Vol. 328: 17–28, 2006 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published December 20

Sample preparation effects on stable C and N isotope values: a comparison of methods in Arctic marine food web studies

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ABSTRACT: We compared effects of different sample preparation techniques on the stable carbon and nitrogen isotope values of fish muscle, whole crustaceans and particulate organic matter (POM). Comparisons were also made to untreated (i.e. dried and homogenised) samples. Relatively carbonate-rich samples treated with weak acid (0.1 N HCl), either by quickly wetting or acid dampening, were on average 1.3‰ more enriched in ^{13}C than duplicates soaked in 2 N HCl for 5 min, indicating incomplete carbonate removal with the weaker acid. In comparison, no differences in $\delta^{15}N$ values were found between acid treatments, and a following water rinse had no effect on the $\delta^{13}C$ or $\delta^{15}N$ values. Chloroform-methanol (2:1 by volume) extraction overnight removed less lipids than Soxhlet extraction with 7% methanol in dichloromethane for 2 h, resulting in ~1.2‰ difference in $\delta^{13}C$ values between treatments of lipid-rich duplicates. Different lipid-extraction methods did not lead to consistent differences in $\delta^{15}N$ values, however. Depending on the lipid and carbonate content, untreated samples were depleted in ^{13}C by 0.8 to 4.4‰ and in ^{15}N by 0.6 to 1.4‰ compared to treated duplicates. We conclude that $\delta^{13}C$ and $\delta^{15}N$ values of samples with low lipid and carbonate content are highly comparable among studies regardless of pre-treatment methods, whereas the $\delta^{13}C$ values of relatively lipid- and/or carbonate-rich samples must be carefully considered based on the pre-treatment applied to samples. In comparison, $\delta^{15}N$ values are relatively robust to differences in carbonate and lipid-removal methods, and $\delta^{15}N$ values of untreated vs. carbonate- and lipid-treated samples are comparable within ±1.0‰.

KEY WORDS: Sample pre-treatment · $\delta^{13}C$ · $\delta^{15}N$ · Lipids · Carbonates · C:N

INTRODUCTION

Stable isotope measurements of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are commonly applied in Arctic marine ecosystem investigations to determine food web structure and carbon sources (e.g. Dunton et al. 1989, Hobson & Welch 1992, Hobson et al. 1995, 2002, Iken et al. 2005, Lovvorn et al. 2005, Tamelander et al. 2006). However, no standard sample preparation methods have been established in marine food web studies, and this may hamper the comparability among stable isotope data from different studies.

Inorganic carbon is typically isotopically heavy compared to organic carbon and will positively skew the $\delta^{13}C$ values of animals and algae (e.g. coccolithophorids) if they contain carbonate-rich hard parts (Haines & Montague 1979, Fry 1988). For instance, shells of molluscs are known to primarily reflect the $\delta^{13}C$ value of HCO_3^- in water rather than that of the diet (Keith et al. 1964, Fritz & Poplawski 1974). In order to remove inor-

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ganic carbon prior to stable isotope analysis, samples have traditionally been dried, powdered, and soaked in 1 to 2 N HCl for 5 to 60 min, depending on when bubbling ceases, and then rinsed in distilled water to remove acid waste (e.g. Rau et al. 1983, Dunton et al. 1989, Hobson & Welch 1992, Dahl et al. 2003, Tamelander et al. 2006). Some studies have shown that this relatively strong acid treatment may also remove some proteins and thereby affect the nitrogen isotope values (Bunn et al. 1995, Jacob et al. 2005). However, the effects of acid treatment on $\delta^{15}N$ values are inconsistent and vary from insignificant (Bosley & Wainright 1999, Carabel et al. 2006) to as much as 3‰ (Bunn et al. 1995). It is also hypothesized that the distilled water rinse after acid treatment affects the $\delta^{15}N$ values by removing acid soluble amino acids (Bosley & Wainright 1999, Jacob et al. 2005, Carabel et al. 2006). As a precaution, some researchers use more gentle carbonate removal methods, which do not involve water rinse, such as wetting powdered samples in weak acid (0.1 N HCl; e.g. Hobson et al. 2002) or dampening the samples in a HCl atmosphere (e.g. Lovvorn et al. 2005). These relatively gentle acid treatments may reduce the risk of removing proteins, but may not remove carbonates sufficiently from samples rich in carbonate structures. Some reduce this latter problem by using a strong acid treatment on carbonate-rich organisms and a weaker, gentler acid treatment on organisms with little carbonate structures (e.g. Iken et al. 2005).

Polar marine organisms are often lipid-rich and can vary considerably in their lipid content depending on feeding and life-history strategy, season and developmental stage (e.g. Falk-Petersen et al. 1990, Lee et al. 2006). For instance, herbivorous zooplankton accumulate large lipid stores during food excess to survive the long non-vegetative winter, whereas carnivores, with continuous access to food, store less lipids (Hagen & Auel 2001, Lee et al. 2006). Lipids have a relatively high turnover (Graeve et al. 2005) and are depleted in ^{13}C relative to proteins and carbohydrates (DeNiro & Epstein 1977, Griffiths 1991, Focken & Becker 1998, Sotiropoulos et al. 2004). In order to reduce differences in stable isotope composition due to variations in body lipid content, lipids are commonly removed from animal tissue samples prior to stable isotope analysis in Arctic marine food web studies (e.g. Hobson et al. 1995, 2002, Hop et al. 2002, Tamelander et al. 2006), but not always (Dunton et al. 1989, Iken et al. 2005). Algae also vary in their lipid content and particularly at the end of the growth season they may build up large lipid stores in response to nutrient stress (Fahl & Kattner 1993, Lindqvist & Lignell 1997, Mock & Gradinger 2000). However, lipid extraction of samples of particulate organic matter (POM) prior to stable isotope analysis has only been done in 2 recent Arctic marine food web studies (Søreide et al. 2006, Tamelander et al. 2006).

Lipids are commonly removed by extracting powdered samples in chloroform:methanol 2:1 by volume (e.g. Hobson et al. 2002) or by using Soxhlet extraction with 7% methanol in dichloromethane (e.g. Søreide et al. 2006, Tamelander et al. 2006). Soxhlet extraction with petrol-ether is also sometimes used (Lovvorn et al. 2005). Similar to the acid treatment, lipid extraction may affect the $\delta^{15}N$ values due to possible removal of some N-containing compounds (e.g. Sotiropoulos et al. 2004, Sweeting et al. 2006) but to what extent is little known.

We compared stable isotope preparation techniques commonly used in Arctic marine food web studies to assess the comparability of data among different studies. Two stable isotope laboratories, which applied different sample preparation methods, were involved. Duplicate samples of fish muscle and crustaceans were analysed at both laboratories (inter-laboratory comparison). We focused on weak vs. strong acid treatment followed (or not) by a water rinse, and the 2 common lipid removal methods outlined above. Cross-combinations of these different lipid and carbonate removal methods were also compared (cross-comparison) and contrasted to untreated (i.e. dried and homogenized) controls. Finally, we compared the stable isotope composition of lipid-intact and lipid-extracted POM samples that were all carbonate treated.

MATERIALS AND METHODS

Sample material. In the inter-laboratory comparison, fish dorsal muscle tissue, free of skin and bones, was analysed from 5 specimens of the flounder long rough dab *Hippoglossides platessoides*. These specimens, 24 to 30 cm long, were obtained from a bottom trawl catch northwest of Svalbard (79° 46′ N, 8° 46′ E) in May 2003. Whole individuals of the ice amphipod *Gammarus wilkitzkii* (body size: >25 mm) and krill *Thysanoessa inermis* (body size: ~20 mm), collected in the northern Barents Sea (75–80° N, 25–30° E) in July 2003, were also analysed.

The cross-comparison of sample preparation methods involved whole individuals of *Gammarus wilkitzkii* (body size: >25 mm) collected in the northern Barents Sea (79° 50′ N, 29° 46′ E) in July 2003, and whole individuals of *Thysanoessa inermis* (body size: 15 to 28 mm) sampled from Smeerenburgfjorden (79° 43′ N, 11° 07′ E) and Rijpfjorden (80° 05′ N, 22° 14′ E) in northern Svalbard, during August–September 2003. The ice amphipods were sampled from the underside of the sea ice by SCUBA divers, using electrical suction pumps (Lønne 1988), and krill were sampled by oblique trawl hauls (0–100–0 m) or vertical net hauls (0–300 m) using a Tucker trawl or WP3 net, respectively, both with 1 m^2

mouth opening and 1 mm mesh size. The ice amphipods and krill were kept alive in filtered sea water in screened, false-bottom chambers at ambient temperatures for 24 to 48 h, until evacuation of gut contents, then quickly rinsed in distilled water in order to remove salts, and stored at –20°C until analysis.

Particulate organic matter (POM) was sampled in the northern Barents Sea (77° 08′ N, 29° 28′ E) in May 2004. POM consisting primarily of the ice algae *Nitzschia frigida* (Ice-POM) was collected from the underside of first-year sea ice by SCUBA divers using electrical suction pumps equipped with fine nets (20 µm mesh). Suspended POM from the upper 50 m (Pelagic-POM), consisting of a mixture of *Phaeocystis pouchetii* and diatoms, was collected by vertical hauls using a WP2 net (180 µm mesh size) and then filtered through ca. 40 µm mesh to remove zooplankton. All POM samples were filtered onto pre-combusted (450°C for 4 h) Whatman GF/F filters. Zooplankton visible at 40× magnification was removed from the filter surface after which the filters were frozen at –20°C. Samples were stored frozen for <6 mo prior to analysis.

Sample pre-treatments. Fish muscle, crustaceans and POM-filter samples were dried at 60°C in a laboratory oven for 24 to 48 h, depending on sample size. The animal tissue samples were ground to fine powder using glass mortar and pestle and split into duplicate samples, whereas the filter samples were cut into 2 halves, after which the samples were sent to laboratories for further preparation and analyses.

The Institute for Energy Technology (IFE) in Kjeller, Norway and the Environment Canada Laboratory (ECL) in Saskatoon, Canada were involved in the inter-laboratory comparison. These 2 laboratories applied different carbonate and lipid removal methods prior to stable isotope analysis. IFE removed lipids before removing carbonates, and extracted lipids with a Soxhlet apparatus with ca. 100 ml of a solvent consisting of 7% methanol in dichloromethane by volume (Soxhlet 7%M DCM) for ca. 2 h, after which the samples were dried at 80°C in a laboratory oven. When dry, the samples were soaked in 2 N HCl for ca. 5 min to remove carbonates and rinsed with small portions of distilled water until pH 6–7 to wash away acid and acid-waste. Thereafter, the samples were dried again at 80°C in a laboratory oven. This method is based on Hobson & Welch (1992) with modifications.

ECL removed carbonates prior to lipids. Powdered samples were wetted in 0.1 N HCl and then air dried under a fume hood at room temperature for ca. 24 h. Lipids were removed by extracting the samples in chloroform-methanol 2:1 by volume (2:1 CM) overnight at room temperature. The samples, situated in small glass vials with lids, were covered by the 2:1 CM solvent during extraction. After extraction, samples were quickly rinsed in new 2:1 CM and then air dried under a fume hood at room temperature.

In the inter-laboratory comparison, duplicate samples of fish muscle, ice amphipods and krill were prepared and analysed at IFE and ECL as described above (see Fig. 1 for overview). In addition, lipid extracts from the krill samples were kept and analysed at both laboratories.

A cross-comparison of sample preparation methods was conducted at IFE, in which duplicate samples of ice amphipods and krill were prepared after IFE and ECL methods and a cross-combination of these (see Fig. 1 for overview). However, to remove carbonates, the ECL method (quick wetting) was replaced by dampening the samples in a 0.1 N HCl vacuum atmosphere for 24 h. Untreated duplicates (i.e. samples that only were dried and homogenised) were also analysed in the cross-comparison. Comparison of lipid-intact and lipid-extracted POM samples was conducted at ECL, applying the carbonate and lipid removal methods of this laboratory (see Fig. 1 for overview).

Stable isotope analysis. Stable carbon and nitrogen isotope analyses were performed at both laboratories on ~1 mg of the residual sample material, which was packed into tin cups and analysed at IFE with a ThermoQuest NCS 2500 elemental analyser coupled to a Micromass Optima IRMS, and at ECL on an interfaced Europa 20:20 continuous-flow isotope ratio mass spectrometer (CFIRMS), situated at the Department of Soil Science, University of Saskatchewan.

Stable isotope ratios were expressed in δ notation as the deviation from standards in parts per thousand (‰) according to the following equation:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. International standards—USGS-24 calibrated against PeeDee Belemnite (Vienna) for ^{13}C and IAEA-N-1 and IAEA-N-2 calibrated against atmospheric N_2 for ^{15}N—were used to determine $R_{standard}$ at both laboratories. Replicate measurements of the international standards indicated measurement errors of ±0.2‰ and ±0.1‰ for $\delta^{13}C$ at IFE and ECL, respectively, and ±0.3‰ for $\delta^{15}N$ at both laboratories. Replicate measurements of internal biological standards indicated measurement errors similar to those of the international standards at both laboratories, and were used to calibrate the system and compensate for drift over time.

The total amounts of carbon (C) and nitrogen (N) (only measured at ECL) and the percentages of N and C of total sample dry weights, with corresponding C:N ratios, are presented along with the stable isotope values.

Statistical analyses. Statistical tests were performed using STATISTICA 6.1: *t*-tests were used when comparing 2 independent groups, and 1-way ANOVA fol-

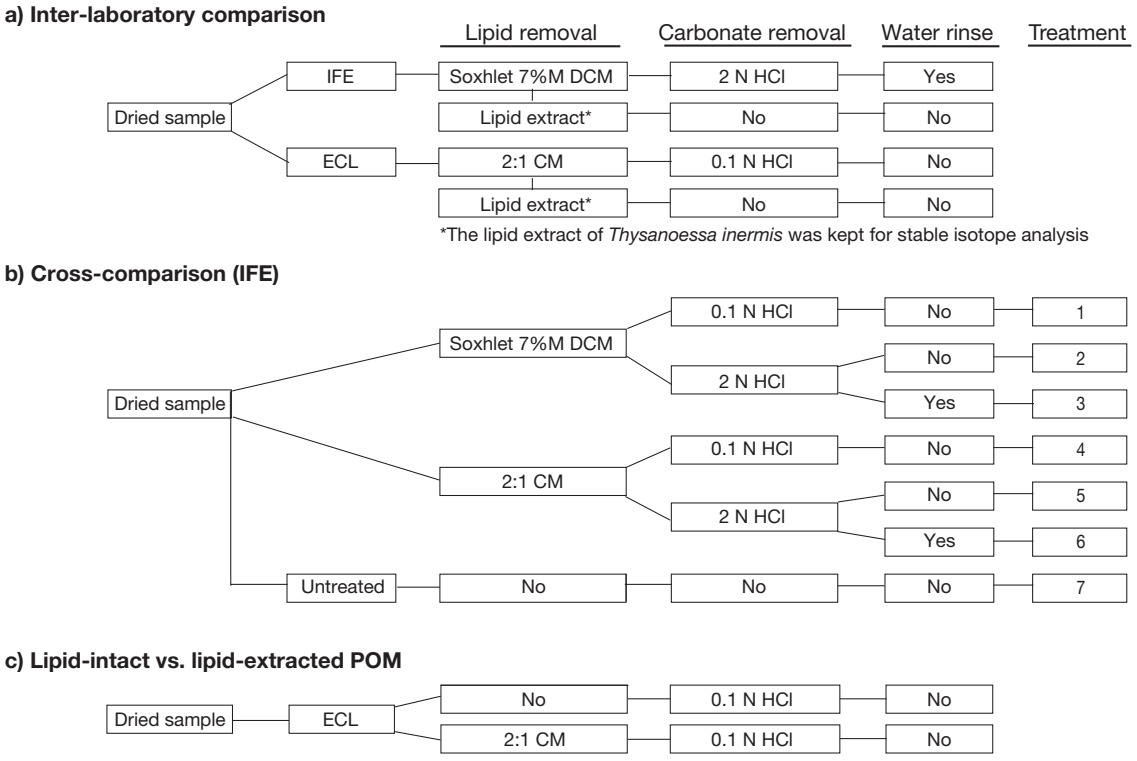

Fig. 1. Overview of different sample pre-treatments: (a) inter-laboratory comparison performed at the Institute of Energy Technology (IFE) and the Environment Canada Laboratory (ECL) using their respective methods; (b) cross-comparison of IFE and ECL methods, as well as a cross-combination of these methods; (c) comparison of lipid-intact and lipid-extracted filter samples of particulate organic matter (POM), all carbonate treated, using ECL methods. M DCM: methanol in dichloromethane; CM: chloroform-methanol

lowed by the post hoc tests Tukey HSD and unequal Tukey HSD were used when comparing multiple groups with similar or unequal number of replicates per group, respectively (Winer et al. 1991). If the variance between independent groups was not homogenous (i.e. Levene's Test, $p \leq 0.05$), we used the Mann-Whitney *U*-test (MWU-test) and Kruskal-Wallis Median test (KW-test) followed by a post-hoc test for multiple comparisons of mean ranks for all groups (Siegel & Castellan 1988). The significance level was set to $p \leq 0.05$ in all tests.

RESULTS

Inter-laboratory comparison

The stable isotope values of flounder muscle did not differ between the 2 laboratories (Table 1, Fig. 2). However, differences in stable isotope values were found for the crustacean samples between the 2 laboratories (Table 1, Fig. 2). At IFE, whole organisms of *Gammarus wilkitzkii* were depleted in ^{13}C by 0.9 to 1.7‰ (mean 1.3‰) compared to duplicates analysed at ECL, whereas whole organisms of *Thysanoessa inermis* were enriched in ^{13}C by 0.5 to 0.9‰ (mean 0.7‰) at IFE relative to ECL. No differences in $\delta^{15}N$ values of whole organisms of *G. wilkitzkii* were found between laboratories, but the $\delta^{15}N$ values of whole *T. inermis* were 0.9 to 1.6‰ higher (mean 1.1‰) at IFE than at ECL.

The total lipids extracted from whole organisms of *Thysanoessa inermis* were depleted in ^{13}C by 5.5 to 6.1‰ and in ^{15}N by 5.5 to 7.2‰ compared to the remaining defatted and carbonate-removed organism samples (Table 1, Fig. 2). The $\delta^{13}C$ values of *T. inermis* lipid extracts were similar at the 2 laboratories, but the $\delta^{15}N$ values were depleted by 1.3 to 3‰ (mean 2.3‰) at IFE compared to ECL. Significantly different %C, %N and C:N ratios were found in the inter-laboratory comparison, except for %N and C:N ratios of *T. inermis* lipid extracts.

Cross-comparison of sample pre-treatment methods

Gammarus wilkitzkii

Untreated whole organisms of *Gammarus wilkitzkii* were depleted in ^{13}C by 0.8 to 2.8‰ (mean 1.8‰) and in ^{15}N by 0.7 to 1.1‰ (mean 0.8‰) relative to the residual material after removal of lipids and carbonates (Table 2, Fig. 3). The 2 different lipid removal meth-

Table 1. Inter-laboratory comparisons (*t*-tests) of $\delta^{13}C$ and $\delta^{15}N$ values, percentage C and N (of dry weights) and the carbon:nitrogen (C:N) ratios (mean ± SE) of duplicate samples prepared and analysed at the Institute of Energy Technology (IFE) and the Environment Canada Laboratory (ECL) using their respective methods (see 'Materials and methods'). The samples consisted of fish *Hippoglossides platessoides* muscle tissue and the crustaceans *Gammarus wilkitzkii* and *Thysanoessa inermis* analysed whole, as well as total lipid extract from *T. inermis*. Significance levels: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$; ns = not significant ($p > 0.05$)

Species	Tissue	n	$\delta^{13}C$ (‰)				$\delta^{15}N$ (‰)				%C			%N			C:N		
			IFE	ECL	Diff.	p	IFE	ECL	Diff.	p	IFE	ECL	p	IFE	ECL	p	IFE	ECL	p
H. platessoides	Muscle	5	−19.6 ± 0.3	−19.5 ± 0.3	0.1	ns	14.3 ± 0.4	14.1 ± 0.4	0.2	ns	48.8 ± 0.6	43.5 ± 1.1	***	15.5 ± 0.2	13.4 ± 0.4	***	3.1 ± 0.0	3.3 ± 0.0	**[a]
G. wilkitzkii	Whole ind.	6	−21.0 ± 0.1	−19.7 ± 0.1	1.3	***	7.4 ± 0.2	7.2 ± 0.2	0.2	ns	44.0 ± 1.3	31.5 ± 0.2	***	11.5 ± 0.5	7.6 ± 0.1	***	3.8 ± 0.1	4.2 ± 0.0	***
T. inermis	Whole ind.	3	−21.3 ± 0.1	−22.0 ± 0.1	0.7	***	9.9 ± 0.1	8.8 ± 0.1	1.1	**	50.6 ± 0.5	42.7 ± 1.4	**	14.8 ± 0.1	11.2 ± 0.1	***	3.4 ± 0.0	3.8 ± 0.1	**
T. inermis	Lipid extract	3	−27.4 ± 0.1	−27.5 ± 0.2	0.1	ns	2.7 ± 0.1	5.0 ± 0.4	2.3	**	72.3 ± 0.2	64.1 ± 2.5	*	2.0 ± 0.0	2.9 ± 0.5	ns	35.9 ± 0.5	23.7 ± 4.6	ns

[a]Mann-Whitney *U*-test

Table 2. The different sample preparation treatments in the cross-comparison with corresponding stable isotope values, percentages of carbon and nitrogen (of dry weight) and the respective C:N ratios of duplicate tissue samples (mean ± SE) of *Gammarus wilkitzkii* and *Thysanoessa inermis*. Comparisons among $\delta^{13}C$ and $\delta^{15}N$ values (ANOVA, Tukey HSD test) from Treatments 1 to 6 are shown. For significance levels see Table 1, ns = not significant ($p > 0.05$). Lipids were removed using a Soxhlet apparatus with 7 % methanol in dichloromethane for ca 2 h (7 %M DCM) or chloroform-methanol (2:1 by volume) overnight (2:1 CM). Carbonates were removed by dampening the samples in 0.1 N HCl for 24 h and letting them air dry (0.1 N HCl) or by soaking the samples in 2 N HCl for 5 min before drying them at 80°C (2 N HCl). Samples that were rinsed in distilled water (Dist. H_2O) after acid treatment were rinsed until pH 6 to 7. Untreated samples were only dried and homogenised prior to analysis. n = number of samples. All samples were prepared and analysed at the Institute of Energy Technology (IFE)

Treatment	Lipids	Carbonates	Dist. H_2O	n	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	%C	%N	C:N	ANOVA $\delta^{13}C$ (‰)					ANOVA $\delta^{15}N$ (‰)				
										1	2	3	4	5	1	2	3	4	5
G. wilkitzkii																			
1	7 %M DCM	0.1 N HCl	No	3	−19.9 ± 0.2	8.5 ± 0.2	30.9 ± 0.6	7.7 ± 0.1	4.0 ± 0.0										
2	7 %M DCM	2 N HCl	No	3	−21.0 ± 0.1	8.3 ± 0.1	26.8 ± 0.4	7.0 ± 0.1	3.8 ± 0.0	**					ns				
3	7 %M DCM	2 N HCl	Yes	3	−21.4 ± 0.1	8.5 ± 0.1	46.6 ± 1.0	11.6 ± 0.2	4.0 ± 0.0	***	ns				ns	ns			
4	2:1 CM	0.1 N HCl	No	3	−20.1 ± 0.2	8.7 ± 0.2	31.2 ± 0.2	7.1 ± 0.1	4.4 ± 0.0	ns	*	**			ns	ns	ns		
5	2:1 CM	2 N HCl	No	3	−21.3 ± 0.4	8.3 ± 0.3	30.5 ± 0.4	7.2 ± 0.1	4.2 ± 0.0	***	ns	ns	**		ns	ns	ns	ns	
6	2:1 CM	2 N HCl	Yes	3	−21.9 ± 0.1	8.5 ± 0.3	43.4 ± 0.1	10.2. ± 0.1	4.2 ± 0.0	***	*	ns	***	ns	ns	ns	ns	ns	ns
7	Untreated	Untreated	No	14	−22.7 ± 0.2	7.6 ± 0.2	41.2 ± 0.1	6.8 ± 0.1	6.1 ± 0.1										
T. inermis																			
1	7 %M DCM	0.1 N HCl	No	3	−22.3 ± 0.2	9.4 ± 0.0	39.4 ± 0.3	11.9 ± 0.1	3.3 ± 0.0										
2	7 %M DCM	2 N HCl	No	3	−22.0 ± 0.0	9.0 ± 0.1	35.9 ± 0.7	10.9 ± 0.1	3.3 ± 0.0	ns					ns				
3	7 %M DCM	2 N HCl	Yes	3	−22.2 ± 0.1	9.7 ± 0.1	49.0 ± 0.1	14.4 ± 0.1	3.4 ± 0.0	ns	ns				ns	*			
4	2:1 CM	0.1 N HCl	No	3	−23.3 ± 0.1	9.4 ± 0.1	38.3 ± 0.2	10.6 ± 0.1	3.6 ± 0.0	***	***	***			ns	ns	ns		
5	2:1 CM	2 N HCl	No	3	−23.3 ± 0.1	9.8 ± 0.2	37.3 ± 0.6	9.3 ± 0.0	4.0 ± 0.1	***	***	***	ns		ns	**	ns	ns	
6	2:1 CM	2 N HCl	Yes	3	−23.7 ± 0.2	9.7 ± 0.1	45.1 ± 0.1	11.6 ± 0.1	3.9 ± 0.1	***	***	***	ns	ns	ns	*	ns	ns	ns
7	Untreated	Untreated	No	18	−26.4 ± 0.1	8.4 ± 0.1	52.4 ± 0.4	7.2 ± 0.1	7.3 ± 0.1										

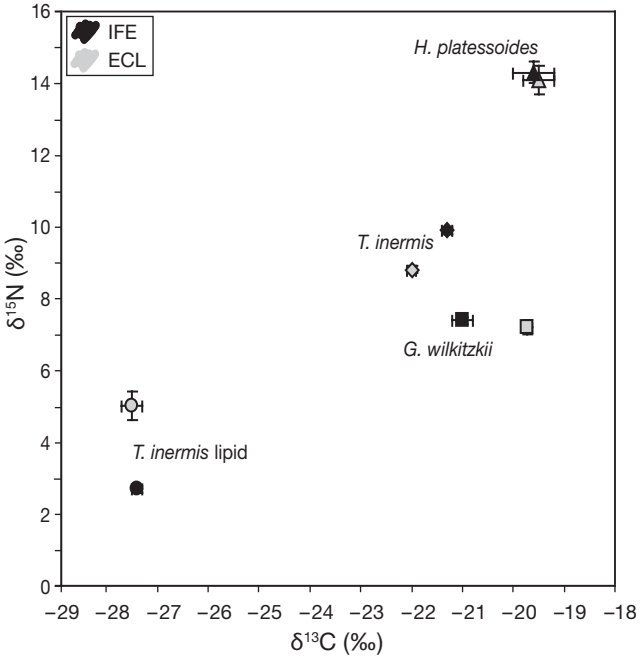

Fig. 2. Inter-laboratory comparison of $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SE) for duplicate samples analysed at the Institute of Energy Technology (IFE) and the Environment Canada Laboratory (ECL) using their respective methods. Duplicate samples consisted of muscle tissue from *Hippoglossides platessoides*, whole individuals of *Gammarus wilkitzkii* and *Thysanoessa inermis*, and lipid-extract from *T. inermis* samples

ods did not lead to different $\delta^{13}C$ values of *G. wilkitzkii*, but the different carbonate removal methods did. The $\delta^{13}C$ values of samples treated with weak acid were 1.1 to 2‰ (mean 1.4‰) higher than in samples treated with strong acid (Table 2). The following water rinse after the strong acid treatment did not affect the $\delta^{13}C$ and $\delta^{15}N$ values. The sample variances did not differ among treatments and were low (SE: <0.2‰) for both the $\delta^{13}C$ and $\delta^{15}N$ values, except for the more variable $\delta^{13}C$ values (SE: 0.4‰) in Treatment 5 (Table 2).

Significant differences in %C, %N and C:N were found among treatments of whole organisms of *Gammarus wilkitzkii* (KW-test and ANOVA, $p \leq 0.001$). Untreated samples and those rinsed with distilled water (Treatments 3 & 6) had ca. 10% higher %C than the other treatments (Table 2). The %N was also higher in samples rinsed with distilled water (~10 to 11%), whereas untreated samples had %N comparable to the other treatments (~7 to 8%). The C:N ratios of untreated samples (6.1) were higher than those of the treated samples, all of which had similar C:N ratios (3.8 to 4.2, ANOVA, Tukey HSD test, $p \geq 0.3$) except for a slightly higher C:N ratio (4.4) in Treatment 4 ($p = 0.008$).

Thysanoessa inermis

Untreated whole organisms of *Thysanoessa inermis* were depleted in ^{13}C by 3 to 4.4‰ (mean 3.6‰) and in ^{15}N by 0.6 to 1.4‰ (mean 1.1‰) relative to the residual material after removal of lipids and carbonates (Table 2, Fig. 3). There were no differences in $\delta^{13}C$ values among different acid treatments for whole organisms of *T. inermis*, regardless of the lipid-removal procedure. The 2 different methods for removing lipids, however, gave significantly different $\delta^{13}C$ values with 2:1 CM being depleted by 0.5 to 1.7‰ (mean 1.2‰) compared to the Soxhlet procedure. The different lipid removal methods did not result in different $\delta^{15}N$ values, except for the slightly lower (0.4 to 0.8‰) $\delta^{15}N$ values of samples treated with Soxhlet 7%M DCM and 2 N HCl (Treatment 2). The sample variances did not differ among treatments and were low (<0.2‰) for both $\delta^{13}C$ and $\delta^{15}N$ values.

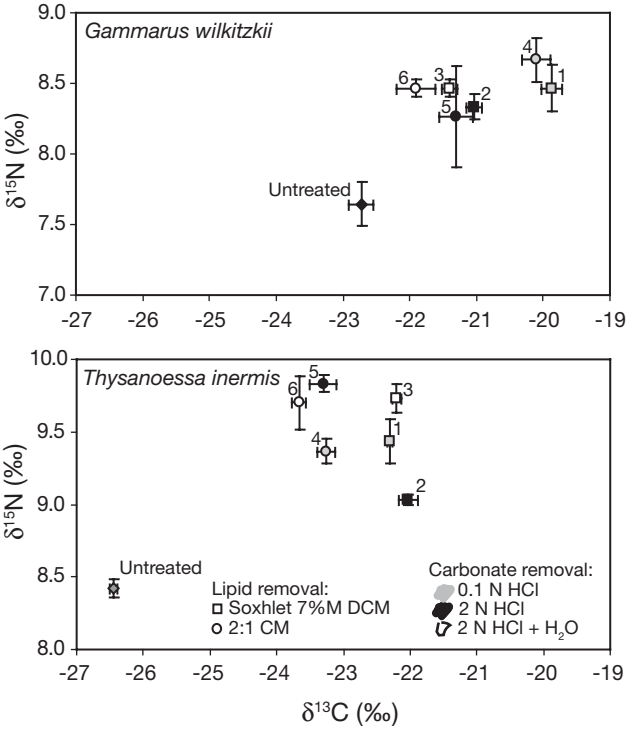

Fig. 3. Cross-comparison of $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SE) of duplicate samples of *Gammarus wilkitzkii* (upper panel) and *Thysanoessa inermis* (lower panel) treated by different lipid and carbonate removal methods (Treatments 1 to 6, see Table 2). Lipids were removed using a Soxhlet apparatus with 7% methanol in dichloromethane for 1 to 2 h (Soxhlet 7%M DCM) or with chloroform-methanol (2:1 by volume) overnight (2:1 CM). Carbonates were removed by soaking the samples in 2 N HCl for 5 min, followed by a water rinse (H_2O) or not, or by dampening the samples in 0.1 N HCl for 24 h. Untreated samples were only dried and homogenised prior to stable isotope analysis. Numbers refer to treatments in Table 2

Significant differences in %C, %N and C:N were found among treatments of whole organisms of *Thysanoessa inermis* (ANOVA, $p \leq 0.001$). In particular, the untreated samples had high %C (52%), followed by those rinsed in distilled water (45 to 49%) and then the others (35 to 39%) (Table 2). Samples treated with Soxhlet 7%M DCM had 1 to 3% higher %N than those treated with 2:1 CM, when comparing samples that otherwise were treated alike (ANOVA, Tukey HSD, $p \leq 0.001$). The C:N ratios were similarly low (3.3) in samples treated with Soxhlet 7%M DCM (ANOVA, Tukey HSD, $p \geq 0.507$). Samples treated with 2:1 CM had also similar C:N ratios (ANOVA, Tukey HSD, $p \geq 0.507$), which were 0.5 units higher than the C:N ratios of samples treated with the Soxhlet procedure (ANOVA, Tukey HSD, $p \leq 0.032$). Untreated samples had C:N ratios that were roughly twice as high as those in treated samples (*t*-tests, $p < 0.001$).

Comparison of lipid-extracted and lipid-intact POM samples

Ice-POM

Lipid-extracted Ice-POM samples were 1.3‰ (range 0.8 to 2.1‰) and 0.4‰ (range 0.1 to 0.6‰) more enriched in ^{13}C and ^{15}N, respectively, compared to the lipid-intact duplicates (Table 3, Fig. 4). The %C content and the C:N ratio were considerably lower in the lipid-extracted Ice-POM samples, whereas only minor, although statistically significant differences were found regarding the %N content (Table 3). The sample variance in $\delta^{13}C$ values was higher in lipid-extracted than lipid-intact Ice-POM samples, but was consistently low for the $\delta^{15}N$ values.

Pelagic-POM

No differences in $\delta^{13}C$, %C or C:N values were found between lipid-extracted and lipid-intact samples of Pelagic-POM (Table 3, Fig. 4). However, lipid-intact and lipid-extracted samples had significantly different $\delta^{15}N$ values. Samples containing little N (< 8 µg total N) became 2.5‰ enriched in ^{15}N after lipid removal, whereas samples with twice or higher N content (14.9 to 24.1 µg) became only 0.5 to 0.6‰ enriched in ^{15}N after lipid removal. The differences could not be tested statistically since only 1 replicate of each sample was analysed. Both samples showed the same trend, however. The variances in $\delta^{13}C$ and $\delta^{15}N$ values were similarly low in both lipid-extracted and lipid-intact samples.

DISCUSSION

Fish muscle

The muscle samples of *Hippoglossides platessoides* most likely contained low amounts of carbonates, since no skin or bones were present (e.g. Goering et al. 1990), and low amounts of lipids (<2.5%; Budge et al. 2002), which can explain why no differences in $\delta^{13}C$ or $\delta^{15}N$ values were found among the different carbonate and lipid removal methods used (Sotiropoulos et al.

Table 3. Comparison of stable carbon and nitrogen values (mean ± SE) of lipid-intact (intact) and lipid-extracted (extr.) duplicate particulate organic matter samples collected from the underside of the sea ice (Ice-POM) and the water column (Pelagic-POM). All samples were quickly wetted in 0.1 N HCl and air dried at room temperature for ca. 24 h prior to stable isotope analysis. Lipids were removed by extracting the samples in chloroform-methanol (2:1 by volume) overnight. Total (Tot.) C and N dry weights, percentage of C and N (of sample dry weights) and the respective carbon:nitrogen ratio (C:N) are given (mean ± SE). All samples were prepared and analysed at the Environment Canada Laboratory. For significance levels see Table 1; ns = not significant ($p > 0.05$). –: too few replicates for statistical testing

$\delta^{13}C$ (‰)			$\delta^{13}C$ (‰)			Tot. C (µg)		%C			C:N		
Sample	Sample ID	n	intact	extr.	p	intact	extr.	intact	extr.	p	intact	extr.	p
Ice-POM	3001–3003	3	−17.6 ± 0.0	−16.4 ± 0.4	*	382.1	263.7	28.4 ± 0.8	19.4 ± 1.0	**	10.9 ± 0.1	6.7 ± 0.4	***
Pelagic-POM	3004–3005	2	−23.6 ± 0.1	−23.2 ± 0.1	ns	34.4	45.8	0.7 ± 0.0	0.7 ± 0.1	ns	5.8 ± 0.1	5.9 ± 0.1	ns
Pelagic-POM	3006	1	−24.6	−24.5	–	86.5	179.4	4.3	4.6	–	5.8	6.3	–
Pelagic-POM	3007	1	−24.5	−23.5	–	142.2	188.0	6.9	8.2	–	5.9	6.6	–
$\delta^{15}N$ (‰)			$\delta^{15}N$ (‰)			Tot. N (µg)		%N			C:N		
Sample	Sample ID	n	intact	extr.	p	intact	extr.	intact	extr.	p	intact	extr.	p
Ice-POM	3001–3003	3	2.6 ± 0.1	3.0 ± 0.1	**	34.9	39.5	2.6 ± 0.1	2.9 ± 0.0	*	10.9 ± 0.1	6.7 ± 0.4	***
Pelagic-POM	3004–3005	2	3.0 ± 0.0	5.5 ± 0.0	***	5.9	7.7	0.1 ± 0.0	0.1 ± 0.0	ns	5.8 ± 0.1	5.9 ± 0.1	ns
Pelagic-POM	3006	1	3.7	4.3	–	14.9	28.4	0.7	0.7	–	5.8	6.3	–
Pelagic-POM	3007	1	4.3	4.8	–	24.1	28.3	1.2	1.2	–	5.9	6.6	–

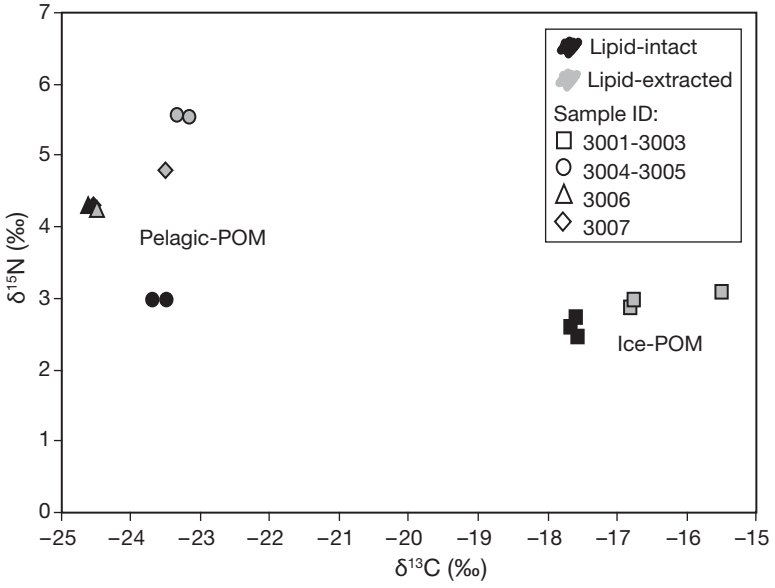

Fig. 4. $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SE) of lipid-extracted and lipid-intact parallel samples of particulate organic matter from underside of sea ice (Ice-POM) and suspended in the upper water column (Pelagic-POM). All filter samples were quickly wetted in 0.1 N HCl and air dried at room temperature prior to stable isotope analysis. Lipids removed by extracting samples in chloroform-methanol (2:1 by volume) overnight

2004, Carabel et al. 2006). Bunn et al. (1995) showed effects of acid treatment on $\delta^{15}N$ values, but the present results revealed no changes in $\delta^{15}N$ of the *H. platessoides* muscle tissue. Our results and findings by Bosley & Wainright (1999) and Carabel et al. (2006) suggest that carbonate removal has no significant effects on the $\delta^{15}N$ values of fish muscles as long as the carbonate content is low. Relatively large differences in $\delta^{13}C$ values, however, have been found between lipid-intact and lipid-extracted samples of whole fish or fish liver, which are often very lipid-rich (Focken & Becker 1998, Sotiropoulos et al. 2004, Sweeting et al. 2006). Lipids are depleted in ^{13}C by 4 to 8.5‰, with weighted mean of 6.5 to 7.0‰, relative to proteins (Sweeting et al. 2006 and references therein). Changes in $\delta^{15}N$ values after lipid removal have also been found, but they are generally small compared to changes in $\delta^{13}C$ values. Sweeting et al. (2006) found that the $\delta^{15}N$ values were negatively related to sample lipid content, but lipid-removal only had a significant impact on the $\delta^{15}N$ values of lipid-rich samples (45 to 57% lipids), leading to an enrichment of 0.9‰.

The C:N ratios can provide a good estimate of the samples lipid content, and can to some extent be used to correct $\delta^{13}C$ values for variations in lipid content (Sweeting et al. 2006). Much higher %C and %N were found in samples that had been water rinsed than those which had not, but this did not influence the C:N ratios. The difference in C:N ratios of fish muscle between laboratories (0.2) was statistically significant, yet small. This could have been a result of differences in lipid and carbonate removal methods. The higher %C and %N of water-rinsed vs. unrinsed samples, showed that acid remnants were removed, but this did not affect the $\delta^{13}C$ or $\delta^{15}N$ values.

Crustaceans

Crustaceans deposit carbonate in their exoskeletons as $CaCO_3$ by extracting isotopically heavy HCO_3^- from seawater, which causes carbonates to have high $\delta^{13}C$ values (Roer & Dillaman 1993, Yokoyama et al. 2005). We expected that the removal of carbonates was more efficient when using 2 N HCl than 0.1 N HCl, not only because of the differences in acid strengths, but also because we soaked the samples for a longer time in the strong acid than in the weak. Acid dampening is also likely to be more gentle than soaking. However, we only found differences in $\delta^{13}C$ values between carbonate-removal methods for *Gammarus wilkitzkii*. Whole organisms of *G. wilkitzkii* treated with weak acid were in both the inter-laboratory and the cross-comparison enriched in ^{13}C by ~1.3‰ compared to duplicates treated with strong acid, regardless of whether a following water rinse was performed or not (Tables 1 & 2). For whole organisms of *Thysanoessa inermis* the different carbonate-removal methods did not lead to differences in $\delta^{13}C$ values, but the 2 different lipid-removal methods did (Tables 1 & 2). These inconsistent $\delta^{13}C$ results are most likely explained by a relatively high proportion of carbonate-rich cuticle in samples of whole *G. wilkitzkii* compared to samples of whole *T. inermis*, which has a much thinner cuticle and a higher lipid content than *G. wilkitzkii*. Lipids may comprise about 28% and 50% of the dry matter in *G. wilkitzkii* and *T. inermis*, respectively (Sargent & Falk-Petersen 1981, Scott et al. 2001, Lee et al. 2006), which corresponded well with the ~30% and ~50% reduction in C:N ratios after lipid removal for the 2 respective species (Table 2). Unacidified exoskeletons of mysids and shrimp are enriched in ^{13}C by 1.2 to 3.8‰ compared to the whole organism (Gorokhova & Hansson 1999, Yokoyama et al. 2005), but when the proportion of exoskeleton to body mass is small (Bunn et al. 1995), effects of carbonate removal on the $\delta^{13}C$ values may not be detected. For whole individuals with high proportions of hard parts, however, untreated samples can be enriched in ^{13}C by 2.5‰ compared to carbonate-treated duplicates (Carabel et al. 2006). If *G. wilkitzkii* and *T. inermis* only con-

tained insignificant carbonate structures, lipid-intact samples of whole organisms would be expected to be depleted in ^{13}C by ca. 2.1‰ and 3.5‰, respectively, compared to lipid-extracted duplicates (e.g. Sweeting et al. 2006). That untreated whole organisms of *G. wilkitzkii* and *T. inermis* were depleted in ^{13}C by 1.8 and 3.7‰, respectively, compared to treated duplicates showed that isotopically light lipids influenced the $\delta^{13}C$ values more than isotopically enriched carbonates in both species.

The 2 different lipid-removal methods were not equally efficient in extracting lipids when samples were very lipid-rich, which was somewhat surprising since the more polar 2:1 CM was expected to extract total lipids more efficiently than the less polar 7%M DCM (Smedes & Askland 1999). However, it is possible that the extraction at room temperature with only 2 rinses with 2:1 CM was not sufficient for complete removal of lipids of the particularly lipid-rich *Thysanoessa inermis*. In comparison, the Soxhlet extraction for 2 h was conducted at ca. 40°C and involved 10 to 12 solvent rinses.

Untreated samples of *Gammarus wilkitzkii* and *Thysanoessa inermis* were depleted in ^{15}N by ~0.8‰ and ~1.1‰, respectively, compared to treated duplicates. Whether it was the carbonate or lipid removal that mostly affected the $\delta^{15}N$ values was not specifically tested, but findings of Bosley & Wainright (1999) and Carabel et al. (2006) show no effects of carbonate removal on the $\delta^{15}N$ values in a wide spectrum of zooplankton size fractions, crabs and fish muscles. We strongly suggest that differences in the $\delta^{15}N$ values were caused by the lipid-removal, which is in agreement with Sotiropoulos et al. (2004) and Sweeting et al. (2006). The lipid-extracts in our study contained N (Table 1), which indicated that both lipid extraction methods removed some proteins. Polar lipids are constituents of membranes where they occur in close association with proteins (Christie 1982). The higher the polarity of the lipid solvent, the higher is the risk of incidental leaching of proteins from tissue during lipid extraction (Manirakiza et al. 2001, Sotiropoulos et al. 2004). The more enriched $\delta^{15}N$ values of the lipid-extract from the polar 2:1 CM method indicated that this solvent extracted more or other N-compounds than the less polar 7%M DCM solvent (i.e. DCM:M ≥ 13:1 by volume). This was also supported by the $\delta^{15}N$ values and the C:N ratios of the defatted whole organism samples of *T. inermis* in the inter-laboratory comparison (Table 1, Fig. 2). However, in the cross-comparison we found no significant differences in $\delta^{15}N$ values between lipid extraction (or carbonate removal) methods (Table 2, Fig. 3), indicating that the 2 different lipid extraction methods affected the $\delta^{15}N$ values of bulk tissue material similarly. One treatment (*T. inermis* Treatment 2, Table 2) gave slightly lower $\delta^{15}N$ values, but the reason for this is not known. Water has a high polarity and could potentially wash away some polar N-compounds (Christie 1982, Jacob et al. 2005), but besides this 1 treatment no significant differences in $\delta^{15}N$ or $\delta^{13}C$ values were found between water-rinsed and unrinsed samples that otherwise were treated alike, suggesting little or no effects of the water-rinsing-step on $\delta^{13}C$ and $\delta^{15}N$ values of animals. Also, the C:N ratios were unaffected by the water-rinsing-step, indicating that it did not selectively remove C or N compounds.

Bunn et al. (1995) observed a 2 to 3 fold increase in sample variance for both $\delta^{13}C$ and $\delta^{15}N$ values after carbonate removal, while lipid extraction did not seem to affect sample variance, at least not for fish (Sotiropoulos et al. 2004). In our study, untreated and treated fish and crustacean samples had similarly low $\delta^{13}C$ and $\delta^{15}N$ variance, which suggests that neither carbonate nor lipid removal increase the sample variance notably.

Particulate organic matter

Effects of lipid removal on the $\delta^{13}C$ values of POM were only detected in the Ice-POM samples, which after being lipid-extracted became markedly depleted in carbon, from 28.4 to 19.4 %C of dry matter, with a respective reduction in the C:N from 10.9 to 6.7 (Table 3). This reduction in%C indicates that Ice-POM contained ca. 9% lipids (of dry matter), which is in accordance with the lipid content that Fahl & Kattner (1993) found in ice algae. The average difference in the $\delta^{13}C$ values between lipid-extracted and lipid-intact Ice-POM samples was 1.2‰, which suggests 0.13‰ enrichment of ^{13}C for every 1% lipid removed. Lipid-extraction did not alter the $\delta^{13}C$ values,%C or C:N ratios of the Pelagic-POM samples. However, these samples were collected during a spring bloom situation before nutrients became limiting and, thus, the algae had good conditions, favouring growth rather than lipid accumulation (Mock & Gradinger 2000). Lipid storage in algae is related to unfavourable growth conditions, such as nutrient limitation, low temperature and low irradiance (Fahl & Kattner 1993, Lindqvist & Lignell 1997, Mock & Gradinger 2000). Algae with C:N ratios >7.0 are normally nutrient limited (Mayzaud et al. 1989 and references therein) and such high C:N ratios were only found in our Ice-POM samples (Table 3). Ice-POM was collected from a 2 to 3 cm thick algal mat on the underside of the ice and most likely experienced nutrient limitation because of a relatively thick boundary layer, consistent with its enriched $\delta^{13}C$ values (McMinn et al. 1999).

Pelagic-POM which contained very little N (<8 µg total N) became markedly enriched in ^{15}N after lipid-removal, whereas only a slight increase in the $\delta^{15}N$ values was found in Pelagic-POM with larger N-pools (≥15 µg total N) after lipid-removal, comparable to the increase in ^{15}N of Ice-POM after lipid removal (Table 3). However, the enrichment in ^{15}N by 0.4 to 0.6‰ after lipid removal was small and only slightly larger than the analytical error for $\delta^{15}N$ values (±0.3‰). As previously mentioned, lipid extraction may potentially remove some N-compounds, and the increase in $\delta^{15}N$ values can be explained by a combination of a small N-pool and selective removal of some N-compounds, i.e. N that is closely associated with membranes. The particularly large increase in $\delta^{15}N$ values after lipid removal for those samples containing as little as 6 to 8 µg N, however, can also be explained by a possible source linearity problem which may arise on CFIRMS machines if particularly small amounts of N are analysed (Fry et al. 1992, Bunn et al. 1995). The stable isotope composition of POM samples containing very little organic material should therefore be carefully considered when they are used, and the total N and C content of samples can be useful additional information for determining the reliability of the results. The sample variance, however, did not seem too be much affected by sample amount or lipid-removal. The relatively large variability in $\delta^{13}C$ values of lipid-extracted Ice-POM samples was caused by 1 high $\delta^{13}C$ value (Fig. 4), which may be regarded as an outlier.

Comparability among studies in a pan-Arctic perspective

Different methods for removing lipids and carbonates seem to have minor impacts on the overall $\delta^{13}C$ and $\delta^{15}N$ values of lipid- and carbonate-poor samples (Focken & Becker 1998, Bosley & Wainright 1999, Sotiropoulos et al. 2004, Jacob et al. 2005, Carabel et al. 2006, this study), and the comparability among samples of this kind is most likely high among studies. For relatively lipid- and/or carbonate-rich samples, however, the sample pre-treatment methods must be carefully considered before comparisons are being made, in order to be able to infer that apparent $\delta^{13}C$ differences are not just a result of methodological differences. Relatively small differences in $\delta^{13}C$ values can lead to important trophic inference since the enrichment of ^{13}C from one trophic level to the next is small (e.g. 0.4 to 0.6‰; Post 2002, Søreide et al. 2006). In comparison, larger differences in ^{15}N values are needed before significant trophic inference can be assumed, since the enrichment of ^{15}N from one trophic level to the next is much larger, generally between 3 and 4‰ (Hobson & Welch 1992, Post 2002, Søreide et al. 2006). Comparisons of $\delta^{13}C$ values between treated and untreated samples that are lipid-rich and/or relatively carbonate-rich are not recommended, but for the $\delta^{15}N$ values it is possible within ±1‰ (Sweeting et al. 2006, this study). However, for organisms with more carbonate structures, e.g. brittle stars (Ophiuroidea), weak vs. strong acid treatment may lead to even larger differences in the $\delta^{13}C$ values than those found in this study (e.g. Carabel et al. 2006). No direct comparisons of $\delta^{13}C$ values of ophiuroids treated with weak vs. strong acid have been made, but studies using weak acid treatments report up to twice as high $\delta^{13}C$ values (–17 to –10‰ vs. –23 to –18‰) for Arctic ophiuroids (Hobson et al. 2002, Tamelander et al. 2006) compared to studies that used relatively strong acid treatment (Hobson et al. 1995, Iken et al. 2005), which may indicate insufficient carbonate removal by the weaker acid. In contrast, the $\delta^{15}N$ values of Arctic ophiuroids reveal much smaller differences among these studies. The often high lipid content in Arctic zooplankton precludes comparisons of $\delta^{13}C$ values between studies that analyse lipid-intact vs. lipid-extracted samples, as well as between lipid-intact zooplankton and lipid-intact benthic organisms, since zooplankton generally have much higher lipid content than benthic organisms in the Arctic (Graeve et al. 1997). For instance Iken et al. (2005) removed carbonates, but not lipids in their study in the Canada Basin, which gave mean $\delta^{13}C$ values of –26.8‰ for zooplankton (n = 20) and mean $\delta^{13}C$ values of –20.0‰ for benthic organisms (n = 25). To what extent isotopically depleted lipids could explain the difference of 6.7‰ in mean $\delta^{13}C$ values between zooplankton and benthic organisms is not possible to estimate since C:N data were not presented. The surface sediment, however, was not significantly more enriched in ^{13}C than Pelagic-POM or Ice-POM in the Canada Basin (Iken et al. 2005). We highly recommend removing both carbonates and lipids if $\delta^{13}C$ values are used for tracing organisms' carbon sources, particularly if the proportion of Pelagic- vs. Ice-POM is quantified, as is done with a 2-source food web model (e.g. Søreide et al. 2006). Considering pre-treatment of POM samples for estimating representative food web baseline values it may be appropriate to remove lipids, since algae also vary in their lipid content (Doucette & Fryxell 1985, Mock & Gradinger 2000, this study). The increase in $\delta^{15}N$ values after lipid-removal for Ice-POM was only slightly larger than the analytical precision level for $\delta^{15}N$ values in this study, and should thus not lead to any ecologically significant implications for inferences of carbon sources. Differences in carbonate-removal methods were not tested on POM, but all POM samples should be carbonate-

treated prior to stable isotope analysis since POM can contain significant proportions of carbonates. For instance, Carabel et al. (2006) found that untreated POM (i.e. just dried prior to analysis) was >3‰ enriched in ^{13}C compared to carbonate-treated POM. The largest challenge for estimating representative $\delta^{13}C$ or $\delta^{15}N$ values of phytoplankton and ice algae, however, is to collect sufficiently large samples that are representative for these sources and at the same time avoid contamination by heterotrophic microplankton and detritus (e.g. Hamilton et al. 2005, Søreide et al. 2006).

The optimum stable isotope method may be to analyze $\delta^{13}C$ and $\delta^{15}N$ values in separate samples in order to avoid effects of carbonate and lipid removal on $\delta^{15}N$ values. This, however, would require more sample material and would make the analyses more costly and time consuming. When possible, muscle tissue should be prioritized for stable isotope analyses in food web studies. Isolating muscle tissue from small animals may not always be feasible, and analyses are thus often performed on whole organisms. However, typically low $\delta^{13}C$ values of lipids (Sotiropoulos et al. 2004, Sweeting et al. 2006, this study) and potentially low $\delta^{15}N$ values of exoskeletons relative to muscle tissue (Macko et al. 1990, Gorokhova & Hansson 1999) suggest that the interpretation of $\delta^{13}C$ and $\delta^{15}N$ values in crustaceans rich in these compounds may improve from compound-specific analyses. The choice of sample preparation methods and the type of tissue used for analyses, as well as the quality of the POM sample used for determining carbon sources and food web baseline values, are therefore important issues to consider when using stable isotope techniques for determining food web structures.

Acknowledgements. This research was funded by the Norwegian Research Council Norklima Programme, projects: On Thin Ice? 150356-S30 and CABANERA 155936/700. We greatly acknowledge S. Siegle for preparation of samples at IFE and B. Mano and M. Stocki for preparing and running the stable isotope analysis as part of the ECL protocol. We also thank 3 anonymous reviewers for their helpful comments.

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Editorial responsibility: Otto Kinne (Editor-in-Chief), Oldendorf/Luhe, Germany

Submitted: July 20, 2006; Accepted: October 4, 2006
Proofs received from author(s): November 30, 2006