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Correction of the isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of preserved Baltic and North Sea macrozoobenthos and their trophic interactions

Jacqueline Umbricht[1,*], Joachim W. Dippner[1], Brian Fry[2], Ingrid Kröncke[3], Iris Liskow[1], Petra Nehmer[3], Franziska Thoms[1], Maren Voss[1]

[1]Leibniz Institute for Baltic Sea Research Warnemünde, Seestr. 15, 18119 Rostock, Germany
[2]Australian Rivers Institute, Griffith University Brisbane, Queensland, Australia
[3]Senckenberg am Meer, Dept. for Marine Research, 26382 Wilhelmshaven, Germany

ABSTRACT: To better understand food webs and the trophic relationships of marine animals studied by means of preserved material requires knowledge of the effects of preservation on the organic materials of interest. We examined the effects of formalin and ethanol preservation on the stable isotope values of carbon and nitrogen in the bivalves *Mya arenaria* and *Tellina fabula* and in the polychaetes *Magelona* spp. and *Hediste diversicolor*. Samples of these organisms were collected in the southern North Sea and the southern Baltic Sea, and were preserved with formalin, ethanol, and by freezing. The stable isotope patterns of carbon and nitrogen in the chemically preserved samples were related to those in the frozen samples. The influence of different preservation methods on the stable isotope composition was analyzed using a correction model that is independent of the molar C:N ratio. For most samples from the North Sea, significant correction factors were obtained. In contrast, none of the samples from the Baltic Sea were impacted by the preservatives with respect to their $\delta^{15}N$ values, and, for *H. diversicolor*, also with respect to the $\delta^{13}C$ values. In these cases, corrections of the $\delta^{13}C$ and the $\delta^{15}N$ values were not necessary. Quantitative metrics using a phenotype-based approach were computed to characterize aspects of the trophic structure of the benthic community. Phenotypic clustering in isotopic diversity indicates no competition for food between the dominant species. Our results indicate that a representative dominant mollusk and polychaete species have a higher trophic position in the Baltic Sea than in the North Sea.

KEY WORDS: Macrozoobenthos · Preservation effects · Stable isotopes · Phenotype model · North Sea · Baltic Sea

INTRODUCTION

Stable isotope ratios of carbon and nitrogen are routinely used to quantify food sources and the structure of food webs (Vander Zanden et al. 1999, Fry 2006), sources of energy and organic carbon (Peterson & Fry 1987), and trophic position (DeNiro & Epstein 1978, 1981, Minagawa & Wada 1984, Post 2002) as well as to identify the anthropogenic impact on ecosystems (Cabana & Rasmussen 1996, Fry 1999). Stable isotope analysis can also provide valuable insight into ecosystem processes, species interactions, and community responses to anthropogenic and natural disturbances (Vander Zanden et al. 2003, González-Bergonzoni et al. 2015). In combination with phenotypic community structure models (Cornwell et al. 2006, Layman et al. 2007), it can shed light on the trophic diversity within a food web, trophic redundancy, niche diversification, and the distribution of trophic niches.

*Corresponding author:
jacqueline.umbricht@io-warnemuende.de

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Further potential applications of stable isotope analysis are the identification of biological regime shifts and/or a determination of the impact of climate variability on biodiversity, including changes in trophic position and in food web structure. In the North Sea and Baltic Sea, climate variability, especially that manifesting as the North Atlantic Oscillation, strongly influences the abundance, biomass, and species numbers of macrozoobenthos (Kröncke et al. 1998, Tunberg & Nelson 1998, Hagberg & Tunberg 2000, Dippner & Ikauniece 2001, Dippner & Kröncke 2003). The impact of climate regime shifts is also reflected in the feeding modes, distribution types (temperate, arctic-boreal, warm-temperate, cold-temperate), taxonomic groups, and species of the dominant benthic species (Dippner et al. 2014).

Important information on the response of benthic animals to climate variability, especially the impact of climate changes on marine biodiversity, trophic position, and food web structure, might be gained by analyzing the isotopic composition of these organisms. However, because preservation in formalin or ethanol changes the isotopic composition of a preserved specimen (e.g. Kaehler & Pakhomov 2001, Edwards et al. 2002, Kelly et al. 2006), an expected, but not necessarily mandatory, prerequisite of stable isotope analysis is a correction of the isotopic values.

The most common preservation methods currently in use are freezing, drying, freeze-drying (Krab et al. 2012), conservation in salt (Arrington & Winemiller 2002), and fixation or preservation in Lugol's iodine (Ventura & Jeppesen 2009), formalin, ethanol, formalin–ethanol (Hobson et al. 1997, Arrington & Winemiller 2002, Sarakinos et al. 2002), and lipid-extracted formalin or ethanol (Kelly et al. 2006). Most of the samples archived in natural history museums or in research institutes such as Senckenberg am Meer, Wilhelmshaven, Germany, are preserved with formalin or ethanol.

The potential impact of formalin (CH_2O) or ethanol (C_2H_6O) on the isotopic composition of carbon and nitrogen in preserved samples and a correction for the respective effects was the subject of this study. Using samples from the North Sea and Baltic Sea, we tested the effect of preservation over a time period of days to several months. Our analyses also considered the diversity in the stable isotope values of single species. After applying a correction model that is independent of the molar C:N ratio, we used a phenotypic community structure model to analyze the corrected isotopic values with respect to the geographical area and dominance of the studied species. This enabled us to gain some insight into the trophic interactions and diversity in the studied areas.

MATERIALS AND METHODS

Sampling and treatment of macrozoobenthos

Macrofauna samples were collected from the southern North Sea off the island of Norderney and from the Breitling, a bay of the Warnow River close to its entrance into the Baltic Sea (Fig. 1). Two of the most abundant marine species, the bivalve *Tellina fabula* (suspension and deposit feeder), a native warm-temperate species, and the polychaetes *Magelona* spp. (deposit feeder), a native cold-temperate species, were obtained in June 2015 and 2016 from the North Sea at a water depth of 12 m using a 0.2 m^2

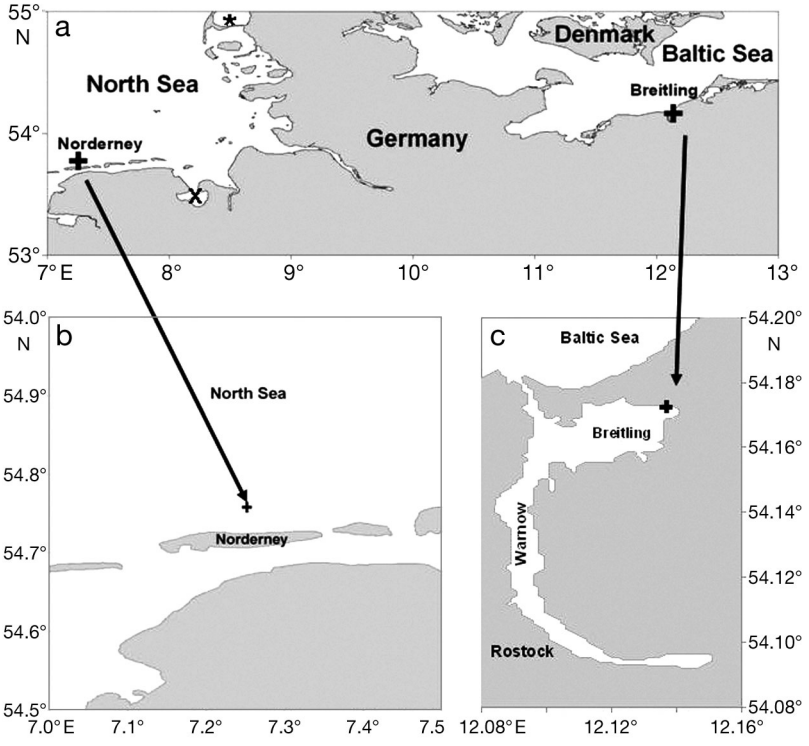

Fig. 1. (a) Study areas in the southern North Sea and southern Baltic Sea. The asterisk marks the southern Sylt-Rømø Bight, and the 'X' marks Jade Bay. (b) Location of the monitoring station off the island of Norderney (modified after Kröncke et al. 2013). (c) Location of the sampling station in the Breitling, a bay at the entrance of the Warnow River into the Baltic Sea, close to the city of Rostock, Germany

van Veen grab (Kröncke et al. 1998, 2013). Both species are interface feeders (Dippner et al. 2014). Immediately after collection, at least 3 individuals per species were either frozen or preserved in 4% formalin buffered with sodium borate or in 70% ethanol (Table S1 in the Supplement at www.int-res.com/articles/suppl/m595p001_supp.xls). Neither *Magelona* spp. nor *T. fabula* were held in filtered seawater, because only tissue of the foot of *T. fabula* (no gut or intestine) was used for stable isotope analysis and the low mass of the gut contents relative to the biomass of *Magelona* spp. (compared to *Hediste diversicolor*; see below) justified the neglect of its gut content in stable isotope analysis. In the lab, subsamples were taken weekly for 6 wk. For the analysis, the preservative was removed by simply letting the liquid drip off the sample and, in the case of *T. fabula*, the shells were detached. All samples were oven-dried for 12–24 h at 60°C and then homogenized using a mortar and pestle. In 2015, 3 individual organisms were prepared in parallel, whereas in 2016, 3 subsamples were taken from a pool of several homogenized organisms.

Two dominant brackish water species, the bivalve *Mya arenaria* (suspension feeder) and the polychaete *H. diversicolor* (omnivorous, often predatory), were collected in October 2015 from the Breitling, using a piercing tube in a water depth of ~0.7 m. These samples were kept in filtered seawater for 24 h to ensure that the organisms had completely emptied their guts. After 24 h, the empty gut of *H. diversicolor* was clearly visible and therefore, whole worms could be used for stable isotope analysis. Six individuals of each species were frozen as reference samples. Thirty individuals of each species were preserved in 70% ethanol and a further 30 from each species were preserved in 4% formalin. After 2 and 7 d, 3 individuals of each species were removed from the preservative. The subsampling was continued every third week for another 18 wk. All samples were freeze-dried and then homogenized with a mortar and a pestle. None of the samples from either the Baltic or the North Sea were rinsed with distilled water, as this procedure could have altered the isotopic composition of the samples due to damage of proteins in the tissues (Sarakinos et al. 2002). The shells of *M. arenaria* were detached.

Stable isotope analysis

For stable $\delta^{15}N$ and $\delta^{13}C$ analyses, 1 ± 0.18 mg of the freeze-dried or oven-dried organism was weighed into a tin capsule and then analyzed using a Thermo Scientific Delta V Advantage mass spectrometer (combined with a Conflo IV interface and a Flash 2000 elemental analyzer) and a Thermo Finnigan Delta plus mass spectrometer (combined with a Conflo III Interface and a Flash 1112 elemental analyzer). Both mass spectrometers have a precision ≤ ±0.2‰. Values of ^{13}C, ^{12}C, ^{15}N, and ^{14}N were calculated using the standard δ notation and expressed in ‰, as shown in Eq. (1) (Peterson & Fry 1987):

$$\delta X = \left[\frac{R_{sample}}{R_{standard}} - 1 \right] \cdot 1000 \quad (1)$$

where δX is $\delta^{13}C$ or $\delta^{15}N$ and R is the corresponding ratio of ^{13}C:^{12}C or ^{15}N:^{14}N, respectively.

Statistical methods and calculations

ANOVA and Tukey's posterior test with $\alpha = 0.05$ were used to determine significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of the subsamples. For each time series, a least square fit was calculated according to Eq. (2) (see Fig. 2):

$$\delta X(t) = \delta X(0) + a(1 - e^{-bt}) \quad (2)$$

where $\delta X(t)$ is the measured $\delta^{13}C$ or $\delta^{15}N$ time series, $\delta X(0)$ is the isotopic value at $t = 0$ of the fresh material, t is the preservation time in days, a is the potential correction factor for the impact of preservation, and b is the inverse of an adjustment time in 1/days. The advantage of this approach over the model of Fry et al. (2003) is the independence from the molar C:N ratio. The correction factor a must be significantly larger than the 95% confidence level of the organisms: $|a| > 2\sigma$, where σ is the standard deviation (SD) measured in the frozen control sample (Fig. 2). In this case, the correction is meaningful. The correction applied to the value determined in Eq. (2) was computed as:

$$\delta X(t)_{corr} = \delta X(t) - a(1 - e^{-bt}) \quad (3)$$

The quality of the correction was estimated based on the deviation of the measured and corrected values from the expected value $\delta X(0)$, the SD σ, the accuracy $(\sigma\sqrt{2\pi})^{-1}$ of the deviations, and the improvement of the accuracy, defined as the quotient of the corrected to measured accuracy.

The Layman model (Layman et al. 2007), a phenotype-based approach for analyzing community structure (Pausas &Verdu 2010, Karlson et al. 2015), was applied to the corrected isotopic values to analyze the difference between geographic regions and the dominant species. The Layman model consists of 6 community-wide metrics that reflect important aspects of

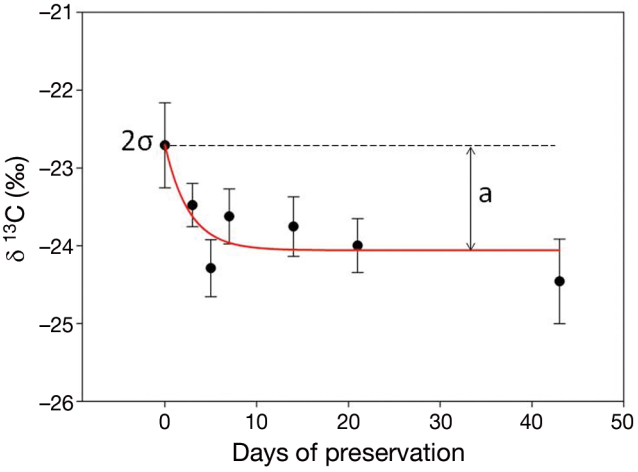

Fig. 2. Example of means ± SD (σ) of observations and of their least square fit (red line) to determine the potential correction factor *a*. Graph shows the performed least square fit for values of ethanol-preserved *Mya arenaria*

trophic structure: (1) the range of $\delta^{15}N$ data (NR), as a measure of the diversity of trophic levels; (2) the range of $\delta^{13}C$ data (CR), as a measure of the diversity of consumed food sources (González-Bergonzoni et al. 2015); (3) the total area (TA) of all species in the convex hull area, as a measure of the total trophic diversity; (4) the mean Euclidean distance to the centroid (CD), as a measure of the averaged degree of trophic diversity; (5) the mean nearest neighbor distance (NND), as a measure of the overall density of species packing; and (6) the SD of the mean NND (SDNND), as a measure of the evenness of species packing. TA, or the convex hull area, is a measure of the total trophic diversity in a food web, and is the smallest convex polygon containing all species in the $\delta^{13}C$– $\delta^{15}N$ biplot space. It was constructed as follows. The minimum $\delta^{13}C$ value, which, by definition (De Berg et al. 2008), is part of the convex hull, served as the starting point. From this point, the slope to every other data point was computed and then sorted in an ascending order, excluding the starting point, using the sorting algorithm indexx (Press et al. 1992). The end points of the sorted slopes form a polygon. The 'three penny algorithm' (Graham 1972) is used to compute from the polygon the vertices, which define the convex hull. From the vertices, the convex hull area (TA) can be computed in an *x*-*y* plane as:

$$TA = 0.5 \cdot \left[\sum_{i=1}^{N-1} (x_i - x_{i+1}) \cdot (y_i + y_{i+1}) + (x_N - x_1) \cdot (y_N + y_1) \right] \quad (4)$$

RESULTS

Effects of preservation on stable isotope composition

During the experiments, we did not observe any further significant effects of the preservation on stable isotope composition after approximately 2 wk of preservation. Neither ANOVA nor Tukey's posterior test indicated any differences between data points in each time series ($p < 0.5$ always). All of our measured isotopic values are presented in Table S1). The isotopic values measured in bivalves ranged from −25 to −17‰ for $\delta^{13}C$ and from 10 to 13‰ for $\delta^{15}N$. For polychaetes, the isotopic values for $\delta^{13}C$ were between −20 and −17‰ and between 10 and 15‰ for $\delta^{15}N$ (Table 1). Ethanol preservation decreased the $\delta^{13}C$ level of *Mya arenaria* significantly ($p < 0.0.5$), while it

Table 1. Range of measured isotope values (‰, mean ± SD of 3 replicate samples). All species were sampled in 2015, and *Tellina fabula* and *Magelona* spp. were also sampled in 2016

Species and sampled isotope	Formalin preservation			Ethanol preservation		
	Control	Minimum	Maximum	Control	Minimum	Maximum
Mya arenaria $\delta^{13}C$	−22.71 ± 0.55	−24.48 ± 0.59	−23.21 ± 0.46	22.71 ± 0.55	−24.29 ± 0.37	−22.56 ± 0.16
M. arenaria $\delta^{15}N$	12.24 ± 0.63	10.74 ± 1.48	12.85 ± 0.35	12.24 ± 0.63	11.68 ± 0.37	12.85 ± 0.27
Hediste diversicolor $\delta^{13}C$	−17.64 ± 0.55	−19.26 ± 0.47	−18.35 ± 0.06	−17.64 ± 0.55	−17.56 ± 0.22	−17.13 ± 0.37
H. diversicolor $\delta^{15}N$	14.30 ± 0.59	12.59 ± 0.86	14.64 ± 0.39	14.30 ± 0.59	12.68 ± 1.23	14.25 ± 0.84
Tellina fabula $\delta^{13}C$ (2015)	−18.26 ± 0.50	−21.10 ± 0.20	−20.50 ± 0.22	−18.26 ± 0.50	−17.57 ± 0.78	−17.35 ± 0.14
T. fabula $\delta^{15}N$ (2015)	9.70 ± 0.21	8.95 ± 0.29	9.68 ± 1.01	9.70 ± 0.21	9.17 ± 0.51	10.47 ± 0.41
T. fabula $\delta^{13}C$ (2016)	−17.67 ± 0.41	−20.75 ± 012	−20.05 ± 0.29	−17.67 ± 0.41	−17.15 ± 0.45	−16.80 ± 0.21
T. fabula $\delta^{15}N$ (2016)	10.25 ± 0.14	9.81 ± 0.50	10.38 ± 0.13	10.25 ± 0.14	10.70 ± 0.32	11.04 ± 0.26
Magelona spp. $\delta^{13}C$ (2015)	−18.13 ± 0.50	−20.78 ± 0.38	−19.62 ± 0.34	−18.13 ± 0.50	−17.18 ± 0.54	−16.45[a]
Magelona spp. $\delta^{15}N$ (2015)	11.86 ± 0.22	10.86 ± 0.2	13.14 ± 1.59	11.86 ± 0.22	11.86 ± 0.65	12.41[a]
Magelona spp. $\delta^{13}C$ (2016)	−19.19 ± 0.09	−21.38 ± 0.42	−20.78 ± 0.07	−19.19 ± 0.09	−18.26 ± 0.26	−17.90 ± 0.19
Magelona spp. $\delta^{15}N$ (2016)	10.38 ±0.14	10.39 ± 0.50	10.73 ± 0.30	10.38 ±0.14	11.02 ± 0.12	11.30 ± 0.04

[a]Material was only sufficient for a single measure

significantly increased the $\delta^{13}C$ level of *Magelona* spp. in both the 2015 and 2016 samples, but only in 2016 for *Tellina fabula*. $\delta^{15}N$ values increased significantly in *Magelona* spp. from both years and in the samples of *T. fabula* from 2016. As observed for the $\delta^{13}C$ values, no significant change in $\delta^{15}N$ values of *T. fabula* from 2015 were detected. Formalin preservation led to a significant decrease in the $\delta^{13}C$ values of *M. arenaria*, and *T. fabula* and *Magelona* spp. from both years. The $\delta^{15}N$ values of *T. fabula* collected in 2015 were significantly increased, while those of *Magelona* spp. significantly decreased. By contrast, formalin had no significant effect on the $\delta^{15}N$ values of *M. arenaria* and *Hediste diversicolor*. Apparently, ethanol preservation caused a decrease in the molar C:N ratio of all species, whereas formalin preservation led to an increase in the molar C:N ratios of bivalves and a decrease in those of polychaetes. In the North Sea samples from 2016, the change in the molar C:N ratio was reversed, i.e. formalin decreased the C:N ratio in the bivalve, whereas the C:N ratio in the polychaete was increased (Table 2). However, the changes in the molar C:N ratio were not significant with the exception of the C:N ratio of formalin-preserved *H. diversicolor* ($r = 0.65$, $p < 0.05$) and ethanol-preserved *Magelona* spp. from 2016 ($r = 0.84$, $p < 0.05$).

Table 2. Effect of preservation on the molar C:N ratio. Upward (downward) arrows indicate increases (decreases) in the molar C:N ratio. Given values are C:N ratio in control samples with alteration during preservation. **Bold** numbers indicate significant ($p < 0.05$) changes. SD of the C:N ratios of the 3 replicate samples was ≤0.2 for the whole time series, except *Magelona* spp., where SD was ≤0.45. All species were sampled in 2015, and *Tellina fabula* and *Magelona* spp. were also sampled in 2016

Species	Formalin	Ethanol
Tellina fabula (2015)	C:N↑5.11+0.40	C:N↓5.11–0.60
Tellina fabula (2016)	C:N↓5.14–0.06	C:N↓5.14–0.74
Mya arenaria	C:N↑4.73+0.04	C:N↓4.73–0.71
Hediste diversicolor	C:N↓5.31–0.52	**C:N↓5.31–1.41**
Magelona spp. (2015)	C:N↓4.90–0.12	C:N↓4.90–1.11
Magelona spp. (2016)	**C:N↑4.72+0.14**	C:N↓4.72–0.48

Correction of preservation effects

The least square fit showed similar results for the North Sea samples from 2015 and 2016. Isotope values were approximately 1‰ higher in the 2016 samples (Table 1). Although the 2016 samples were pooled (i.e. 3 individual organisms were homogenized together and parallels were taken out of the homogenized powder), there was no appreciable difference between the SD of the 2016 data and that of the 2015 data, where 3 individual organisms were analyzed. The values of the least square fit according to Eq. (2) are displayed in Table 3 for the $\delta^{15}N$ values and in Table 4 for the $\delta^{13}C$ values. The use of correction factors is only meaningful if $|a| > 2\sigma$ (Fig. 2), but this was not the case for

Table 3. Results of the least square fit for $\delta^{15}N$ values (where '0' indicates the values of frozen samples) according to Eq. (2): Expt 1: Breitling, Expt 2: North Sea 2015, and Expt 3: North Sea 2016. The following species (Sp.) were analyzed: *Mya arenaria* (Ma), *Hediste diversicolor* (Hd), *Tellina fabula* (T), and *Magelona* spp. (M). The effects of the preservatives (Pres.) ethanol (E) and formalin (F) were determined. *a*: potential correction factor for the impact of preservation, *b*: inverse of an adjustment time in 1/days, R: coefficient of determination, n: number of data points analyzed, σ: standard deviation. **Bold** numbers in the last column indicate significant ($p < 0.05$) changes necessitating correction

Expt.	Sp.	Pres.	δX(0)	*a*	*b*	R	n	σ	$\lvert a\rvert/2\sigma$
1	Ma	E	12.20	0.26	0.06	0.25	7	0.63	0.21
1	Ma	F	12.24	−0.63	27.48	0.37	6	0.63	0.50
1	Hd	E	14.30	−0.55	6.65	0.53	8	0.59	0.47
1	Hd	F	14.37	−0.96	0.31	0.46	12	0.59	0.81
2	T	E	9.84	0.04	0	0.00	7	0.21	0.10
2	T	F	9.70	0.58	4.30	0.65	7	0.21	**1.38**
2	M	E	11.82	0.96	0.02	0.89	6	0.22	**2.18**
2	M	F	11.86	−0.96	0.07	0.98	6	0.22	**2.18**
3	T	E	10.25	0.59	19208	0.8822	7	0.14	**2.11**
3	T	F	10.25	−0.25	379888	0.37	7	0.14	0.89
3	M	E	10.37	0.92	0.17	1	7	0.14	**3.29**
3	M	F	10.38	0.23	869	0.59	7	0.14	0.82

Table 4. Results of the least square fit for $\delta^{13}C$ values. Other details as in Table 3. (–) No material available

Expt.	Sp.	Pres.	δX(0)	*a*	*b*	R	n	σ	$\lvert a\rvert/2\sigma$
1	Ma	E	−22.70	−1.36	0.38	0.85	7	0.55	**1.24**
1	Ma	F	−22.69	−1.52	0.21	0.83	8	0.55	**1.38**
1	Hd	E	−17.64	0.31	3230.42	0.56	12	0.55	0.28
1	Hd	F	−17.64	−1.06	30.78	0.76	12	0.55	0.96
2	T	E	−18.26	0.82	0.27	0.99	7	0.50	0.82
2	T	F	−18.26	−2.50	0.34	0.98	7	0.50	**2.50**
2	M	E	−18.13	1.61	0.11	0.98	6	0.50	**1.60**
2	M	F	–	–	–	–	–	–	–
3	T	E	−17.68	0.77	0.24	0.92	7	0.09	**4.28**
3	T	F	−17.67	−2.87	1.03	0.98	7	0.09	**15.94**
3	M	E	−19.2	1.2	0.23	0.99	7	0.41	**1.46**
3	M	F	−19.21	−2.08	0.18	0.99	7	0.41	**2.54**

the $\delta^{15}N$ values of *M. arenaria* and *H. diversicolor*, the $\delta^{13}C$ values of *H. diversicolor*, the formalin-preserved 2016 samples from the North Sea, and the 2015 sample of ethanol-preserved *T. fabula*. In these cases, the effects of the preservatives were negligible, presumably due to the high intra-specific variability in the samples.

Similarly, using ANOVA and Tukey tests, we could not identify significant differences between the data points of each species and both treatments used in this study because of high SDs within the time series. The regression line for the preserved and control data of the isotopic composition was not parallel to the 1:1 lines of the N and C isotopes; this applied to both preservatives tested (Fig. 3). This suggests systematic bias in the measurements of both isotopes. Despite the high SD within the treatments, bivalves and polychaetes from the North Sea vs. the Baltic Sea formed distinct groups, as did samples preserved in formalin vs. ethanol (Fig. 4). The measured and corrected values determined from the preserved vs. frozen samples were statistically analyzed (Table 5, Fig. 5). In addition to a clear decrease in both the SD and the bias of the corrected values, the accuracy was improved, especially with respect to the $\delta^{13}C$ values of *T. fabula*.

Trophic interactions and diversity

All corrected data were grouped according to geographic area (North Sea and Baltic Sea) and species group (bivalves and polychaetes). The Layman model was applied to each of the 4 species groups (Table 6). The band width of the diversity of trophic levels ($\delta^{15}N$ range) was similar in all 4 groups, whereas the diver-

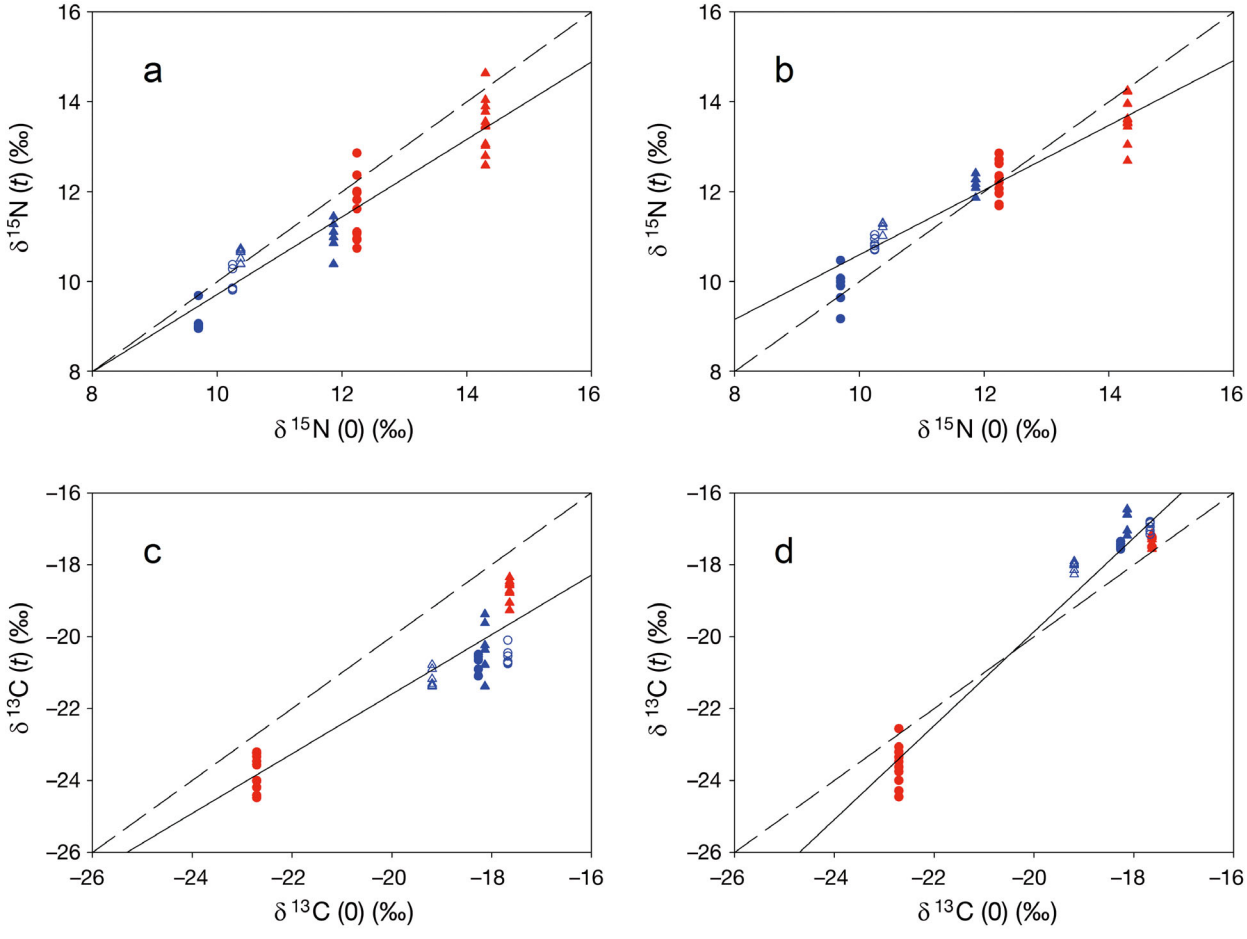

Fig. 3. Isotopic composition of preserved [δX(*t*)], where *t* is the preservation time in days vs. control (frozen) [δX(0)] samples: (a,c) formalin preservation, (b,d) ethanol preservation. The solid line is the regression line, and the dashed line is the 1:1 line. Blue symbols represent data from the island of Norderney (North Sea), red symbols show data from the Breitling (Baltic Sea); circles indicate bivalves (blue: *Tellina fabula*, red: *Mya arenaria*) and triangles are polychaetes (blue: *Magelona* spp., red: *Hediste diversicolor*). For *T. fabula* and *Magelona* spp., the open symbols represent data from 2016

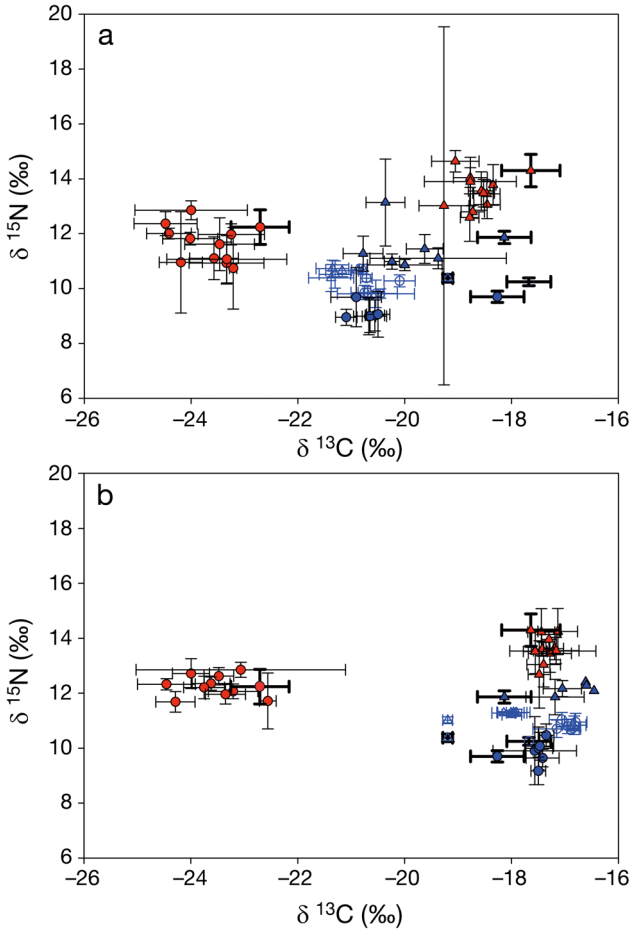

Fig. 4. Stable isotope biplot showing the means and SD of carbon and nitrogen isotope values. Symbols are coded as in Fig. 3. **Bold** error bars mark the t_0 (control (frozen)) values. Preservatives: (a) formalin and (b) ethanol

Table 5. Error analysis of the measured (index *t*) and the corrected (index *c*) isotopic values. Rows 1–4: averaged (< >) deviation of the measured and corrected values from the expected value δX(0). Rows 5–8: SD (σ) of the measured and corrected values. The accuracy $(\sigma\sqrt{2\pi})^{-1}$ is used to compute the improvement of accuracy (IA), defined as the quotient of corrected to measured accuracy, which gives an estimate of the quality of the correction

	Tellina fabula	*Magelona* spp.	*Mya arenaria*	*Hediste diversicolor*
$<\Delta\delta^{13}C_t>$	−0.95	−0.87	−0.92	−0.38
$<\Delta\delta^{13}C_c>$	0	−0.21	0.40	0
$<\Delta\delta^{15}N_t>$	−0.02	0.38	−0.32	−0.80
$<\Delta\delta^{15}N_c>$	0.04	0.15	−0.10	−0.08
$\sigma(\delta^{13}C_t)$	1.97	1.75	1.06	0.81
$\sigma(\delta^{13}C_c)$	0.16	0.45	0.66	0.21
$\sigma(\delta^{15}N_t)$	0.52	0.84	0.70	0.95
$\sigma(\delta^{15}N_c)$	0.29	0.53	0.54	0.51
IA(δ^{13}C)	12.2	3.9	1.6	3.9
IA(δ^{15}N)	1.8	1.6	1.3	1.9

sity of consumed food sources and of total trophic diversity of Baltic Sea species and the bivalves was much higher than it was for the North Sea species and the polychaetes. The same was true for the averaged degree of trophic diversity. Both the overall density of species packing (NND) and the evenness of species packing (SDNND) were slightly higher for bivalves and polychaetes from the Baltic Sea than from the North Sea. The arrangement of groups is shown in Fig. 6, together with the convex hulls, a measure of the niche space occupied (Layman et al. 2007) and constructed to compute the total trophic diversity.

DISCUSSION

Influence of preservation on isotopic composition

Formalin denatures proteins and integrates itself into tissues, forming new bonds among cellular components (Lang 2013). Thus, the resulting loss of amino acids, and hence nitrogen, may change the $\delta^{15}N$ composition of preserved samples. Similarly, formalin, which contains carbon, integrates into tissues, but its carbon signature differs from that of the sample: this may result in a change in the $\delta^{13}C$ value of the sample. In the case of ethanol preservation, ethanol extracts water from tissues and leads to lipid extraction (Lang 2013), which would increase the $\delta^{13}C$ signal, as lipids are isotopically lighter than other cellular components (Abelson & Hoering 1961, Park & Epstein 1961).

Consistent with the results of previous studies (see Table 7), in our formalin-preserved samples the $\delta^{13}C$ values decreased and the $\delta^{15}N$ values increased, except for the $\delta^{15}N$ values of *Magelona* spp. from 2015. However, ethanol preservation increased both the $\delta^{13}C$ and $\delta^{15}N$ values except those of *Mya arenaria* (Table 7). Our study and the other studies included in Table 7 show an average increase in the $\delta^{13}C$ values of the ethanol-preserved samples of 1‰ and in the $\delta^{15}N$ values of 0.6‰. Formalin preservation led to an average decrease in the $\delta^{13}C$ values of 1.3‰ and to an average increase in the $\delta^{15}N$ values of 0.3‰. For both treatments, the $\delta^{13}C$ values of the samples were more strongly affected than the $\delta^{15}N$ values (Table 7).

Trophic interactions and diversity

Two findings from our study are of particular interest (Fig. 3). Firstly, the $\delta^{15}N$ values were significantly

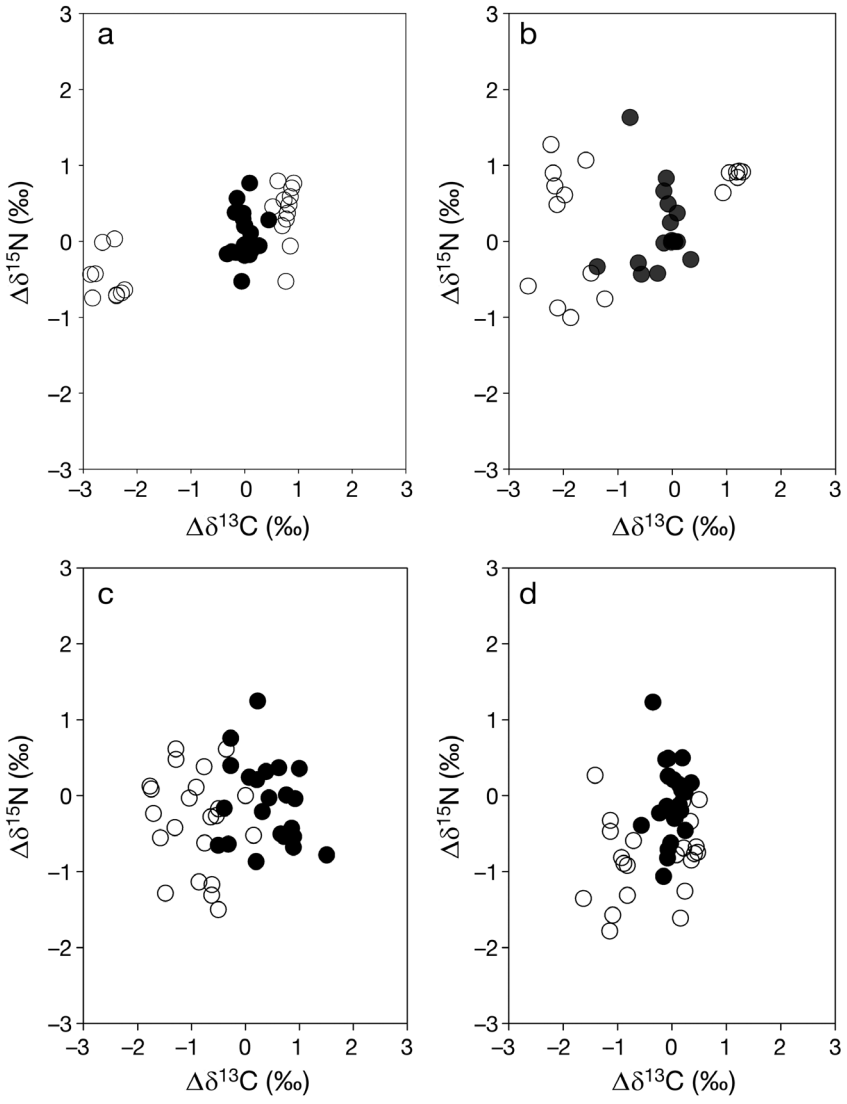

Fig. 5. Stable isotope biplot of the differences between the measured (open circles) and corrected (filled circles) values of 2 chemical preservation treatments minus the control (frozen) values for (a) *Tellina fabula*, (b) *Magelona* spp., (c) *Mya arenaria*, and (d) *Hediste diversicolor*

Table 6. Results of the Layman model applied to geographical areas and to dominant species. NR: $\delta^{15}N$ range of the data, CR: $\delta^{13}C$ range of the data, TA: total area of all species in the convex hull area, CD: mean Euclidean distance to the centroid, NND: mean nearest neighbor distance, and SDNND: SD of the NND

Metric	North Sea	Baltic Sea	Bivalves	Polychaetes
NR	4.33	4.23	4.32	5.24
CR	2.28	5.94	5.99	2.23
TA	5.23	13.40	11.02	6.15
CD	0.89	2.62	2.45	1.78
NND	1.19	2.98	2.81	2.13
SDNND	0.03	0.07	0.06	0.06

higher for organisms from the Breitling (Baltic Sea) than from the southern North Sea. In addition, the $\delta^{15}N$ values of the Breitling samples were not significantly impacted by the tested preservatives, in contrast to both the $\delta^{13}C$ and the $\delta^{15}N$ values of the North Sea samples. In the latter, except for ethanol-preserved *Tellina fabula* in 2015, formalin-preserved *Magelona* spp. in 2016, and $\delta^{15}N$ of formalin-preserved *T. fabula*, the effects were significant. Higher $\delta^{15}N$ values might reflect the more intense eutrophication in the Baltic Sea than in the North Sea and the very large difference in the water residence time: 30 to 60 yr in the Baltic Sea (Winsor et al. 2001) vs. 1 to 3 yr in the North Sea (Maier-Reimer 1979). This impact from eutrophication at the sampling site might be even more intense because the samples were taken in an embayment impacted by the Warnow River draining agricultural land (Deutsch et al. 2006). Eutrophication increases the $\delta^{15}N$ values of macrophytes (Cole et al. 2004, Deutsch & Voss 2006) that serve as a food source for benthic suspension and deposit feeders. Consequently, the $\delta^{15}N$ values of animals feeding on them increase as well. Furthermore, depending on the meteorological forcing, the Breitling station is influenced by terrestrial runoff from the Warnow River, and by brackish water inflow from the Baltic Sea. Consequently, more isotopically different food sources are supplied to the benthic organisms in the Baltic Sea than in the North Sea. The changing environmental conditions, especially the food availability at Breitling station, might account for the high natural variability in the isotopic composition of the respective samples, resulting in a significant overlap with that attributed to preservation.

Secondly, the $\delta^{13}C$ values of *M. arenaria* were lower (Fig. 3) than those of the other tested species. Particulate organic matter from terrestrial sources has a much lower $\delta^{13}C$ value than that of marine origin (Middelburg & Nieuwenhuize 1998), which would explain the lower $\delta^{13}C$ values of *M. arenaria* from the Breitling vs. those of animals from the North Sea. In

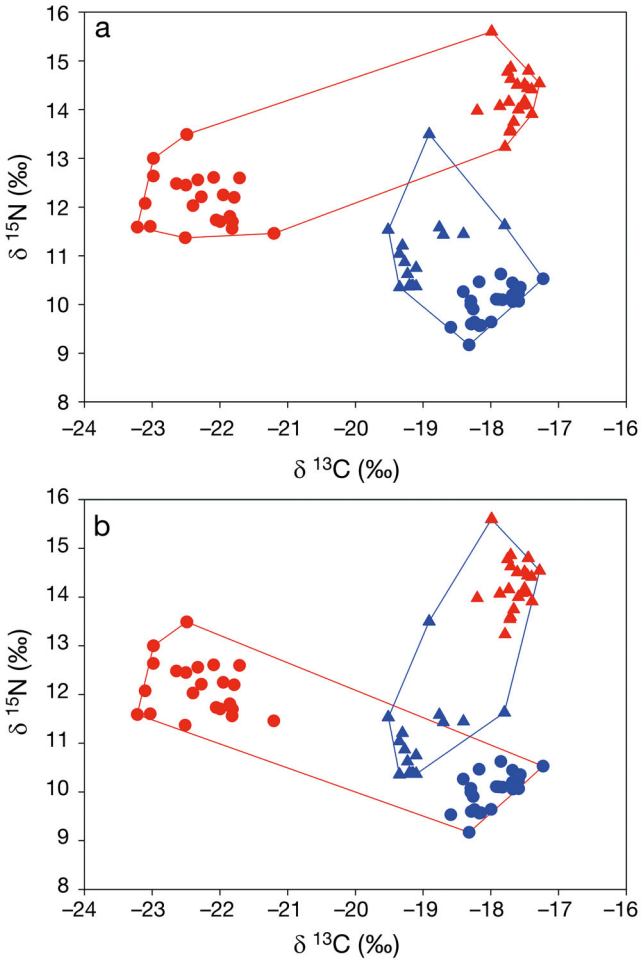

Fig. 6. Stable isotope biplot showing mean of corrected carbon and nitrogen isotope values. Symbols are coded as in Fig. 3. Convex hulls are shown for (a) the North Sea (blue line) and Baltic Sea (red line) and (b) bivalves (red line) and polychaetes (blue line)

their study of food webs at the Westerschelde estuary (southern North Sea), Herman et al. (2000) also measured the lowest $\delta^{13}C$ values in *M. arenaria*. As a suspension feeder, the bivalve relies on pelagic algae as a food source and therefore reflects the isotopic signature of phytoplankton, which has lower $\delta^{13}C$ values than benthic algae. Also, Nordström et al. (2016) found $\delta^{13}C$ values to be most depleted in the filter feeder *Mytilus* spp. in the northern Baltic Sea and explained this observation with the reliance of suspension feeders on primary production as a food source. *Hediste diversicolor* had the highest $\delta^{15}N$ values and also high $\delta^{13}C$ values (Fig. 3), with the latter being consistent with this species being more carnivorous than the others. Furthermore, polychaetes are more mobile than bivalves and can access different food sources both in and above the sediment, whereas bivalves are restricted to food particles at or above the sediment surface. As a result, the isotopic signature of *H. diversicolor* resembles more that of benthic algae, which is enriched in $\delta^{13}C$ compared to that of pelagic algae. *T. fabula* is able to suck food particles from the sediment surface, unlike *M. arenaria*, which must achieve a stable position before it can filter suspended particles out of the water column. Thus, the food sources, and therefore the isotope values, of the less mobility-restricted *T. fabula* will probably more closely resemble those of the polychaetes, i.e. of benthic algae, than those of *M. arenaria*.

The strong difference in the $\delta^{13}C$ values of *M. arenaria* and *H. diversicolor* indicated a much higher calculated trophic diversity at the Breitling than at the North Sea station (see TA in Table 6). Herman et al. (2000) reported that suspension feeders depend on pelagic food sources, whereas deposit feeders rely on microphytobenthic production. France (1995) showed that benthic and pelagic algae differ in their isotopic signatures. Together, these findings can explain the difference in the isotope values between suspension- and deposit-feeding organisms.

Overall, the North Sea samples contrasted remarkably with samples from the Baltic Sea. The difference between the $\delta^{13}C$ values of *M. arenaria* and *H. diversicolor* was representative of the dependence of the bivalve on pelagic food sources, whereas the polychaete relies on benthic food sources. Concerning the North Sea, values in *T. fabula* and *Magelona* spp. suggested either a reliance on benthic food sources or a high rate of resuspension that prevented a distinction between benthic and pelagic food particles based on their $\delta^{13}C$ values. In this study, bivalves and polychaetes from the North Sea and Baltic Sea were compared using the Layman model and by the construction of convex hulls (Fig. 6), to achieve a link between isotopic diversity and trophic diversity in the dominant species of the 2 regions. In general, species distribution in a trait space might be phenotypically overdispersed, random, or clustered (Pausas & Verdu 2010). Our isotopic data points in the convex hull (Fig. 6) indicated clustering and clear isotopic differences in the dominant species at the 2 sampling stations. In addition, environmental filtering leads to phenotypic clustering, whereas competition results in phenotypic overdispersion (Weiher & Keddy 1995). The observed isotopic diversity suggested the absence of competition for food between the species considered. This was supported by the lower ranges for NR and CR and a smaller occupied trait space (Perry et al. 2002). As expected from the diverse feeding modes, our results showed that bivalves and polychaetes occupy

Table 7. Effects of different preservation methods on stable isotope values. The differences (Δ) between the preserved and control samples are shown. Upward (downward) arrows indicate increases (decreases) in the isotopic composition. Values for *Tellina fabula* (this study) are 2015 and 2016 averaged values. The difference between fish tissue a and b is the length of preservation. Values are ±SD

Method/Species	$\Delta\delta^{13}C$(‰)	$\Delta\delta^{15}N$(‰)	Source
Formalin			
Octopus vulgaris	↓−0.26 ± 0.1	↑+0.06 ± 0.27	Kaehler & Pakhomov (2001)
Fish tissue	↓−0.47 ± 0.05	↑+0.23 ± 0.49	Kaehler & Pakhomov (2001)
Fish tissue a	↓−2.00	↑+0.4	Edwards et al. (2002)
Fish tissue b	↓−0.8 ± 0.81	↑+0.5 ± 0.81	Edwards et al. (2002)
Fish tissue	↓−1.36 ± 0.23	↑+0.71 ± 0.16	Sweeting et al. (2004)
Fish tissue	↓−2.21 ± 0.36	↑+0.66 ± 0.64	Kelly et al. (2006)
Corbicula fluminea	↑+1.3 ± 0.3	↑+0.9 ± 0.2	Syväranta et al. (2011)
Fish tissue	↓−0.94 ± 0.44	↑+0.33 ± 0.19	González-Bergonzoni et al. (2015)
Freshwater invertebrates	↓−2.0	↑0.0	Rennie et al. (2012)
Tellina fabula	↓−2.69 ± 0.26	Not significant	This study
Magelona spp.	↓−2.08	↓−0.96	This study
Mya arenaria	↓−1.52 ± 0.79	Not significant	This study
Ethanol			
Octopus vulgaris	↑+1.63 ± 0.38	↑+0.20 ± 0.43	Kaehler & Pakhomov (2001)
Fish tissue	↑+0.74 ± 0.15	↑+0.12 ± 0.77	Kaehler & Pakhomov (2001)
Fish tissue	↑+0.42 ± 0.30	↑+0.95 ± 0.27	Sweeting et al. (2004)
Fish tissue	↓−0.78 ± 0.45	↑+0.35 ± 0.63	Kelly et al. (2006)
Freshwater invertebrates	↑+2.2 ± 0.3	↑+1.0 ± 0.2	Syväranta et al. (2011)
Freshwater invertebrates	↑+1.18 ± 0.94	↑+0.39 ± 0.68	Ventura & Jeppesen (2009)
T. fabula	↑+0.77	↑+0.59	This study
Magelona spp.	↑+1.41 ± 0.15	↑+0.94 ± 0.01	This study
M. arenaria	↓−1.36 ± 6.45	Not significant	This study

different positions within the food web in their habitat. The construction of convex hulls with corrected isotopic values helps to visualize the differences in isotope values between analyzed species and supported our observations on uncorrected isotope values discussed above. Overall samples from the North Sea more closely resembled each other than did samples from the Baltic Sea. This again demonstrated the more stable marine influence in the North Sea in contrast to the varying influence at Breitling station, where marine and, of probably greater importance, terrigenous influences alternate.

Correction of preservation effects

The computed correction was significant for the $\delta^{13}C$ values of all species, except for *H. diversicolor*, and for both treatments. This indicates that $\delta^{13}C$ values are less variable. Our data suggest that, in terms of correction, $\delta^{15}N$ values are more susceptible to the high variability of isotope values than $\delta^{13}C$ values, as a correction appeared to be largely unnecessary. Especially regarding the natural isotope fractionation of about 3–4‰ (DeNiro & Epstein 1981, Minagawa & Wada 1984), a correction of $\delta^{15}N$ values seems to be negligible. This partly contradicts the findings of González-Bergonzoni et al. (2015), who recommended the use of correction models when working with preserved samples. A possible explanation for the difference between our study and other studies (Table 7) is that most of the previous studies examined muscle tissue of fish. Samples from the same muscle will have a much lower variability when obtained from the same fish than from different individuals of the same species (Table 7) and will therefore require correction. Both Sarakinos et al. (2002) and Rennie et al. (2012) reported differences in the effects of preservation between mollusks and fish. The application of correction factors to taxonomic groups other than the ones analyzed in this study would seem to be reasonable, as the alteration of isotope values by preservatives is apparently species specific.

An alternative approach to correct the preservation effects, based on mass balance (Fry et al. 2003), was applied to zooplankton by Smyntek et al. (2007) and to freshwater invertebrates by Ventura & Jeppesen (2009). This mass balance approach assumes that carbon in the form of lipids, glycogen, or formalin is added to proteins, increasing C:N ratios. The influence of these additions is corrected by comparisons of C:N ratios of fresh and preserved material. This

mass balance approach was not used in our study for several reasons. First, fresh control samples are lacking for historical samples, so there is no detailed information on their molar C:N ratios. Also, preservation can result in loss of materials as well as additions, resulting in something more akin to exchange than the simple addition implicit in the mass balance model of Fry et al. (2003). Lastly, changes in the C:N ratio in our samples were small (Table 2) so that propagated errors in corrections were large. For these reasons, we used a simpler approach of measuring an offset value between fresh and preserved samples (*'a'* values in Tables 3 & 4) and subtracting these offset values for longer-term corrections.

Combination of correction and phenotype models

Whether organisms in communities from different geographic regions will vary in their isotopic composition, as would be expected because of their different food sources, is still unclear. Our results showed obvious differences in the isotope values between North Sea and Baltic Sea organisms in accordance with their different environments. In contrast to our results, Magni et al. (2013) found no distinguishable isotope values between bivalves from different ecosystems along the European Atlantic coast. The authors concluded that, in terms of the energy flow in a food web, local environmental conditions are more important than the geographic conditions. Karlson et al. (2015) used a modified version of the Layman model to assess the effect of the invasive species *Marenzelleria* spp. on community structure and trophic diversity at 3 Swedish monitoring stations in the Baltic Sea. After constructing isotopic niches to compare the trophic niches of invasive and native species, the authors found that the invasion of new species may positively affect trophic diversity, but the degree of accordance between isotopic niche and trophic niche could not be determined.

An approach that combines the Layman model with our correction model may be useful to investigate the changes in community structure imposed by regime shifts and climate change. Investigations of the macrozoobenthic communities of the North Sea and the Baltic Sea with respect to regime shifts and climate change have revealed changes in the structures of those communities. During cold winters, the macrozoobenthic system has a pronounced resilience but it is also able to establish a new equilibrium level after a climate regime shift (Kröncke et al. 2013, Müller et al. 2016). Cold winters result in an increase in opportunistic species (Neumann & Kröncke 2011). In epifauna, climate variability affects the reproduction rather than other traits but the coastal communities in the German Bight seem to be well adapted to such disturbances (Neumann et al. 2016). Hence, an investigation of the stable isotope patterns in marine organisms may contribute to determining the impact of climate signals on changes in marine biodiversity.

The combined application of the 2 models may also shed light on the ecosystem structure of different geographic regions. For example, a network analysis of the trophic dynamics of the Sylt-Rømø Bight (Fig. 1a) ecosystem in the northern Wadden Sea (southern North Sea) indicated less organized systems with reduced resistance to disturbances because energy is passed through the system less efficiently than in other coastal ecosystems, making the ecosystem sensitive to external forcing (Baird et al. 2004, 2007). In the Jade Bay (Fig. 1a), however, the internal organization of the benthic system is characterized by short trophic pathways (Schückel et al. 2015). A combined approach such as that used in the present study may thus be very useful in the analyses of the internal community structure and the sensitivity of the studied system to external forcing.

Outlook

For a better understanding of trophic positions and food web structure, the application of correction factors to the $\delta^{13}C$ values may be beneficial, whereas in the assessment of the $\delta^{15}N$ values of preserved samples this may not be necessary, at least for benthic invertebrates.

A similar study is planned for the less commonly evaluated stable isotope of sulfur $\delta^{34}S$, which has been used to identify migration patterns (Hesslein et al. 1991) or in pollution studies (Zhao et al. 1998). The shifts in the community structure and biodiversity of near-coastal benthic ecosystems that may occur in response to climate change are still poorly understood. A combination of our correction model and a phenotype-based model (Layman et al. 2007) may contribute to predicting the effects of changing climate on the world's oceans.

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