM with a beige or light brown colour.

3.1. Method comparison for isotopic values of SBOM and CAS

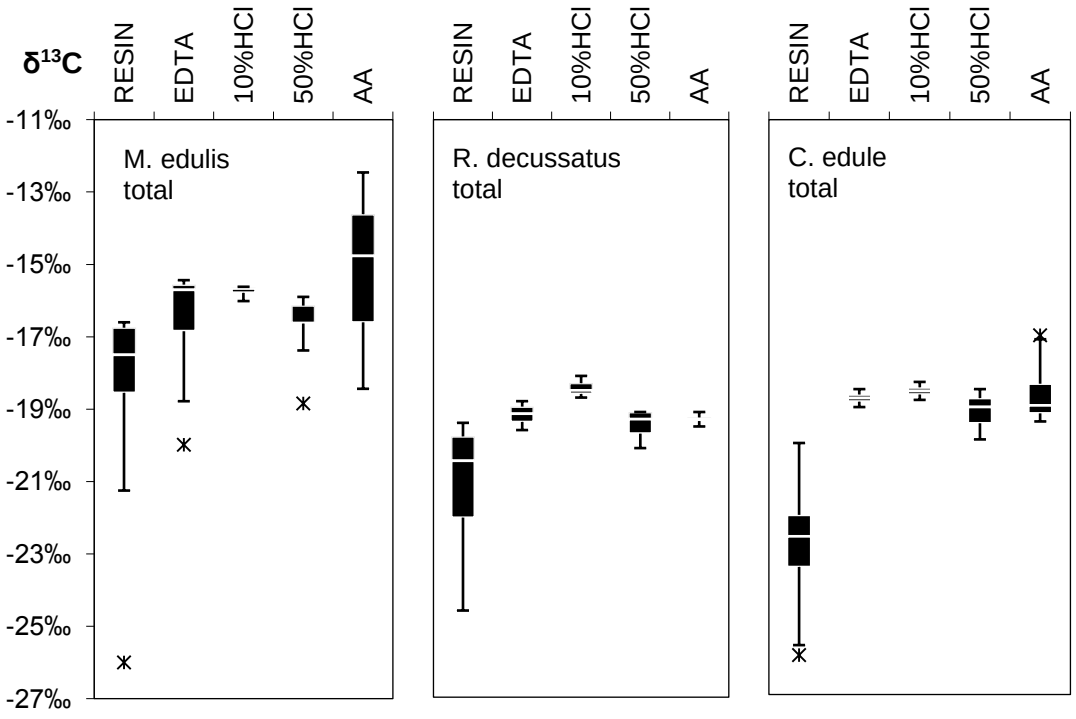
3.1.1. Carbon isotope compositions of total and intra-crystalline SBOM

Total SBOM and intra-crystalline SBOM $\delta^{13}C$ values are shown in Fig. 1, and given in Table A1 of the Supplementary Material. The $\delta^{13}C$ values of shell removal agents are -39.0 ± 0.6‰ (n = 2) for EDTA, and -29.2‰ (n = 1) for cation exchange resin.

Total SBOM isolated using cation exchange resin has lower mean $\delta^{13}C$ values than the other four methods for all test species (Fig. 1 A). The difference is statistically significant for M. edulis versus EDTA (p = 0.0039), 10%HCl (p = 0.0037), and 50%HCl (p = 0.0110), for R. decussatus versus 10%HCl (p = 0.0155), and for C. edule versus EDTA (p = 0.0008), 10%HCl (p = 0.0007), 50%HCl (p = 0.0059), and acetic acid (p = 0.0047). In addition, cation exchange resin $\delta^{13}C$ values are generally the most variable, particularly in R. decussatus and C. edule.

Statistical differences between the other methods are minor: in M. edulis the $\delta^{13}C$ value from the 50%HCl method is significantly lower than EDTA (mean difference of 0.7, p = 0.0409) and 10%HCl (difference of 0.7, p = 0.0339), and in R. decussatus compared to 10%HCl (difference of 0.4, p = 0.0006). In addition, 10%HCl total SBOM $\delta^{13}C$ values are statistically higher compared to those of acetic acid R. decussatus total SBOM samples (difference of 0.9, p = 0.0003). The $\delta^{13}C$ distribution of acetic acid extracted total SBOM is much wider and more positive (when compared to other treatment for the same species) in M. edulis (ranging from -18.4‰ to -12.5‰, n = 3) than other species.

a)



b)

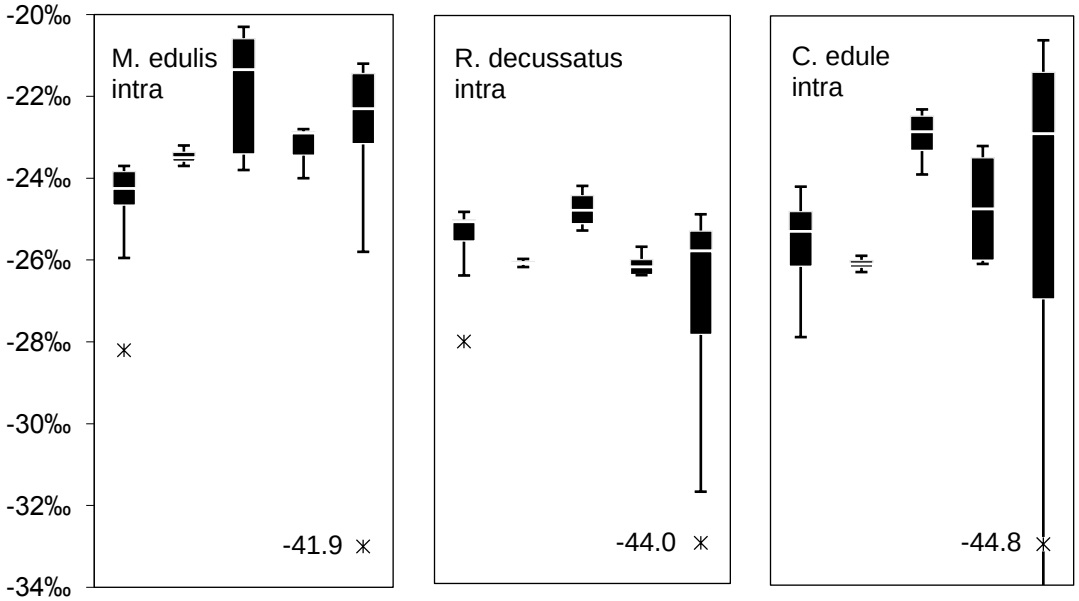


Fig. 1. Box-and-whisker plots of $\delta^{13}C$ values from a) total SBOM and b) intra-crystalline SBOM (intra) for method comparison. Results shown for samples isolated using cation exchange resin (RESIN), EDTA, 10%HCl, 50%HCL and acetic acid (AA).

For all species, intra-crystalline SBOM $\delta^{13}C$ values are significantly lower than total SBOM $\delta^{13}C$ values (note difference in axes for Fig. 1 A and 1 B). Mean differences between the two SBOM pools across the different methods (n = 5) are: $\delta^{13}C$ = 6.9 ± 0.6‰ for M. edulis, 6.1 ± 1.2‰ for R. decussatus, and 5.5 ± 1.6‰ for C. edule.

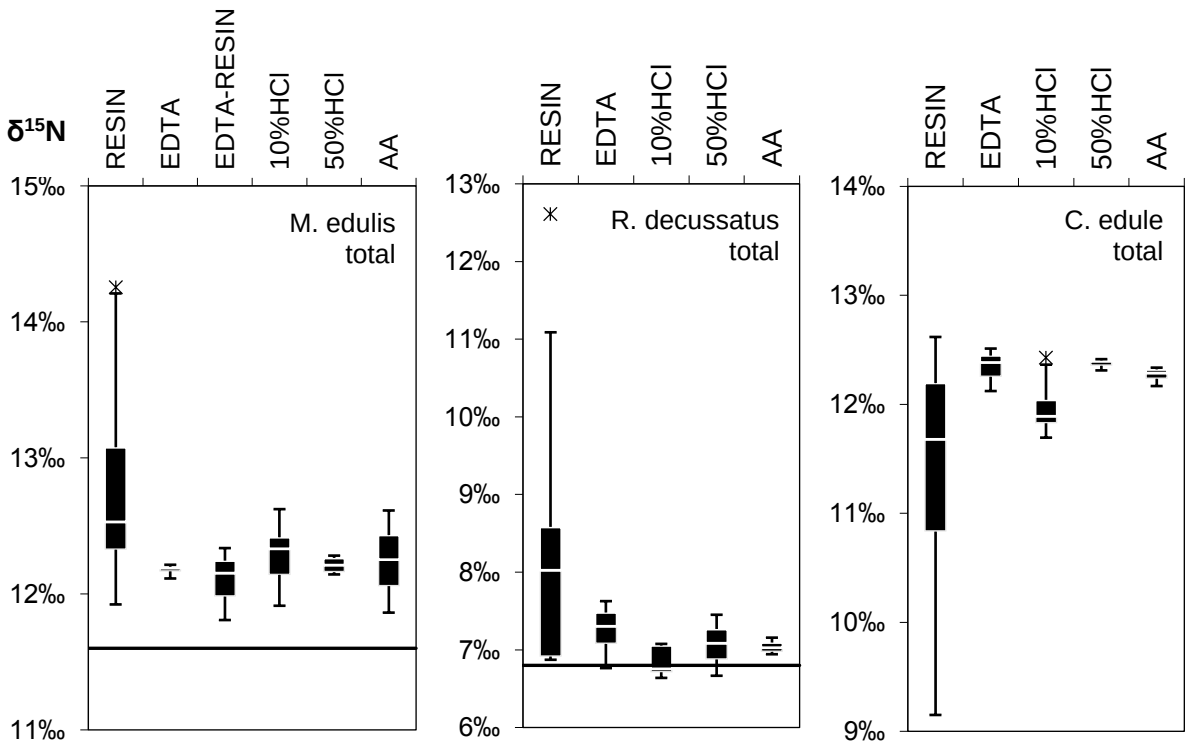
Between the different shell removal techniques used to isolate intra-crystalline SBOM, most notable are the occurrence of some extremely negative values for the acetic acid extraction ($\delta^{13}C$ < -40‰) in each test species. Additionally, in M. edulis intra-crystalline SBOM isolated using cation exchange resin has significantly lower $\delta^{13}C$ values compared to EDTA (p = 0.0001), 10%HCl (p = 0.0030), and acetic acid (p = 0.0001).

For all three species the mean/median values of 50%HCl/EDTA are lower than 10%HCl/acetic acid values. This difference is statistically confirmed using t-tests between M. edulis EDTA vs. acetic acid (p = 0.0346), R. decussatus EDTA vs. 10%HCl (p = 0.0074) and 50%HCl vs. 10%HCl (p = 0.0043), and C. edule EDTA vs. 10%HCl (p = 0.0001) and 50%HCl vs. 10%HCl (p = 0.0054). No statistical differences exist between intra-crystalline samples obtained using EDTA and 50%HCl, or between 10%HCl and acetic acid.

3.1.2. Nitrogen isotopes and concentrations of total and intra-crystalline SBOM

$\delta^{15}N$ results are presented in Fig. 2 and for each analysed aliquot of bulk shell powder the $\delta^{15}N$ value is plotted against %N value in Fig. 3. The $\delta^{15}N$ value of EDTA used for the isolations is 1.5 ± 0.6‰ (n = 2), and in the cation exchange resin no nitrogen was detectable (sample weight analysed was: ~10 mg).

a)



b)

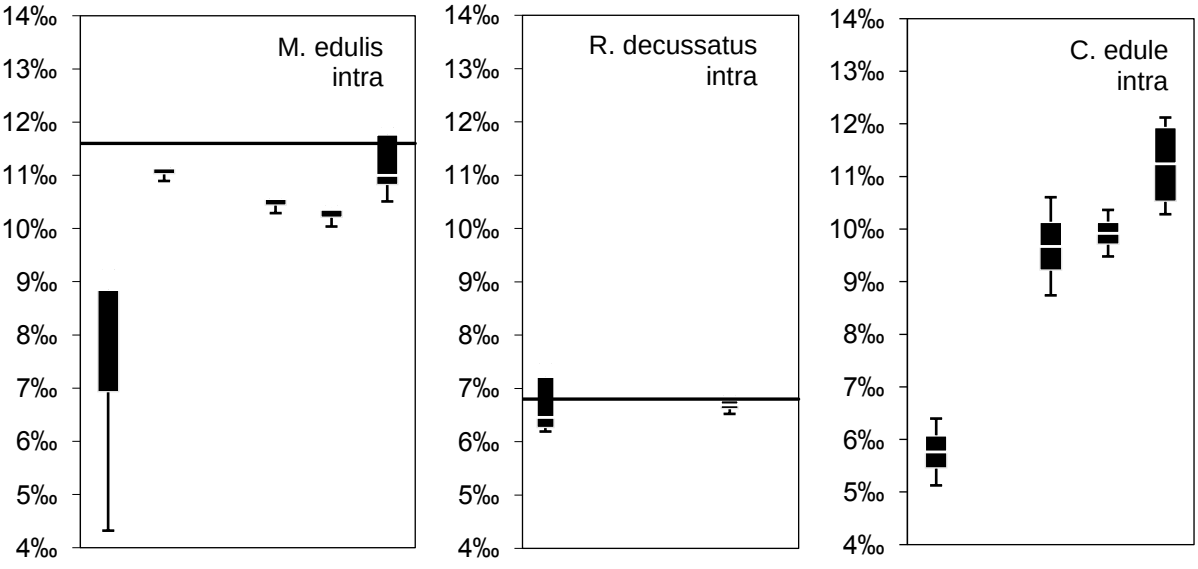


Fig. 2. Box-and-whisker plots of $\delta^{15}N$ values from a) total SBOM and b) intra-crystalline SBOM (intra) for method comparison. Results shown for samples isolated using cation exchange resin (RESIN), EDTA, 10%HCl, 50%HCL and acetic acid (AA). Untreated shell total SBOM results are shown as a solid line in M. edulis and R. decussatus

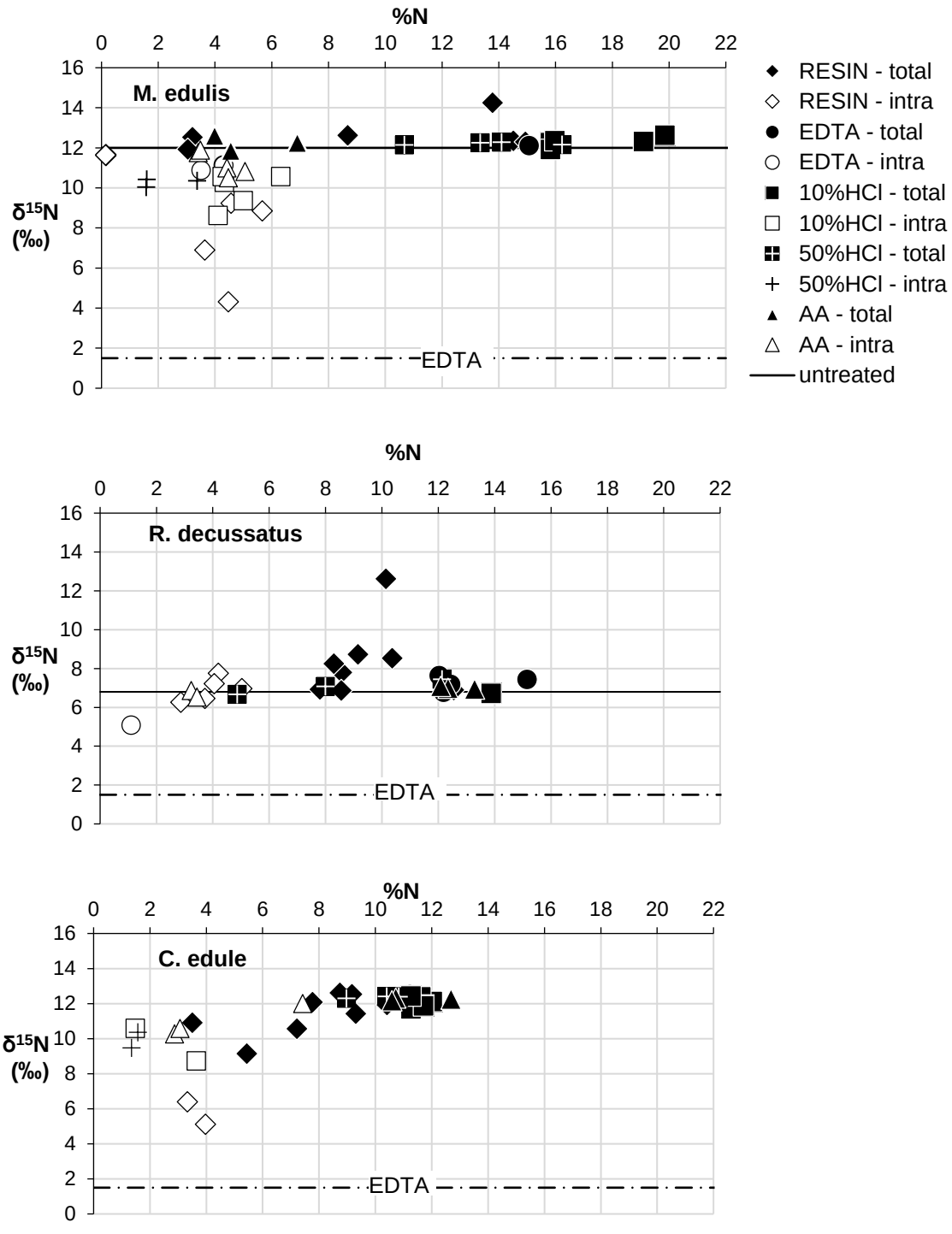


Fig. 3. $\delta^{15}N$ values and nitrogen concentration (%N) of total SBOM and intra-crystalline SBOM (intra) for method comparison. Results shown for samples isolated using cation exchange resin (RESIN), EDTA, 10%HCl, 50%HCL, and acetic acid (AA). Untreated total SBOM results are shown as a solid line in M. edulis and R. decussatus (data unavailable for C. edule). The value of EDTA is also shown.

374 There is no statistical difference between total SBOM $\delta^{15}N$ values obtained using the
375 different shell removal methods, with the exception of lower $\delta^{15}N$ values for 10%HCl
376 samples for R. decussatus and C. edule (~ 0.5‰ lower). To further analyse the nitrogen
377 stable isotopic effect of cation exchange resin isolation on total SBOM, several EDTA
378 extracted samples from M. edulis were re-extracted using resin. This procedure did not
379 change the isotopic signature of the samples (EDTA: $\delta^{15}N = 12.2 \pm 0.1$‰, n = 3, EDTA-resin:
380 $\delta^{15}N = 12.1 \pm 0.3$‰, n = 3), or the nitrogen concentration between EDTA (%N = 14.7 ±
381 0.7%, n = 3) and EDTA-resin samples (%N = 13.1 ± 1.3%, n = 3), although variation slightly
382 increased for both parameters.
383
384 For each test species untreated shell powder was analysed three times for $\delta^{15}N$ composition,
385 with sample weights ranging from 35 to 48 mg. M. edulis samples all gave reliable results
386 due to high %N (=0.15) and high SBOM wt%. Only one analysis for R. decussatus was
387 reliable (%N = 0.07, n = 3), and no measurement for C. edule was reliable (%N = 0.07, n =
388 3). Compared to untreated total SBOM of M. edulis ($\delta^{15}N$: 11.6 ± 0.1‰, n = 3), all other
389 methods have significantly higher $\delta^{15}N$ values (p = 0.005 to 0.0424, n = 5) by ~ +0.7‰.
390 Untreated R. decussatus total SBOM value ($\delta^{15}N = 6.8$‰, n = 1) only overlaps with the
391 majority of 10%HCl obtained values ($\delta^{15}N = 6.8 \pm 0.2$‰, n = 5), samples isolated using
392 EDTA/50%HCl/acetic acid are ~ 0.3‰ higher, and for cation exchange resin ~ 1.0‰ higher.
393 Despite the lack of isotopic differences, the concentration of nitrogen (%N) of total
394 SBOM differs between the methods, as shown in Fig. 3. %N is generally the most variable
395 for samples obtained using cation exchange resin, and also considerably lower for resin
396 compared to other methods: for M. edulis vs. 10%HCl (p = 0.0130), for R. decussatus vs.
397 EDTA (0.0034), vs. 10%HCl (0.0001) and acetic acid (0.0068), and for C. edule vs. EDTA
398 (0.0069), 10%HCl (0.0026), 50%HCl (0.0466), and acetic acid (p = 0.0119). Resin $\delta^{15}N$

399 outliers are not related to a difference in %N, as shown in Fig. 3. The %N of total SBOM
400 samples from M. edulis obtained using acetic acid (5.2 ± 1.5 %, n = 3) is similar to cation
401 exchange resin, and approximately half that of the other methods (ranging from 10.7% to
402 19.9%), but this low concentration is not reflected in the other two test species. In addition,
403 50%HCl samples were statistically lower in %N than 10%HCl for M. edulis (p = 0.0300), to
404 EDTA (0.0369) and 10%HCl (0.0043) for R. decussatus), and to 10%HCl for C. edule (p =
405 0.0307). %N in EDTA extracted total SBOM samples is not significantly higher when
406 compared to samples extracted using 10% HCl or acetic acid, so there is no direct evidence
407 for extensive residual EDTA in the samples.
408
409 Where total and intra-crystalline SBOM could be compared, intra-crystalline SBOM $\delta^{15}N$
410 values are significantly more negative than total SBOM for all methods/species, with the
411 exception of M. edulis SBOM obtained using acetic acid (total SBOM = 12.3 ± 0.4‰, n = 3 /
412 intra-crystalline SBOM = 11.2 ± 0.6‰, n = 3) and R. decussatus using cation exchange resin
413 (total SBOM = 7.7 ± 0.8‰, n = 7 / intra-crystalline SBOM = 7.0 ± 0.6‰, n = 5). The mean
414 difference between the two SBOM pools is around 1-2‰ for M. edulis, 0.5-1‰ for R.
415 decussatus, and 1-2.5‰ for C. edule; for cation exchange obtained samples this difference is
416 much larger for M. edulis (mean difference = 4.9‰) and C. edule (5.5‰). The %N of intra-
417 crystalline SBOM is also significantly lower than the %N of total SBOM (Fig. 3). For M.
418 edulis the difference between the two pools was 7.1% compared to 12.4% respectively (n = 4
419 methods), with the exception of acetic acid (1.0% difference). For R. decussatus there is a
420 statistically significant difference between total and intra-crystalline SBOM %N for resin
421 (5.4%) and AA (9.6%), for the other methods insufficient data are available for comparison.
422 The %N of intra-crystalline SBOM is significantly lower than that of total SBOM for C.

edule in: 10%HCl (-9.5%), 50%HCl (-8.6%), AA (-6.8%), but not for cation exchange resin obtained samples (-3.3%).

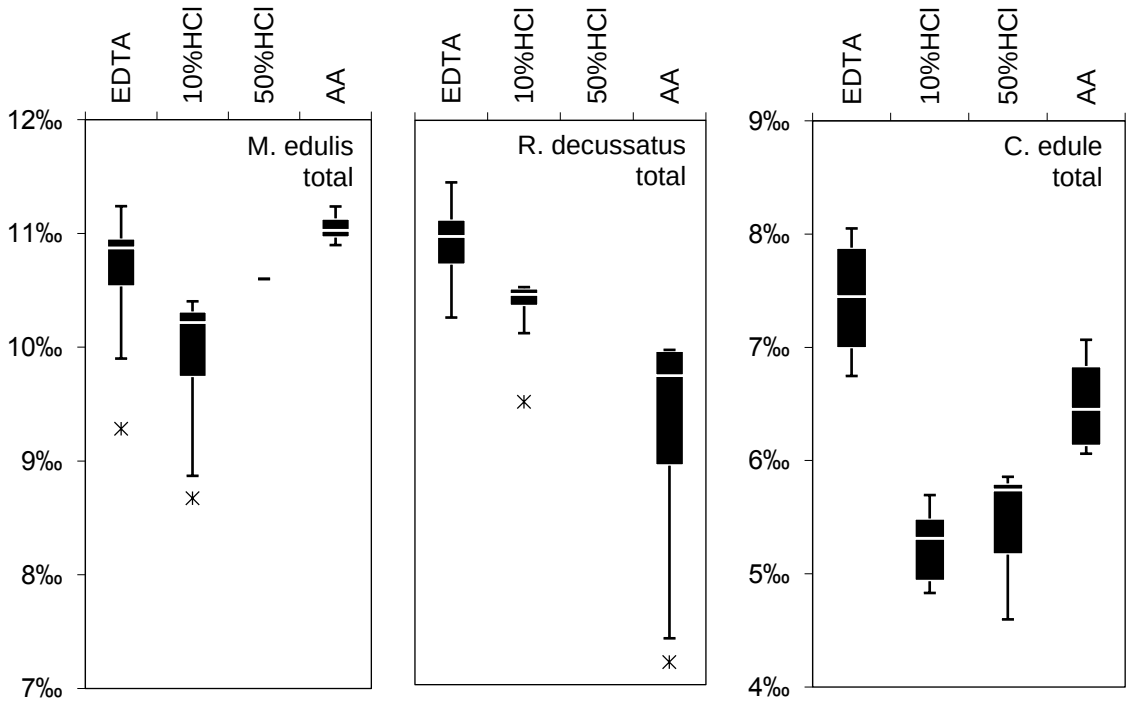
The treated intra-crystalline SBOM samples have more negative $\delta^{15}N$ values compared to untreated total SBOM for all methods ($p = 0.0001$ to 0.0188), with the exception of acetic acid ($p = 0.3183$) (Fig. 2b). The mean difference between the pools ranges from 4‰ for cation exchange resin, to 0.4-1.7‰ for the other methods. For R. decussatus, the $\delta^{15}N$ value of untreated total SBOM (6.8‰) is similar to intra-crystalline SBOM obtained using cation exchange resin (7.0 ± 0.6‰, $n = 5$) and acetic acid (6.7 ± 0.3‰, $n = 2$).

Because of the low shell wt% and the low %N of intra-crystalline SBOM, the number of analyses that produced robust $\delta^{15}N$ values were fewer, making comparison between methods more difficult. The available data show that for M. edulis cation exchange resin intra-crystalline SBOM is ~2‰ lower and more variable than SBOM obtained using the other methods (EDTA, 10%HCl, acetic acid). Similarly, for C. edule cation exchange samples are ~4‰ lower compared to the other available method data (10%HCl, 50%HCl, acetic acid). For R. decussatus $\delta^{15}N$ data for intra-crystalline SBOM is only available for cation exchange resin and acetic acid samples, but there is no statistical difference between these two methods.

Comparison between EDTA, 10%HCl, 50%HCl and acetic acid extracted intra-crystalline SBOM for M. edulis shows that the mean $\delta^{15}N$ values of the methods vary < 1‰. However due to very small intra-method variability (S.D of the methods: 0.2‰ to 0.6‰), 10%HCl samples are statistically more negative than those extracted using EDTA ($p = 0.0002$) and acetic acid (0.0035). 50%HCl samples are also more negative than EDTA samples (0.0080). For intra-crystalline SBOM from C. edule $\delta^{15}N$ intra-method variability is greater (0.6‰ to 1.3‰), and the different shell removal techniques are statistically similar.

448 The %N of intra-crystalline SBOM samples obtained using 50%HCl is lower than all
449 other methods (p = 0.0065 to 0.0384, n = 4) in M. edulis. In C. edule the same is true when
450 compared to resin (p = 0,0054), but not when compared toother methods. No further
451 comparisons for 50%HCl are available, and the data from other methods are not statistically
452 different.
453
454 **3.1.3. Sulfur isotopes and concentrations of total and intra-crystalline SBOM and CAS**
455 The $\delta^{34}S$ results are reported in Fig. 4 and are plotted against %S values in Fig. 5. The $\delta^{34}S$
456 value of the cation exchange resin used for the isolations is -1.5‰ (n = 1), with a %S of
457 2.9%.
458 Total SBOM $\delta^{34}S$ values from M. edulis obtained using resin are significantly lower
459 (2.4 ± 3.4‰, n = 6) and show a significant increase in %S (resin average = 3.7 ± 3.5 %, n =
460 6) compared to all other methods (combined values for other isolations, incl. outliers: $\delta^{34}S$ =
461 10.3 ± 3.5‰, n = 13 and %S = 0.7 ± 0.5 %, n = 11). The same pattern is found for R.
462 decussatus resin $\delta^{34}S$ values (1.8 ± 0.5‰, n = 4) and %S (5.4 ± 1.4%, n = 4) compared to
463 other methods ($\delta^{34}S$ = 10.1 ± 1.1‰, n = 10, and %S = 1.9 ± 0.3 %, n = 10) and for C. edule:
464 $\delta^{34}S$ = 0.1 ± 1.7‰ (resin, n = 2) vs. 6.2 ± 1.1‰ (other methods, n = 14), and in concentration:
465 %S = 5.1 ± 2.5% (resin, n = 2) vs. 2.5 ± 0.5% (other methods, n = 14).
466 $\delta^{34}S$ values become lower with increasing %S and are strongly correlated, with R^2
467 values of 0.62, 0.47, 0.33 for M. edulis, R. decussatus, and C. edule respectively. . M. edulis
468 EDTA samples that underwent a second treatment using resin show a similar shift, with a
469 lower isotopic value ($\delta^{34}S$ before resin treatment = 10.1 ± 1.1‰, n = 2; after resin treatment =
470 3.2 ± 0.1‰, n = 2) and a higher concentration (%S before resin treatment= 0.8 ± 0.2 %, n =
471 2; after resin treatment = 2.8 ± 0.1 %, n = 2).
472

473 a)



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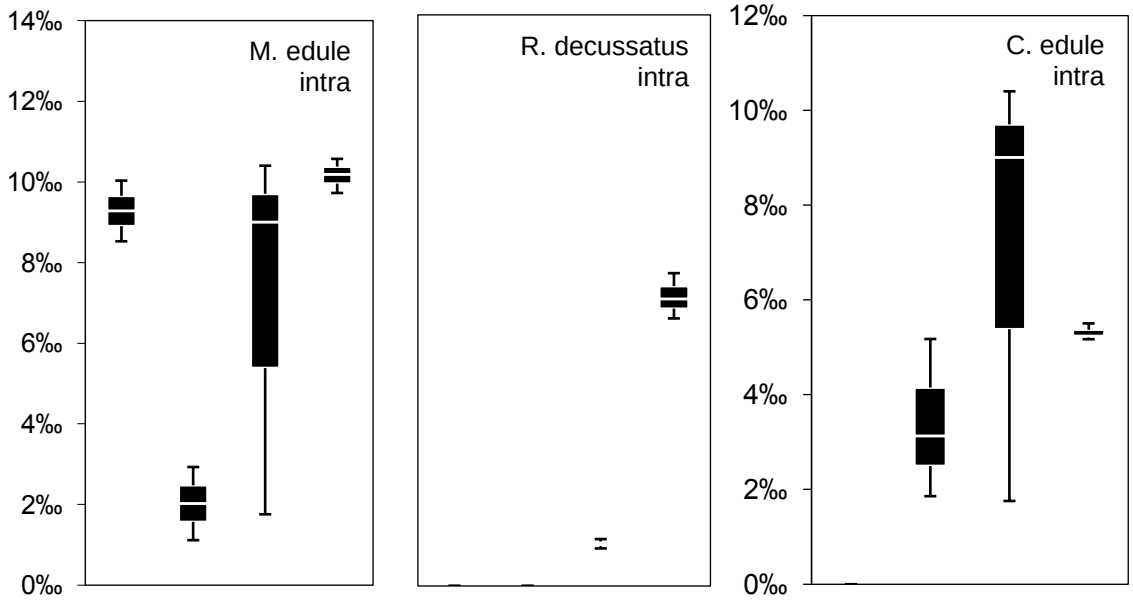
475 b)



476

477 **Fig. 4**. Box-and-whisker plots of $\delta^{34}S$ values from (a) total SBOM and (b) intra-crystalline

478 SBOM (intra) for method comparison. Results shown for samples isolated using EDTA,

479 10%HCl, 50%HCL and acetic acid (AA). Cation exchange resin data is excluded.

480

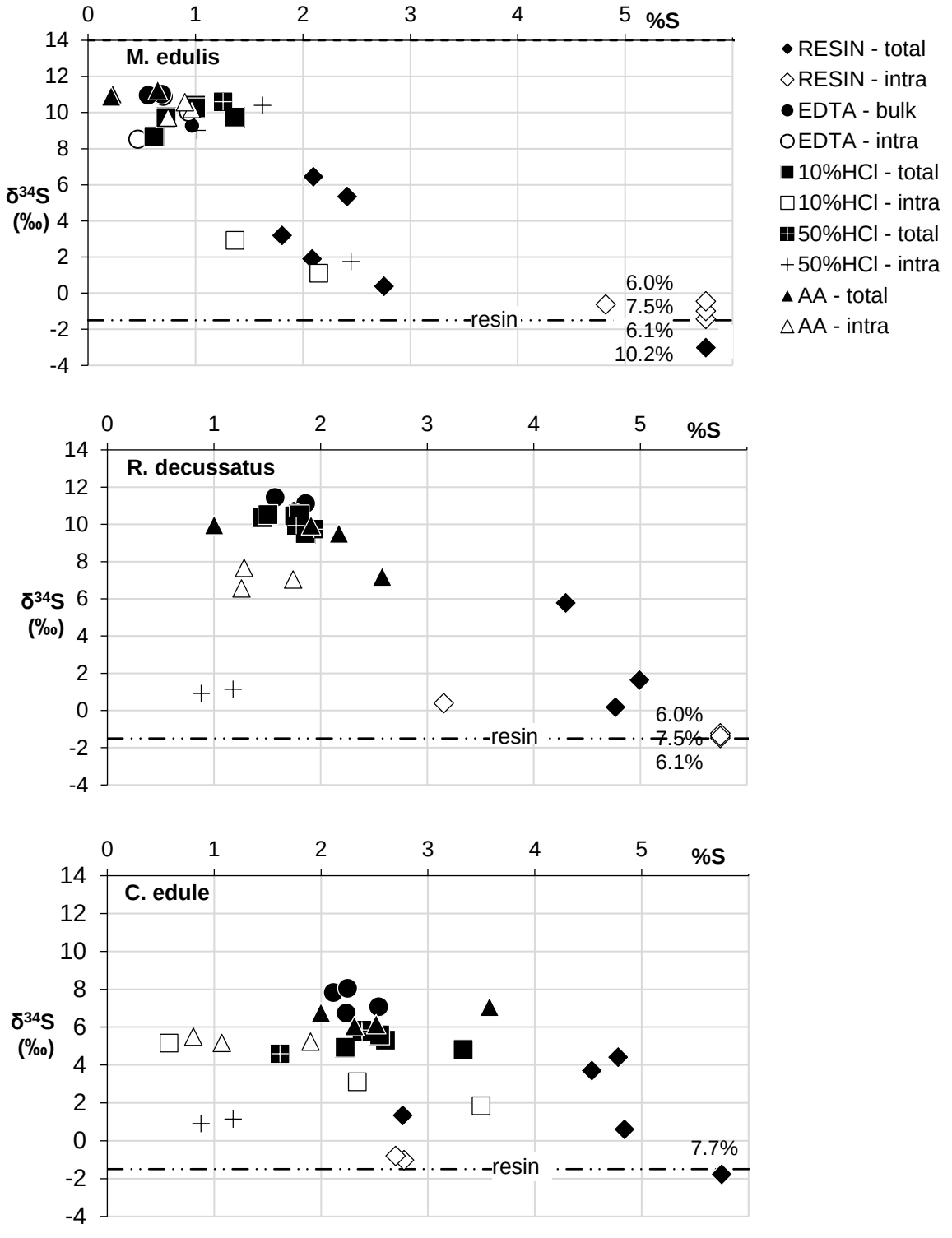


Fig. 5. $\delta^{34}S$ values and sulfur (%S) concentration of bulk total SBOM and intra-crystalline SBOM (intra) for method comparison. Results shown for samples isolated using cation exchange resin (RESIN), EDTA, 10%HCl, 50%HCL, and acetic acid (AA). The $\delta^{34}S$ value of the cation exchange resin is given.

EDTA extracted total SBOM generally has more positive $\delta^{34}S$ values compared to the acidification methods. This difference is significant for M. edulis between EDTA vs. 10%HCl ($p = 0.0164$), R. decussatus EDTA vs. acetic acid (0.0030), and C. edule EDTA vs. 10%HCl (0.0009) and 50%HCl (0.0095). The relationship between the different acidification methods is variable, but significant differences exist between 10% HCl and acetic acid for all three test species ($p = 0.044$ to 0.0121, $n = 3$). Multiple $\delta^{34}S$ measurements of total SBOM obtained using 50%HCl could only be done for C. edule, and results are statistically similar to 10%HCl. %S of total SBOM are not statistically different between any of the shell removal techniques for the three test species.

Untreated shell powder of the three test species was also analysed for sulfur isotopes, but because of low %S (0.02-0.04%) the peak heights were below our normal minimum for reliable analyses (<1nA), therefore the $\delta^{34}S$ data has increased uncertainty (up to ±1‰): M. edulis (18.1-18.7‰, $n = 2$), R. decussatus (17.6-17.9‰, $n = 3$), C. edule (18.2-19.2‰, $n = 3$).

Method comparison for intra-crystalline SBOM shows that resin samples exhibit the same systematic relationship observed in total SBOM resin $\delta^{34}S$ and %S values (Fig. 5). M. edulis has very low $\delta^{34}S$ values for resin (-0.9 ± 0.4‰, $n = 4$) compared to all other methods (7.2 ± 4.0‰, $n = 13$), as well as an increase in %S between resin (6.6 ± 1.1‰, $n = 4$) and the other methods (1.2 ± 0.6‰, $n = 11$). The same relationship can be found for R. decussatus: $\delta^{34}S$ = -0.9 ± 0.9‰ (resin, $n = 4$) vs. 5.0 ± 3.7‰ (other isolation methods, $n = 5$); and %S = 5.1 ± 1.3 % (resin, $n = 4$) vs. 1.3 ± 0.2 % (other, $n = 5$), as well as for C. edule: $\delta^{34}S$ = -0.9 ± 0.1‰ (resin, $n = 2$) vs. 4.9 ± 0.9‰ (other, $n = 8$), and %S = 2.8 ± 0.1 % (resin, $n = 2$) vs. 1.5 ± 0.8 % (other, $n = 8$).

For other methods used to isolate intra-crystalline SBOM, several very low $\delta^{34}S$ values were observed for 10%HCl and 50%HCl in M. edulis, R. decussatus and C. edule. In

M. edulis these lead to a significant difference of ~ 7‰ between 10%HCl vs. EDTA ($p = 0.0075$) and acetic acid ($p = 0.0005$), and very large variation in 50%HCl $\delta^{34}S$ values. Unfortunately EDTA values could not be obtained for the other two test species, because of low %S in the samples. Acetic acid total SBOM samples also have significantly higher $\delta^{34}S$ values compared to 50%HCl in R. decussatus ($p = 0.0007$) by ~ +6‰, but there are no statistical differences between the acidification methods for C. edule. With the exception of resin extracted material, the sulfur concentrations of total SBOM are not statistically different between the other methods.

A comparison between total SBOM and intra-crystalline SBOM of the different shell removal methods (where available) does not give a consistent relationship for $\delta^{34}S$ or %S between the two SBOM pools. Acetic acid SBOM data could be compared for all three test species: both pools are similar in isotopic composition and concentration for M. edulis, but for the other two species the intra-crystalline SBOM $\delta^{34}S$ is statistically lower by 1-2‰ compared to total SBOM ($p = 0.0013$ and 0.0181), and also statistically lower in concentration ($p = 0.0363$ and 0.0479). EDTA obtained SBOM could only be compared for M. edulis and intra-crystalline SBOM has a statistically significant lower $\delta^{34}S$ value (~ -1‰, $p = 0.0385$) compared to total SBOM, and a statistically significantly higher %S (+1%). 10%HCl samples from M. edulis have lower $\delta^{34}S$ values for intra-crystalline SBOM (~ -8‰) than total SBOM, but both pools have a similar %S. For 10% HCl obtained total and intra-crystalline SBOM from C. edule, both $\delta^{34}S$ and %S are statistically similar. This was also found for 50%HCl SBOM samples from the same species.

The $\delta^{34}S$ values of CAS obtained in this study are shown in Table 1. C. edule CAS obtained using HCl (20.3 ± 0.3‰. n = 2) has significantly higher $\delta^{34}S$ values than C. edule CAS (total SBOM) obtained from resin water (11.9 ± 0.7‰, n = 2, $p = 0.0041$), with a mean difference

of 8.4‰. C. edule CAS HCl is also significantly different from all resin CAS data (combined: 11.0 ± 2.3‰, n = 11, excluding outlier shown in Table 1), with a p-value of 0.0002.

Species	**Method**	**SBOM**	**$\delta^{34}S$** (‰ ±SD)
M. edulis	RESIN	total	14.5 (n = 1)
		intra	11.1 ±1.5 (n = 3), outlier 0.7
R. decussatus	RESIN	total	12.6 (n = 1)
		intra	5.4 (n = 1)
C. edule	RESIN	total	11.9 ±0.7 (n = 2)
		intra	10.5 ±0.6 (n = 3)
	10%HCl	total	20.3 ±0.3 (n = 2)

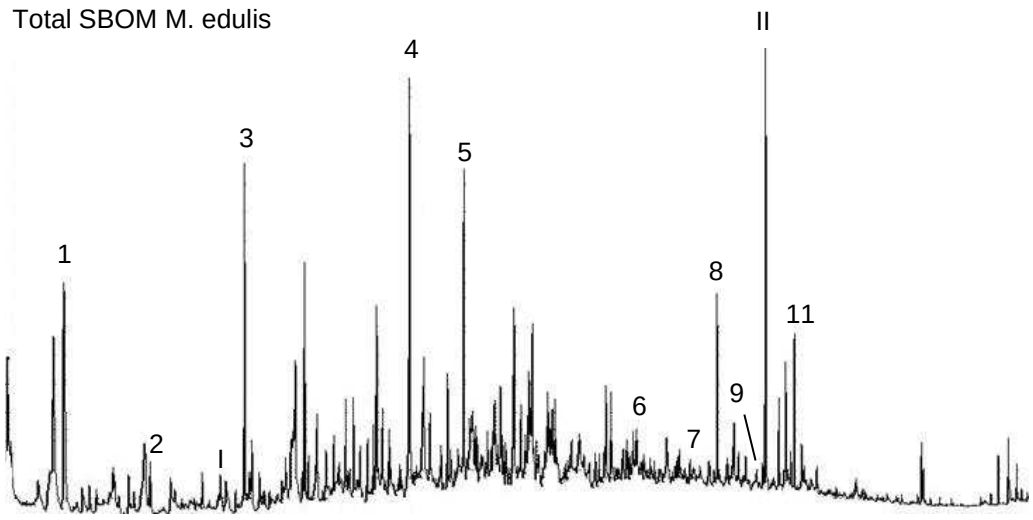
Table 1. $\delta^{34}S$ values of CAS obtained using cation exchange resin and HCl.

3.2. THM of total and intra-crystalline SBOM in the presence of TMAH (THMAH thermochemolysis)

The molecular composition of total and intra-crystalline SBOM (isolated using cation exchange resin) from M. edulis was analysed using TMAH thermochemolysis, whereby the molecular components are separated using gas chromatography and identified using mass spectrometry (Fig. 6).

TMAH thermochemolysis results show that the majority of SBOM consists of proteins, and that lipids (in the form of saturated fatty acids) are also present, which is in agreement with published literature (Goulletquer & Wolowicz et al., 1989; CoBabe & Pratt, 1995). Comparison between the two samples shows that intra-crystalline SBOM has a relatively simple chromatogram with only several high intensity peaks, when compared to the more complex total SBOM sample.

554 a)



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556 b)

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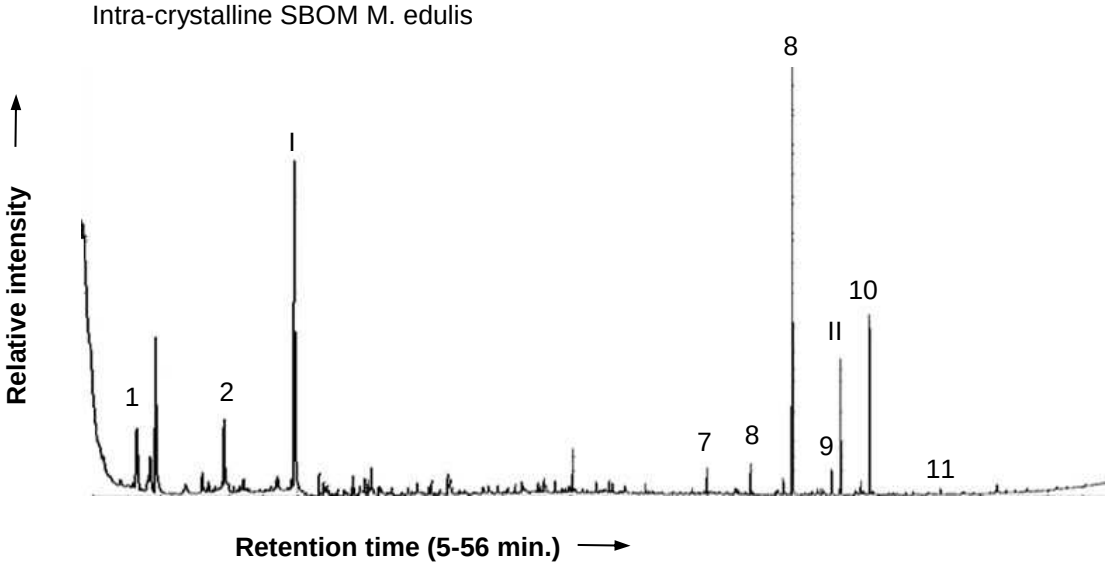

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1	Toluene	**6**	$C_{14:0}$ saturated fatty acid methyl ester
2	Styrene	**7**	$C_{15:0}$ saturated fatty acid methyl ester
3	Phenol	**8**	$C_{16:0}$ saturated fatty acid methyl ester
4	1-Methylindole	**9**	$C_{17:0}$ saturated fatty acid methyl ester
5	1,3-Dimethyl indole	**10**	$C_{18:0}$ saturated fatty acid methyl ester
		11	$C_{20:0}$ saturated fatty acid methyl ester

561

562

563

564

565 **Fig. 6**. Total ion chromatograms from TMAH thermochemolysis for a) total SBOM and b)

566 intra-crystalline SBOM of M. edulis. Identified pyrolysis products are numbered and listed in

567 the table, the derivatisation agent TMAH (I) and internal standard androstane (II) are

568 labelled.

The most abundant component of intra-crystalline SBOM is the lipid C16:0 (palmitic acid), and there are also clear peaks for other saturated fatty acids (C14:0, C15:0, C17:0, C:18:10). These fatty acids are common compounds of living organisms, and have previously been identified in invertebrate shells (CoBabe & Ptak, 1999). Other major thermochemolysis products are components of proteins, and toluene. Toluene, phenol and indole are all abundant in the total SBOM sample, the generation of these compounds is associated with the presence of aromatic amino acids in proteins (Moldoveanu, 1998). The C16:0 peak has a much lower relative abundance in total SBOM than in the intra-crystalline SBOM sample. No identifiable cation exchange resin products were detected in the samples.

4. Discussion

4.1. Method comparison of shell removal techniques

With the exception of total SBOM $\delta^{15}N$ values, it is not possible to assess the effects of shell removal methods based on untreated samples. The success of the different methods is therefore primarily assessed on the assumption of low isotopic variation, because the extensive homogenization of the shell powder is expected to have a uniform isotopic signal. This assumption was confirmed by the $\delta^{15}N$ analysis of untreated total SBOM from M. edulis ($\delta^{15}N$ = 11.6 ±0.1‰, n = 3), that showed isotopic variation within measurement error and absence of outliers. In addition to data distribution, consistent isotopic differences between shell removal techniques are paid particular attention, because these could be indicative of method-specific effects on isotope values. Soft tissues of the test species were analysed as a potential metric to assess method precision, but these generally produced a wide range of values and could not be used for method comparison (Supplementary Information, Table A3).

4.1.1. Comparison of sulfur and nitrogen isotope data between total extracted SBOM and untreated-shell analyses.

Total SBOM samples obtained from all shell removal methods have statistically significant higher $\delta^{15}N$ values than the data from untreated M. edulis shell powders. However, the untreated samples have low variation in $\delta^{15}N$ values, and mean differences between untreated SBOM and treated SBOM are generally small, ranging from -0.6‰ to -1.2‰ for the different methods. These results are therefore broadly consistent with those of Darrow et al. (2017, reporting no statistical difference between untreated shell material and HCl extracted SBOM) as the isotopic effect of isolation methods is minor. Our work does suggest that all SBOM isolation methods remove a ^{15}N depleted component, likely protein, from the total SBOM pool. Identifying the precise mechanism and source of this change is outside the scope of this study, but is a possible target for future work in this area. We therefore recommend simple combustion of shell powder for the $\delta^{15}N$ analysis of SBOM, as previously suggested by e.g. Versteegh et al. (2011) and Gillikin et al. (2017). If isolation of SBOM is necessary we recommend the use of EDTA, acetic acid, and HCl, as discussed below.

Interestingly, the $\delta^{15}N$ value of intra-crystalline SBOM is generally 0.4-1.7‰ lower than the untreated SBOM, and the untreated SBOM is intermediate between the two treated SBOM pools. It's likely that intra-crystalline SBOM is affected by the chemical isolation methods the same way as total SBOM, meaning that the "true" $\delta^{15}N$ value of intra-crystalline SBOM is likely to be lower. This hypothesis could be tested by analysing untreated bleached samples using analytical equipment specialized for low %N samples.

The $\delta^{34}S$ values obtained from untreated shell powder represent a mixed signal from both SBOM and CAS $\delta^{34}S$ compositions. Although the data has increased uncertainty, the untreated $\delta^{34}S$ values (17.6 to 19.2‰, n = 8) suggest lower $\delta^{34}S$ values for SBOM than those reported for CAS (~20‰), consistent with the values measured directly on extracted SBOM

(SBOM $\delta^{34}S$ values are ~5-11‰ from all species and all methods except resin). The data are also consistent with previous reports on the isotopic relationship between SBOM and CAS, e.g. Newton et al. (2018), where SBOM was found to be substantially more ^{34}S depleted than CAS

4.1.2. Cation exchange resin

Cation exchange resin was tested as an alternative method to isolate SBOM for stable isotope analysis. However, a significant method-specific effect was found for $\delta^{34}S$ SBOM values, whereby increasing %S result in lower $\delta^{34}S$ values. This negative correlation suggests that the lower values are caused by an accumulating residual component from the cation exchange resin ($\delta^{34}S$ value = -1.5‰). Although in a very limited number of samples resin beads were observed, the physical size of the resin should make it impossible for it to penetrate the dialysis bag (3500 dalton). Therefore a different mechanism involving the release, transport and trapping a dissolved species is necessary to explain the ^{34}S depletion in all of the samples. It is likely that the sulfonic acid functional groups of the resin are the source of the sulfur contamination (Albalat et al., 2015). If resin extraction is necessary for other analytical reasons, we suggest that a blank extraction to determine the amount and isotopic composition of leachable sulfur is essential sulfur.

Soluble residual resin compounds also effect CAS $\delta^{34}S$ values obtained from resin water ($\delta^{34}S$ = 11.0 ± 2.3‰, n = 11), that are significantly lower than the expected seawater sulfate $\delta^{34}S$ value (20.3‰). C. edule CAS $\delta^{34}S$ values obtained using HCl (20.3 ± 0.3‰, n = 2) accurately reflect seawater sulfate, and confirm this conclusion. Because the resin water is filtered before precipitation, either the contamination is precipitated with CAS from inorganic sulfate, or is present as organic sulfur and co-precipitated with $BaSO_4$.

In summary, the cation exchange resin leaches sulfur into solution during the extraction process. The isotopic data are consistent with this soluble-sulfur having a $\delta^{34}S$ value of close to -1.5‰ (measured in the solid resin). Because both the %S of samples and the $\delta^{34}S$ value of the contaminant are known, it should still be possible to identify large ^{34}S differences in SBOM despite this contamination.

The use of cation exchange resin also results in lower $\delta^{13}C$ values for total and intra-crystalline SBOM compared to the other methods. It's likely that these lower values are also caused by similar resin-leachable components as those that affect SBOM $\delta^{34}S$, because resin has a $\delta^{13}C$ value of -29.2‰. For intra-crystalline SBOM of *R. decussatus* and *C. edule* resin samples are not significantly lower compared to other methods. This is very likely related to the difference in mean intra-crystalline $\delta^{13}C$ values of *M. edulis* (-22.7‰, mean of the four other methods) compared to *R. decussatus* (-25.8‰) and *C. edule* (-25.0‰), as the latter two species are closer in value to cation exchange resin. The effect on the total and intra-crystalline SBOM $\delta^{13}C$ less negative than -23‰ is limited to minus 1-2‰.

Lastly, cation exchange resin showed the largest variation in $\delta^{15}N$ total SBOM values, as well as lower %N. For two of the test species intra-crystalline SBOM $\delta^{15}N$ values are significantly lower compared to other methods, and have higher %N. This suggests that the cation exchange resin extraction maybe able to both remove and add nitrogen. Mechanisms for this are currently unknown and amounts of nitrogen in the resin are very small. However, the $\delta^{15}N$ total SBOM values are statistically similar to the other shell removal methods, and can be used to identify trophic levels and differentiate nitrogen sources.

665

666 **4.1.3. EDTA**

667 Several possible disadvantages of using EDTA for SBOM isolation were outlined in the
668 introduction, the primary concern being that residual EDTA (containing carbon and nitrogen)
669 would become incorporated with SBOM and influence its stable isotope values. The $\delta^{13}C$
670 value of EDTA ($\delta^{13}C$ = -39.0‰) was determined, but $\delta^{13}C$ values of total SBOM isolated
671 using EDTA were not lower compared to the other shell removal methods. However, for
672 intra-crystalline SBOM obtained using EDTA the $\delta^{13}C$ values are lower than 10%HCl/acetic
673 acid methods, and mean can be as much as 2‰. Therefore it is possible that the smaller
674 amounts of intra-crystalline SBOM (0.1% of shell weight vs. ~1% in total SBOM) is more
675 strongly affected by residual EDTA. Alternatively, the acidification methods could
676 preferentially remove ^{12}C, and EDTA samples therefore represent the "true" value.

677 The $\delta^{15}N$ data of EDTA obtained SBOM is similar in value and variability to other
678 shell removal methods. The $\delta^{34}S$ values of EDTA SBOM samples are generally higher than
679 those of acidification methods, as discussed in the next section.

680

681 **4.1.4. Acidification methods (10%HCl, 50%HCl, acetic acid)**

682 Statistical differences were found in $\delta^{13}C$ values of SBOM between the different acidification
683 methods: 50%HCl samples are lower for total and intra-crystalline SBOM compared to
684 10%HCl and acetic acid. Loss of acid-soluble organic carbon (amino acids/carbohydrates)
685 has previously been reported, and can cause lower $\delta^{13}C$ values if acid-insoluble lipids are
686 preferentially retained, because lipids are characteristically ^{13}C depleted (Schlacher &
687 Connelly, 2014). Our results suggests that the 50%HCl method is removing the ^{13}C enriched
688 compounds to a greater degree, potentially because the acid is stronger than 10%HCl and
689 acetic acid, and causes volatilization or solubilisation of organic compounds. Nitrogen

isotope and concentration data are also consistent with this hypothesis: the %N of 50%HCl total SBOM samples is lower than for 10%HCl and EDTA samples, and 50%HCl and 10%HCl $\delta^{13}C$ values obtained from intra-crystalline SBOM are lower than those obtained from the weaker acetic acid. These data are consistent with increased lipid content because lipids contain very limited amounts of nitrogen.

Acetic acid extraction resulted in more variable SBOM $\delta^{13}C$ values. Variability towards higher $\delta^{13}C$ values for total SBOM could be explained by the inclusion of unreacted inorganic carbon (which has a very high $\delta^{13}C$ value) due to the weakness of the acid. The extremely low $\delta^{13}C$ values for intra-crystalline SBOM are best explained by residual acid, as there are no other credible mechanisms that could lower $\delta^{13}C$ values by -20‰.

$\delta^{34}S$ SBOM data obtained using acidification has generally lower values when compared to EDTA extracted samples. Moreover, several 10%HCl and 50%HCl extracted samples produced extremely low values ($\delta^{34}S < 2.0$‰, n = 7, species=3) for intra-crystalline SBOM, that are not present in EDTA or acetic acid samples. This suggests strong fractionation of the SBOM sulfur pool. It's known that the sulfur containing amino acids can become unstable, which would could be a potential explanation for this effect (Dreier et al., 2012).

4.2. Comparison between total SBOM and intra-crystalline SBOM

Intra-crystalline SBOM has consistently lower carbon and nitrogen stable isotopic values compared to total SBOM, irrespective of shell removal method and species ($\delta^{13}C$ is -5 to 7‰ lower and $\delta^{15}N$ is -1 to 2‰ lower). In addition, the intra-crystalline fraction generally has a lower %N than total SBOM. These differences have previously been identified as effects of the hypochlorite treatment to remove inter-crystalline SBOM (Darrow et al., 2017), but we suggest they reflect original compositional differences between the SBOM pools. The

isotopic and concentration difference can be explained by the high lipid content of the intra-crystalline fraction observed by TMAH thermochemolysis analysis, because lipids are characteristically depleted in ^{13}C due to enzymatic discrimination (DeNiro & Epstein, 1978). In addition, lipids contain very limited amounts of nitrogen, which would also be consistent with the difference in %N between the two pools. The lower $\delta^{15}N$ values of intra-crystalline SBOM could be due to the presence of e.g. ^{15}N depleted lipoprotein compounds, that have previously been suggested as an explanation for thehigher $\delta^{15}N$ values of soft tissues after lipid removal (Ruiz-Cooley, 2011). Unfortunately, due to the limited amount of sulfur data, it is unclear if there is a consistent difference in sulfur stable isotope values and concentration between the two SBOM pools.

The difference in macromolecular composition between inter- and intra-crystalline SBOM has previously been recognized (e.g. Curry et al., 1991) and shows that they are secreted following different biochemical pathways. Thus it is likely that these differences are related to different functions of the two pools in the biomineralisation process. SBOM in general is thought to play key roles in the nucleation, growth and morphology of crystals (Lowenstam and Weiner, 1989), and intra-crystalline SBOM in particular has been shown to control shell microstructures (Okumara et al., 2013). The intra-crystalline SBOM is characterized by high lipid content, and although lipids have been suggested to be important in controlling the movement of ions, their role in biomineralisation is poorly understood (Farre and Dauphin, 2009; Marin et al., 2012). Phospholipids from coral skeletons have, however, been suggested to act as nucleation sites for the deposition of calcium carbonate (Isa and Okazaki, 1987).

These results are relevant for palaeontological investigations. In general, the intra-crystalline SBOM pool is often preferred for biochemical analysis of fossil specimens because it is

physically protected by the mineral from external diagenetic pathways (Sykes et al., 1995). This study, however, shows that when this pool is used for stable isotope analysis, the results will be very different from total SBOM values, and subsequent reconstruction of the precise nutritional sources of the animal needs to take this into account. In addition, a change in the isotopic off-set between total SBOM and intra-crystalline SBOM in fossil specimens (compared to modern taxa) could indicate degradation or contamination of either the inter-crystalline pool or both SBOM pools. If both pools have the same isotopic value, the SBOM is unlikely to have retained its original isotopic signal. If the off-set between the pools differs in magnitude from modern taxa, the inter-crystalline SBOM is likely to have become diagenetically altered. Therefore the isotopic analysis of both pools can serve as a new proxy for the extent of alteration of the original SBOM.

5. Conclusions

SBOM has great potential as an isotopic proxy for nutrition in biomineralizing organisms, and can be analysed as total SBOM or intra-crystalline SBOM. However, the analysis of SBOM generally requires its isolation from the mineral component of the shell, and this study shows that shell removal techniques can influence the stable isotope values of SBOM to varying degrees. In addition to the isotopic effect of the methods on the SBOM, the choice of shell removal technique will also depend on the required accuracy needed to answer the research questions, as well as differences in costs and duration.

Of the different methods, EDTA, 10%HCl and 50%HCl are most suitable for $\delta^{13}C$ analysis of total and intra-crystalline SBOM. Intra-crystalline $\delta^{13}C$ data obtained using acetic acid falls outside the 2‰-range of variation of the other methods, and cation exchange resin

764 samples are affected by a residual resin component. The use of resin primarily affects $\delta^{34}S$
765 values of SBOM, but can also influence $\delta^{13}C$ and $\delta^{15}N$ data.
766 For $\delta^{15}N$ SBOM analysis simple combustion of shell powder is recommended. All
767 isolation methods remove a ^{15}N depleted component from the organics, but this effect is
768 generally minor (~1‰). Therefore EDTA, 10%HCl, 50%HCL, and acetic acid can also be
769 used for $\delta^{15}N$ total SBOM analysis, whilst intra-crystalline SBOM should preferably be
770 isolated using EDTA or acetic acid, because of outliers reported for HCL samples. EDTA is
771 also the preferred method for $\delta^{34}S$ analysis, as acidification methods generally give 1-2‰
772 lower $\delta^{34}S$ values and can result in extreme outliers.
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911 **Supplementary Material**

912 **Table A1**. $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ values, and carbon (%C), nitrogen (%N) and sulfur (%S)
913 concentrations of total SBOM and intra-crystalline SBOM for all isolation methods.

914

915 SBOM was obtained from aliquots of homogenised shell powder from M. edulis, R.
916 decussatus and C. edule, using cation exchange resin (RESIN), EDTA, acetic acid (AA),
917 10%HCl and 50%HCl. Total SBOM $\delta^{15}N$ data was also obtained from untreated powder for
918 M. edulis and C. edule, and M. edulis. Some total SBOM samples which had been isolated
919 using EDTA were subsequently subjected to the cation exchange resin method and were re-
920 analysed (EDTA-RESIN). For sample sets with outliers (see: Fig. 1, Fig. 2, Fig. 4), mean
921 values excluding outliers are underlined.

922

TOTAL SBOM			
$\delta^{13}C$ (‰, ± SD)	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	-18.7 ± 3.3 (n = 7) -17.5 ± 0.9 (n = 6)	-21.1 ± 2.0 (n = 6)	-22.8 ± 1.8 (n = 7) -22.3 ± 1.3 (n = 6)
EDTA	-16.7 ± 2.2 (n = 4) -15.6 ± 0.2 (n = 3)	-19.2 ± 0.4 (n = 4)	-18.8 ± 0.2 (n = 4)
10%HCl	-15.6 ± 0.1 (n = 3)	-18.4 ± 0.2 (n = 5)	-18.7 ± 0.2 (n = 6)
50%HCl	-16.8 ± 1.2 (n = 5) -16.3 ± 0.4 (n = 4)	-19.5 ± 0.4 (n = 5)	-19.1 ± 0.7 (n = 3)
AA	-15.2 ± 3.0 (n = 3)	-19.3 ± 0.2 (n = 4)	-18.6 ± 1.1 (n = 4) -19.1 ± 0.3 (n = 3)
%C	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	45.8 (n = 1)	43.2 ± 1.4 (n = 3)	41.6 ± 1.6 (n = 4)
EDTA	42.6 ± 1.1 (n = 4)	43.0 ± 3.6 (n = 4) 41.3 ± 0.7 (n = 3)	42.6 ± 1.5 (n = 4)
10%HCl	45.7 ± 0.4 (n = 3)	45.2 ± 0.4 (n = 5)	43.3 ± 1.8 (n = 3)
50%HCl	39.7 ± 7.3 (n = 3)	21.4 ± 7.5 (n = 2)	n/a
AA	24.7 ± 4.0 (n = 4)	42.3 ± 2.5 (n = 4)	40.3 ± 4.3 (n = 3)
INTRA-CRYSTALLINE SBOM			
$\delta^{13}C$ (‰, ± SD)	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	-24.8 ± 1.7 (n = 6) -24.1 ± 0.5 (n = 5)	-25.7 ± 1.3 (n = 5) -25.1 ± 0.3 (n = 4)	-25.7 ± 1.6 (n = 4)
EDTA	-23.5 ± 0.3 (n = 3)	-26.1 ± 0.1 (n = 3)	-26.1 ± 0.2 (n = 4)
10%HCl	-21.9 ± 1.5 (n = 8)	-24.8 ± 0.5 (n = 4)	-22.9 ± 0.7 (n = 5)

50%HCl	-23.2 ± 0.7 (n = 3)	-26.1 ± 0.3 (n = 4)	-25.7 ± 1.4 (n = 3)
AA	-25.2 ± 7.2 (n = 6) -22.0 ± 0.9 (n = 5)	-29.0 ± 7.4 (n = 6) -26.0 ± 1.4 (n = 5)	-28.4 ± 8.8 (n = 6) -25.1 ± 4.1 (n = 3)
%C	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	38.0 ± 3.0 (n = 5)	34.7 ± 4.8 (n = 4)	28.5 ± 3.0 (n = 3)
EDTA	41.6 ± 2.7 (n = 3)	38.7 ± 1.2 (n = 3)	41.4 ± 0.9 (n = 3)
10%HCl	33.4 ± 14.1 (n = 5)	23.7 ± 11.6 (n = 3)	30.8 ± 15.6 (n = 3)
50%HCl	n/a	19.3 ± 5.7 (n = 2)	22.0 ± 14.8 (n = 3)
AA	40.5 ± 2.4 (n = 6) 39.6 ± 1.2 (n = 5)	25.8 ± 8.0 (n = 4) 29.7 ± 2.0 (n = 3)	34.0 ± 5.1 (n = 5)

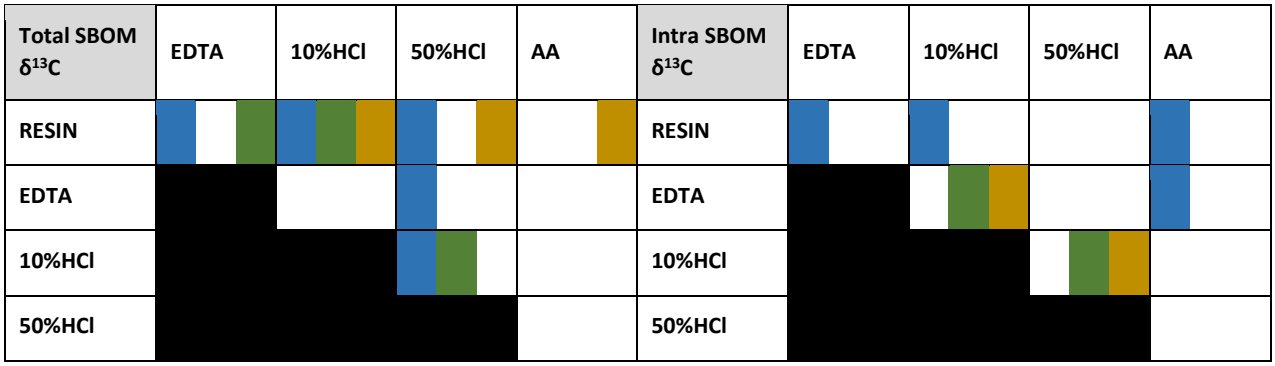
TOTAL SBOM			
δ^{15}N (‰, ± SD)	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	12.8 ± 0.8 (n = 7) 12.5 ± 0.5 (n = 6)	8.3 ± 1.9 (n = 8) 7.7 ± 0.8 (n = 7)	11.3 ± 1.2 (n = 8)
EDTA	12.2 ± 0.1 (n = 4)	7.3 ± 0.3 (n = 4)	12.3 ± 0.2 (n = 3)
EDTA-RESIN	12.1 ± 0.3 (n = 3)	n/a	n/a
10%HCl	12.3 ± 0.3 (n = 6)	6.8 ± 0.2 (n = 5)	12.0 ± 0.3 (n = 4) 11.8 ± 0.1 (n = 3)
50%HCl	12.2 ± 0.1 (n = 4)	7.1 ± 0.4 (n = 3)	12.4 ± 0.1 (n = 3)
AA	12.3 ± 0.4 (n = 3)	7.1 ± 0.1 (n = 3)	12.3 ± 0.1 (n = 4)
UNTREATED	11.6 ± 0.1 (n = 3)	6.8	n/a
%N	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	11.7 ± 3.8 (n = 7)	9.4 ± 1.5 (n = 8)	7.0 ± 2.2 (n = 8)
EDTA	14.9 ± 0.7 (n = 4)	12.9 ± 1.5 (n = 4)	11.6 ± 0.4 (n = 3)
EDTA-RESIN	13.1± 1.3 (n = 3)	n/a	n/a
10%HCl	17.1 ± 1.9 (n = 6)	14.2 ± 0.5 (n = 5)	11.5 ± 0.3 (n = 4)
50%HCl	13.6 ± 2.3 (n = 4)	8.3 ± 3.6 (n = 3)	10.1 ± 0.9 (n = 3)
AA	5.2 ± 1.5 (n = 3)	12.6 ± 0.6 (n = 3)	11.3 ± 1.2 (n = 3)
UNTREATED	0.2 ± 0.1 (n = 3)	0.1	n/a
INTRA-CRYSTALLINE SBOM			
δ^{15}N (‰, ± SD)	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	7.6 ± 2.1 (n = 5)	7.0 ± 0.6 (n = 5)	5.8 ± 0.9 (n = 2)
EDTA	11.1 ± 0.2 (n = 3)	n/a	n/a
10%HCl	9.9 ± 0.2 (n = 5)	n/a	9.7 ± 1.3 (n = 2)
50%HCl	10.3 ± 0.2 (n = 3)	n/a	10.0 ± 0.6 (n = 2)
AA	11.2 ± 0.6 (n = 3)	6.7 ± 0.3 (n = 2)	11.0 ± 0.9 (n = 3)
%N	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	4.6 ± 0.8 (n = 5)	4.0 ± 0.8 (n = 5)	3.7 ± 0.5 (n = 2)
EDTA	4.0 ± 0.5 (n = 3)	n/a	n/a
10%HCl	4.8 ± 0.9 (n = 5)	n/a	2.6 ± 1.5 (n = 2)
50%HCl	2.2 ± 1.0 (n = 3)	n/a	1.5 ± 0.2 (n = 3)
AA	4.2 ± 0.7 (n = 3)	3.3 ± 0.1 (n = 2)	4.5 ± 2.5 (n = 3)

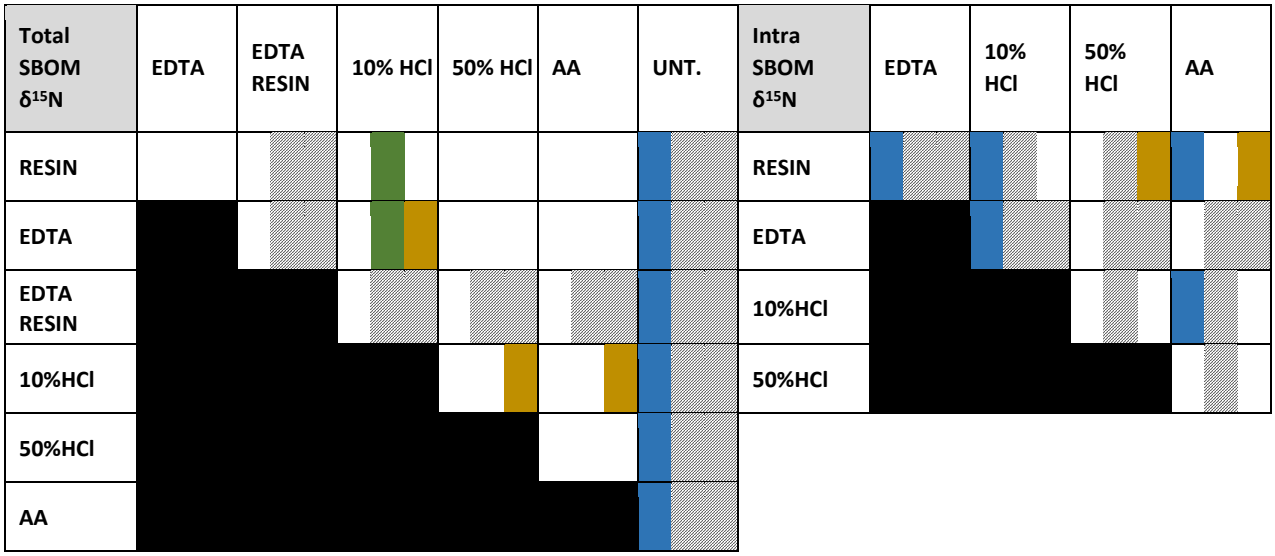
TOTAL SBOM			
$\delta^{34}S$ (‰, ± SD)	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	2.4 ± 3.4 (n = 6)	1.8 ± 2.8 (n = 4)	0.1 ± 1.7 (n = 2)
EDTA	10.6 ± 0.8 (n = 5) 10.9 ± 0.3 (n = 4)	11.0 ± 0.4 (n = 4)	7.4 ± 0.6 (n = 4)
EDTA-RESIN	3.2 ± 0.1 (n = 2)	n/a	n/a
10%HCl	9.7 ± 0.7 (n = 5) 10.2 ± 0.3 (n = 4)	10.3 ± 0.4 (n = 5) 10.5 ± 0.1 (n = 4)	5.2 ± 0.4 (n = 4)
50%HCl	10.6	n/a	5.4 ± 0.7 (n = 3)
AA	11.0 ± 0.2 (n = 3)	9.3 ± 1.3 (n = 5) 9.8 ± 0.3 (n = 4)	6.5 ± 0.5 (n = 3)
%S	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	3.7 ± 3.5 (n = 6)	5.4 ± 1.4 (n = 4)	5.1 ± 2.5 (n = 2)
EDTA	0.8 ± 0.2 (n = 4)	1.8 ± 0.1 (n = 4)	2.3 ± 0.2 (n = 4)
EDTA-RESIN	2.8 ± 0.1 (n = 2)	n/a	n/a
10%HCl	0.9 ± 0.4 (n = 3)	1.6 ± 0.2 (n = 3)	2.7 ± 0.5 (n = 4)
50%HCl	1.3	n/a	2.3 ± 0.2 (n = 3)
AA	0.3 ± 0.7 (n = 3)	2.2 ± 0.4 (n = 3)	2.8 ± 0.7 (n = 3)
INTRA-CRYSTALLINE SBOM			
$\delta^{34}S$ (‰, ± SD)	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	-0.9 ± 0.4 (n = 4)	-0.9 ± 0.9 (n = 4)	-0.9 ± 0.1 (n = 2)
EDTA	9.3 ± 1.1 (n = 2)	n/a	n/a
10%HCl	2.0 ± 1.3 (n = 3)	n/a	4.2 ± 1.5 (n = 2)
50%HCl	7.1 ± 4.6 (n = 3)	1.0 ± 0.1 (n = 2)	5.0 ± 1.1 (n = 3)
AA	10.2 ± 0.5 (n = 3)	7.6 ± 0.6 (n = 3)	5.3 ± 0.2 (n = 3)
%S	**M. edulis**	**R. decussatus**	**C. edule**
RESIN	6.6 ± 1.1 (n = 4)	5.1 ± 1.3 (n = 4)	2.8 ± 0.1 (n = 2)
EDTA	1.8 ± 0.6 (n = 2)	n/a	n/a
10%HCl	1.5 ± 0.6 (n = 3)	n/a	1.5 ± 1.2 (n = 2)
50%HCl	1.7 ± 0.7 (n = 3)	1.1 ± 0.2 (n = 2)	1.7 ± 0.9 (n = 3)
AA	0.9 ± 0.2 (n = 3)	1.4 ± 0.2 (n = 3)	1.3 ± 0.6 (n = 3)

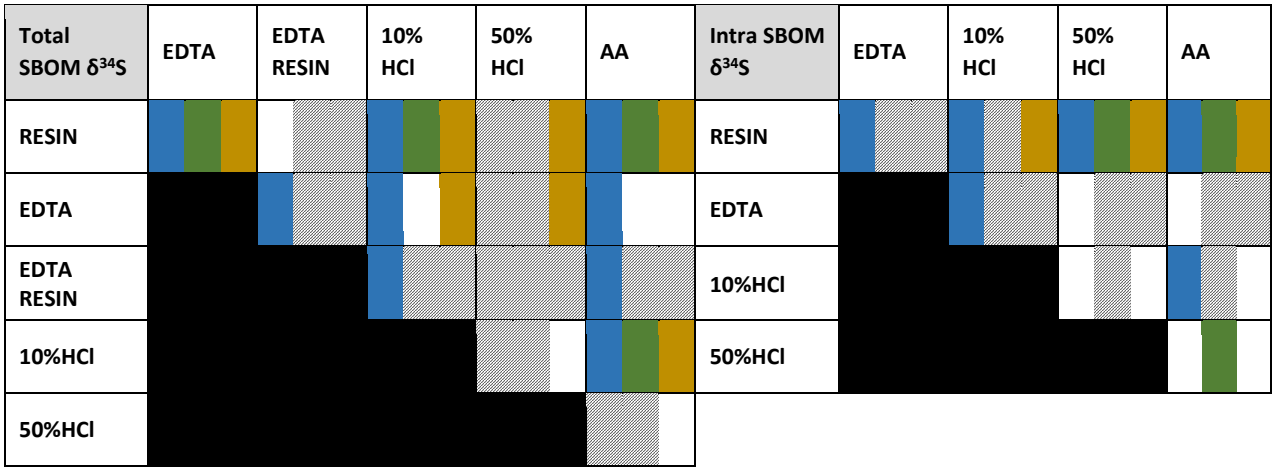
Table A2. Statistical comparison between isolation methods

Stable isotopic data ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) obtained for different SBOM isolation methods (Table A1) are compared using unpaired t-tests (significance threshold: $p < 0.05$), excluding identified outliers. Comparisons are made between cation exchange resin (RESIN), EDTA, acetic acid (AA), 10%HCl, 50%HCl, untreated shell powder (UNT.), and EDTA samples re-treated using cation exchange resin (EDTA-RESIN), for both total SBOM and intra-crystalline (intra) SBOM. Statistically significant differences between methods are colour-

coded per species: M. edulis (blue), R. decussatus (green), and C. edule (yellow). For white cells no statistical difference exists, for shaded cells insufficient data is available.







Supplementary information - Soft tissue data to assess method accuracy

Soft tissues from the three test species were analysed for comparison with SBOM data, to potentially contribute to assessing the accuracy of the different isolation methods. This data is however not useful for method comparison, because the the isotopic range of the soft tissues is either very large compared to SBOM values, and encompasses (most of) the SBOM results of all the methods, or it excludes all SBOM results from the different methods.

Materials & Methods

Because of the difficulty of homogenising soft tissues, particularly for such a large amount of individual animals, it was not possible to obtain "bulk" soft tissue values. Therefore soft tissues were analysed from five randomly selected individuals. Soft tissues were excised from the shells, and separated into gill, mantle, foot, adductor muscle and rest. They were rinsed three times with DI water, freeze-dried, and homogenized (where necessary with liquid nitrogen) with a ceramic mortar and pestle. $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ analyses were performed on the soft tissues as described in the section 2.4 of the manuscript.

Results & Discussion

Table A3. Range of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values for each test species. Including values from the gill, mantle, foot, and adductor muscle of five individual specimens (n = 20 per range).

	$\delta^{13}C$	**$\delta^{15}N$**	**$\delta^{34}S$**
M. edulis	-20.0 to -16.5‰	10.9 to 12.9‰	9.1 to 14.0‰
R. decussatus	-21.1 to -16.9‰	3.2 to 14.8‰	11.0 to 16.3‰
C. edule	-20.3 to -17.1‰	10.2 to 13.6‰	11.3 to 15.0‰

For comparison to total SBOM values we refer to Supplementary Table A1 and the figures in the manuscript.

965 Carbon. For R. decussatus and C. edule all $\delta^{13}C$ values of SBOM obtained using the different
966 methods fall within the soft tissue range, with the exception of some lower cation exchange
967 resin values. This is in agreement with our conclusion that cation exchange resin results in
968 lower $\delta^{13}C$ values compared to other methods. However, for M. edulis only resin values
969 overlap with the soft tissue range. This is likely related to a species-specific difference in the
970 isotopic relationship between SBOM and soft tissues, due to the presence of calcite in the
971 shell of M. edulis (which has previously been noted for $\delta^{15}N$ values, see Gillikin et al., 2017).

972 Nitrogen. The SBOM $\delta^{15}N$ values are included within the soft tissue ranges, with the
973 exception of two higher (M. edulis) and one lower (C. edule) resin $\delta^{15}N$ value. This confirms
974 that the large variation in $\delta^{15}N$ values is due to method-specific effects of the resin.

975 Sulfur. For M. edulis most SBOM $\delta^{34}S$ values overlap with the soft tissue range, with the
976 exception of several low 10HCl values. For C. edule all of the SBOM $\delta^{34}S$ values are lower
977 than the soft tissue range, and for R. decussatus only a minority of the EDTA $\delta^{34}S$ values fall
978 within the soft tissue range (other $\delta^{34}S$ values are lower). These observations are in agreement
979 with the conclusions of the manuscript, recommending EDTA as an isolation method for
980 analysis $\delta^{34}S$. The (large) difference in $\delta^{34}S$ values between soft tissues and SBOM was
981 previously noted by Mae et al. (2007, using EDTA), Dreier et al. (2012, using EDTA), and
982 Feng et al. (2018, using acetic acid). Feng et al. (2018) reports that this bias could in part be
983 caused by the acidification isolation method.

984 Additional references

985 Feng D, Peckmann J, Li N, Kiel S, Qiu JW, Liang Q, Carney RS, Peng Y, Tao J, Chen D
986 (2018) The stable isotope fingerprint of chemosymbiosis in the shell organic matrix of seep-
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