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On the Effects of Acid Pre-treatment on the Elemental and Isotopic Composition of Lightly- and Heavily-calcified Marine Invertebrates

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Abstract – Carbonate removal using acids is a common practice in ecological studies. The effects, however, of acid pre-treatment on the elemental and isotopic composition of marine invertebrates as well as how these effects vary according to species' carbonate content is little known. We examined the effects of acid pre-treatment on the elemental (%C, %N, C:N ratio (%C:%N)) and isotopic composition ($\delta^{13}C$, $\delta^{15}N$) of 28 lightly- and heavily-calcified species from Cnidaria, Mollusca, Arthropoda, Bryozoa, Echinodermata and Chordata. The present study showed that acid pre-treatment modified the elemental and isotopic composition of lightly- and heavily-calcified marine invertebrates. The shifts were clearly seen as a decrease in the %C and $\delta^{13}C$ of heavily-calcified species while we did not detect a clear pattern for %N and $\delta^{15}N$ (in both lightly- and heavily calcified species). Apart from carbonates, acid pre-treatment caused also the loss of organic compounds, thus confounding the interpretation of carbonate proxy (CP) -a widely used proxy for carbonate content. We recommend the use of CP solely with heavily-calcified species. For the first time it was shown that the use of $\delta^{15}N$ values from acidified samples can introduce substantial bias in our perception about the number of trophic levels, the distribution of species and distribution of biomass across the trophic levels in a community. We have uncovered and elucidated previously unknown aspects and highlighted the challenge posed when predicting shifts in elemental and isotopic composition of species following acid pre-treatment. The present findings should be considered in future studies using acid pre-treatment as they can contribute to the optimum use of samples while avoiding bias in the interpretation of findings.

Keywords – acid pre-treatment, carbonate content, elemental composition, stable isotopes, food web

1. Introduction

The elemental and isotopic composition of organisms' organic compounds can provide ecologists with useful information about food-web structure and cycling of organic matter. Specifically, organic carbon and nitrogen content (%C, %N) can be used in biomass estimations while stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$) can reveal species' food sources and relative position in food webs (Frost et al. 2002, 2005; Sterner and Elser 2002; van Oevelen et al. 2009; Layman et al. 2012). In contrast to the organic carbon fractions, the inorganic forms of carbon (i.e. carbonates, $CaCO_3$) do not reflect dietary sources. In addition, they exhibit enriched (i.e. higher) $\delta^{13}C$ values and their presence can confound findings about species' diet and community trophic structure (e.g. Schlacher and Connolly 2014). In consequence, the removal of carbonates is very important when organic %C and $\delta^{13}C$ are investigated while it is not necessary in those studies focusing on organic %N and $\delta^{15}N$ since calcium carbonates do not contain nitrogen (Serrano et al. 2008). For some organisms, carbonate removal can be carried out mechanically (e.g. shells of large-sized bivalves). However, this approach is not always possible for small-sized invertebrates and some Phyla (e.g. echinoderms) (Mateo et al. 2008; Schlacher and Connolly 2014). In these cases, carbonate removal is carried out using acids which leads to the release of carbonates. The most-commonly used acids are hydrochloric (HCl), sulphuric (H_2SO_3) and phosphoric (H_3PO_4) (King et al. 1998; Brodie et al. 2011; Schlacher and Connolly 2014). Carbonate removal using HCl has produced more coherent and reliable $\delta^{13}C$ and C/N data compared to the use of H_2SO_3 and H_3PO_4 (Brodie et al. 2011). Apart from

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the type of acid used, there is also variability in terms of the acid concentration used (e.g. 1–36% in the case of HCl, Brodie et al. 2011 and references therein) and the means of application (e.g. direct application of the acid in the capsule containing the sample vs. acid fumigation, addition of a specific volume of acid vs. the addition of acid until the cessation of effervescence) (Bosley and Wainright 1999; Jacob et al. 2005; Søreide et al. 2006; Jaschinski et al. 2008; Serrano et al. 2008; Vafeiadou et al. 2013).

Apart from the removal of carbonates, acid pre-treatment can affect the organic fractions of carbon and nitrogen (Schlacher and Connolly 2014). This is important since measurements on the elemental and isotopic composition of carbon and nitrogen are often carried out in the same acidified sample to reduce preparation time and analytical cost. The effects of carbonate removal on the organic fractions of carbon and nitrogen are attributed to factors like loss of acid-soluble organic carbon during carbonate dissolution, volatilization of organic compounds, loss of material (e.g. fine particles) during pre-treatment, fractionation of organic matter during acid evaporation, preservation of organic matter with small $\delta^{13}C$ value and limited analytical accuracy due to low C or N recovery in samples (Bunn et al. 1995; Serrano et al. 2008; Schlacher and Connolly 2014 and references therein).

To date, only a few groups of marine invertebrates (i.e. arthropods, molluscs, polychaetes -see Figure 2 in Schlacher and Connolly 2014) have been investigated regarding the effects of carbonate removal on their elemental and isotopic composition. Surprisingly, studies on groups common in marine ecosystems (e.g. echinoderms, bryozoans, ascidians) are absent and thus the extent of changes in their elemental and isotopic composition is unknown. Despite this absence of knowledge, previous works have used the $\delta^{15}N$ values from acidified samples of heavily-calcified organisms (e.g. Mintenbeck et al. 2007; Yokoyama et al. 2009; Iken et al. 2010; Feder et al. 2011; Sokolowski et al. 2014; Divine et al. 2015; Tu et al. 2015). Furthermore, it should be mentioned that previous works examining pre-treatment effects on species' composition were mainly focused on the direction and magnitude of shifts but did not investigate the possible effects on the interpretation of community trophic structure using acidified and non-acidified samples. This is important for $\delta^{15}N$ values since they are used for the calculation of species' trophic level (e.g. Post 2002). In addition, $\delta^{15}N$ values of primary consumers are used as a trophic baseline in food webs (Vander Zanden and Rasmussen 1999; Iken et al. 2010).

The present study is the first one to investigate the effects of acid pre-treatment on the elemental and isotopic composition of marine invertebrates from a large range of carbonate content [from lightly (e.g. arthropods) to heavily-calcified (e.g. echinoderms)] and the possible consequences of these effects on the interpretation of community trophic structure. It was hypothesized that carbonate removal would cause the depletion of %C and $\delta^{13}C$ values -especially in heavily-calcified organisms like echinoderms and calcified bryozoans- while such an effect was not expected for %N and $\delta^{15}N$. In addition, it was hypothesized that no differences in community trophic structure (number of trophic levels, distribution of species' number and biomass across the trophic levels) between the use of acidified and non-acidified $\delta^{15}N$ values would be found.

2. Material and Methods

Study areas and collection of samples

Macro- and megafauna were collected from cold-water coral reefs (CWCRs) in the northeast Atlantic and soft sediments in the east Canadian Arctic (see Table A1 in Appendix). During the cruise "JC073" on board the RRS "*James Cook*" in 2012, fauna living in association with the substratum-forming sponge *Spongosorites coralliophaga* (Stephens, 1915) that colonize coral rubble was collected from Mingulay reef complex and Logachev Mound in the northeast Atlantic using the remotely operated vehicle (ROV) "*Holland I*" (Roberts and Shipboard Party 2013).

The tight sampling schedule during the JC073 "Changing Oceans" expedition (where samples from two cold-water coral reefs were collected) did not allow for immediate taxonomic identification on board. In addition, freezing of specimens was not carried out in order to avoid damage to specimens that would impede the proper taxonomic identification of the specimens. As a response, the collected fauna was preserved in 10% seawater formalin and data were treated following previous studies (Gontikaki et al. 2011; Hunter et al. 2012; Jeffreys et al. 2013; Kazanidis and Witte 2016). Specifically, taking into account the findings from previous studies on the effects of formalin preservation on carbon and nitrogen isotope ratios (Bosley and Wainright 1999; Post 2002; Sarakinos et al. 2002; Fanelli et al. 2010; Ruiz-Cooley et al. 2011; Lau et al. 2012; Rennie et al. 2012; Liu et al. 2013) we acknowledge that the $\delta^{13}C$ and $\delta^{15}N$ values of fauna from cold-water coral reefs may have been distorted due to the chosen method of sample preservation. The possible distortion of stable isotope

ratios due to preservation in formalin could introduce some uncertainty in the examination of a community's trophic structure (i.e. carbon sources, species' trophic level); however, it should be mentioned that a) several studies have shown that the effects of formalin preservation on δ^{15}N values were minor compared to a commonly-used trophic fractionation factor (i.e. +3.4‰, DeNiro and Epstein 1981; Post 2002) enabling thus the allocation of species to trophic levels (Fanelli et al. 2010) and b) a confounding effect on our findings should not be expected since both acidified and non-acidified subsamples have been preserved in formalin. Under these circumstances, we chose to address possible effects of formalin on δ^{13}C values through the addition of 1‰, as in previous studies (Demopoulos et al. 2007; Sweetman and Witte 2008; Gontikaki et al. 2011; Hunter et al. 2012). This approach is in agreement with previous studies where a decrease up to 1‰ in δ^{13}C values due to formalin preservation has been mentioned (Bosley and Wainright 1999; Edwards et al. 2002; Sarakinos et al. 2002; Syväranta et al. 2008; Bicknell et al. 2011; de Lecea et al. 2011; Xu et al. 2011; Lau et al. 2012; Rennie et al. 2012; Liu et al. 2013; González-Bergonzoni et al. 2015).

The species selected for bulk stable isotope analysis were: the cnidarian *Parazoanthus anguicomus* (Norman, 1868), the arthropods *Aristias neglectus* Hansen, 1888, *Janira maculosa* Leach, 1814, *Galathea strigosa* (Linnaeus, 1761), *Scalpellum scalpellum* (Linnaeus, 1767), the molluscs *Asperarca nodulosa* (juvenile) (O. F. Müller, 1776), cf *Tonicella marmorea*, the bryozoans Candidae sp., *Chartella barleei* (Busk, 1860), *Reteporella beaniana* (King, 1846), the echinoderms *Ophiura ophiura* (Linnaeus, 1758), *Ophiothrix fragilis* (Abildgaard, in O.F. Müller, 1789), *Ophioctenella acies* (adult and juveniles) Tyler et al. 1995, *Porania (Porania) pulvillus* (O.F. Müller, 1776) and the ascidian *Polycarpa pomaria* (Savigny, 1816). During expedition "2013 ArcticNet" on board the "CCGS Amundsen" megafauna samples from the east Canadian Arctic were collected using an Agassiz trawl at 5 stations in summer 2013. The stations were located in the Labrador Sea, Baffin Bay, North Water Polynya, Nares Strait and Lancaster Sound. The identification of specimens was feasible on board and thus it was followed by specimens' preservation at –80°C. Selected species included: the cnidarians Anthozoa indet., *Umbellula* sp., the arthropod *Pagurus* sp., the mollusc *Margarites costalis* (Gould, 1841), the echinoderms *Gorgonocephalus* sp., *Ophiacantha bidentata* (Bruzelius, 1805), *Ophiopleura borealis* Danielssen and Koren, 1877, *Ctenodiscus crispatus* (Retzius, 1805), *Pontaster tenuispinus* (Düben and Koren, 1846),

Psilaster andromeda (Müller and Troschel, 1842), *Heliometra glacialis* (Owen, 1833 ex Leach MS) and *Strongylocentrotus droebachiensis* (O.F. Müller, 1776).

Sample preparation for stable isotope analysis

Samples fixed in formalin were dried at 60°C while frozen samples were freeze-dried (0.041 mbar, –49.8°C). The shell of the gastropod *M. costalis* was removed mechanically prior to grinding. However, carbonate removal was applied to the specimens of *M. costalis* due to the difficulty in removing all small shell pieces. Similarly, the shell of *Pagurus* sp. was removed before grinding. Grinding was carried out using the TissueLyser II (Qiagen) or a mortar and pestle according to the size of the organisms. Following grinding, each species was subjected to a preliminary analysis of their C and N content (as % of dry mass) in order to assess the optimal amount of dry mass for dual (δ^{13}C and δ^{15}N) stable isotope ratios analysis. Each sample of macro- and megafauna was divided in two subsamples i.e. subsamples to be acidified or not. Subsamples not to be acidified were placed in tin cups while subsamples to be acidified were placed in silver cups (5 × 8 mm, Elemental Microanalysis UK). Carbonate removal was carried out through the sequential addition of 15 μl of (1M) hydrochloric acid directly in the silver cups using a micropipette; the cessation of the effervescence was used as the criterion that carbonates had been removed (Vafeiadou et al. 2013). The total volume of 1M hydrochloric acid added in each silver cup was recorded (Table A2 in the Appendix). Both acidified and non-acidified samples were dried at 60°C overnight (e.g. Carabel et al. 2006; Jaschinski et al. 2008; Serrano et al. 2008); no washing with distilled water was carried out (Mateo et al. 2008). Overnight drying and no washing with distilled water were also performed for the non-acidified subsamples.

Stable isotope analysis

Samples from cold-water coral reefs were analysed for δ^{13}C and δ^{15}N values at University of California Davis Stable Isotopes Facility (UC Davis SIF) using an Elementar Micro Cube Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples were combusted at 1000°C in a reactor packed with tungsten oxide. During analysis, samples were interspersed with several replicates of at least five laboratory standards which had been previously calibrated against international isotope standards. The long term standard deviation is 0.2‰ for C and 0.3‰ for

N isotopes. Samples from the Canadian Arctic were analysed for C and N isotopes at the James Hutton Institute (JHI, Aberdeen, UK) using a Flash EA 1112 Series Elemental Analyser connected via a Conflo III to a Delta^Plus XP isotope ratio mass spectrometer (all Thermo Finnigan, Bremen, Germany). The isotope ratios were traceable to International Atomic Energy Agency reference materials USGS40 and USGS41 (both L-glutamic acid); certified both for $\delta^{13}C$ (‰$_{VPDB}$) and $\delta^{15}N$ (‰$_{air}$). Long term precisions for a quality control standard (milled flour) were: $\delta^{13}C$ -25.5 ± 0.2‰ and $\delta^{15}N$ $+1.7 \pm 0.4$‰ (mean $\pm$ sd, $n = 200$). The C and N content (as % of dry mass) was calculated based on the area output of the mass spectrometer and the dry mass (mg) analysed. The fact that samples were not preserved in the same way and they were analysed in different laboratories (Mill et al. 2008) does not have a confounding effect on results presented here since effects of carbonate removal were examined through comparisons between subsamples.

Data treatment and statistical analysis

The carbonate content of samples was determined through the carbonate proxy (CP) (Jacob et al. 2005). This proxy is based on the C:N ratio (w:w) of acidified and non-acidified subsamples and is calculated through the following equation:

$$\text{Carbonate proxy} = \frac{[C/N]_{no\ acid}}{[C/N]_{acid}} - 1$$

Statistical differences between acidified and non-acidified subsamples for the parameters %C, %N, C:N ratio, $\delta^{13}C$, $\delta^{15}N$ were examined. The comparisons between acidified and non-acidified subsamples for the elemental and isotopic composition were carried out with the paired Student's t-test or non-parametric Wilcoxon signed rank-test (significance level $p < 0.05$). Beforehand, the normality of the distributions was checked with the Shapiro-Wilk test and equality of variances with the F-test. The correlations between the CP and i) $\delta^{13}C_{no\ acid} - \delta^{13}C_{acid}$, ii) $\delta^{15}N_{no\ acid} - \delta^{15}N_{acid}$, were examined with Spearman's correlation (r_s). Examination of differences and correlations were carried out at the statistical analysis environment R. Only species with at least three replicates were included in the statistical analysis.

Effects of carbonate removal on $\delta^{15}N$ & trophic structure

The effects of carbonate removal were further examined through the comparison of the trophic structure of a benthic community using $\delta^{15}N$ values from acidified and non-acidified samples. The benthic community that was examined is the recently-described association of the cold-water ecosystem engineer *Spongosorites coralliophaga* (Stephens, 1915) and its epifauna (Kazanidis et al. 2016) from cold-water coral reef settings at Mingulay reef complex (Mingulay reef hereafter; Outer Hebrides Sea, North-East Atlantic) and Logachev mound (Rockall Bank, North-East Atlantic) (Table A1 in Appendix). *S. coralliophaga* is a massive sponge that hosts a species-rich epifaunal community of more than 70 species (see Kazanidis et al. 2016 for details). This community is composed from four trophic levels both in Mingulay reef and Logachev mound (Kazanidis and Witte 2016). The trophic level of epifaunal species was calculated using $\delta^{15}N$ values from acidified and non-acidified sub-samples while the $\delta^{15}N$ values of two primary consumers (PC) were used as a trophic baseline. The following equation was used (following Iken et al. 2010):

$$TP_{(PC)} = \left(\frac{\delta^{15}N_{consumer} - \delta^{15}N_{primary-consumer}}{3.4} \right) + 2$$

where +3.4‰ is a commonly-used trophic fractionation factor for $\delta^{15}N$ between successive trophic levels (DeNiro and Epstein 1981; Minagawa and Wada 1984; Post 2002). Specifically, the trophic baselines used were the $\delta^{15}N$ values (acidified vs. non-acidified) from *Parazoanthus anguicomus* ($\delta^{15}N$ values of non-acidified samples from Mingulay reef were +8.6‰, $\delta^{15}N$ values of acidified samples from Mingulay reef were +8.5‰, $\delta^{15}N$ values of non-acidified samples from Logachev mound were +8.5‰, $\delta^{15}N$ values of acidified samples from Logachev mound were +8.3‰) and *Reteporella beaniana* ($\delta^{15}N$ values of non-acidified samples from Mingulay reef were +8.7‰, $\delta^{15}N$ values of acidified samples from Mingulay reef were +7.3‰, $\delta^{15}N$ values of non-acidified samples from Logachev mound were +9.1‰, $\delta^{15}N$ values of acidified samples from Logachev mound were +7.8‰). The effects of carbonate removal on our perception about the trophic structure was examined in terms of a) number of trophic levels and b) species/biomass distribution across the trophic levels (see Kazanidis and Witte 2016).

3. Results and Discussion

Effects of carbonate removal on elemental composition

In 14 taxa there was a statistically-significant decrease in their %C (average values ranging from -1.3 ± 0.4 in *Margarites costalis* to -10.2 ± 0.8 in *Ophiopleura borealis*) while non-statistically-significant shifts were found in 4 taxa (Table 1).

Table 1. Effects of acidification (acidified minus non-acidified) on the elemental composition (%C, %N, C:N)

Species	Phylum	CP	%C shift (mean ± S.D)	Paired test	%N shift (mean ± S.D)	Paired test	C:N shift (mean ± S.D)	Paired test	n
Anthozoa indet.[a]	CNI	0.3 ± 0.1	-5.5 ± 1.7	7.8[†],***	0.0 ± 0.2	0.4[†], ns	-1.4 ± 0.6	6.2[†],**	6
Parazoanthus anguicomus[b]	CNI	0.2 ± 0.1	-2.6 ± 2.8	33[#],*	0.0 ± 0.9	18[#], ns	-0.6 ± 0.5	3.3[†],*	8
Umbellula sp.[a]	CNI	0.2 ± 0.0	-5.1 ± 0.9	NA	-0.3 ± 0.2	NA	-0.7 ± 0.0	NA	2
Phylum average	CNI	0.2 ± 0.1	-4.0 ± 2.6	133[#],***	-0.1 ± 0.7	0.4[†], ns	-0.9 ± 0.6	224[#],***	16
Aristias neglectus[b]	ART	0.2 ± 0.2	-1.9 ± 13.8	0.7[†], ns	+0.3 ± 1.0	-0.1[†], ns	-0.5 ± 1.5	1.0[†], ns	3
Galathea strigosa[b]	ART	0.1 ± 0.2	-1.8 ± 3.8	0.9[†], ns	+0.4 ± 0.6	-1.2[†], ns	-0.6 ± 1.0	1.4[†], ns	4
Janira maculosa[b]	ART	0.1	-13.8	NA	-2.6	NA	-0.4	NA	1
Pagurus sp.[a]	ART	0.1	-4.4	NA	-0.4	NA	-0.4	NA	1
Scalpellum scalpellum[b]	ART	0.4	-1.4	NA	+0.8	NA	-1.7	NA	2
Phylum average	ART	0.2 ± 0.2	-4.2 ± 7.9	1.8[†], ns	0.0 ± 1.2	-0.1[†], ns	-0.9 ± 1.0	2.9[†],*	11
Asperarca nodulosa juv.[b]	MOL	2.6	-11.1	NA	-0.3	NA	-8.9	NA	1
Margarites costalis[a]	MOL	0.1 ± 0.0	-1.3 ± 0.4	6.2[†],*	+0.2 ± 0.3	-0.9[†], ns	-0.2 ± 0.1	6[#], ns	3
cf Tonicella marmorea[b]	MOL	0.0	-19.8	NA	-5.4	NA	0.0	NA	1
Phylum average	MOL	0.6 ± 1.1	-7.0 ± 8.3	15[#], ns	-1.1 ± 2.5	9.5[#], ns	-1.9 ± 3.9	10[#], ns	5
Candidae sp.[b]	BRY	1.7 ± 0.2	-7.2 ± 0.1	124.7[†],***	+0.1 ± 0.2	-1.5[†], ns	-6.3 ± 1.0	10.3[†],**	3
Chartella barleei[b]	BRY	0.2 ± 0.1	-2.9 ± 0.2	25.1[†],**	0.0 ± 0.1	-2.6[†], ns	-0.8 ± 0.3	4.6[†],*	3
Reteporella beaniana[b]	BRY	NA	NA	NA	-0.1 ± 0.2	1[#], ns	NA	NA	3
Phylum average	BRY	0.9 ± 0.8	-5.0 ± 2.4	5.2[†],**	0.0 ± 0.2	-0.2[†], ns	-3.6 ± 3.1	2.8[†],*	9
Ctenodiscus crispatus[a]	ECH	0.8 ± 0.4	-9.1 ± 1.4	15.9[†],***	-0.4 ± 0.4	2.4[†], ns	-3.1 ± 1.1	7.2[†],***	6
Gorgonocephalus sp.[a]	ECH	1.0	-7.6	NA	-0.1	NA	-3.7	NA	2
Heliometra glacialis[a]	ECH	0.5 ± 0.3	-9.9 ± 1.1	20.6[†],***	-1.0 ± 0.5	4.3[†],*	-2.0 ± 1.1	4.2[†],*	5
Ophiacantha bidentata[a]	ECH	0.8 ± 0.2	-10.4 ± 0.4	10[#], ns	-0.8 ± 0.3	6.3[†],**	-2.9 ± 0.7	7.9[†],**	4
Ophioctenella acies[b]	ECH	1.1 ± 0.1	-8.0 ± 0.6	22.6[†],**	0.0 ± 0.1	0[†], ns	-4.4 ± 0.4	6[#], ns	3
Ophioctenella acies juv.[b]	ECH	1.4	-7.9	NA	+0.1	NA	-5.6	NA	1
Ophiopleura borealis[a]	ECH	1.1 ± 0.7	-10.2 ± 0.8	120[#],***	-0.6 ± 0.4	6.3[†],***	-4.3 ± 2.4	120[#],***	15
Ophiothrix fragilis[b]	ECH	1.0 ± 0.1	-6.8 ± 2.3	5.0[†],*	+0.3 ± 0.5	-0.8[†], ns	-4.4 ± 0.8	9.1[†],*	3
Ophiura ophiura[b]	ECH	0.7 ± 0.2	-9.4 ± 2.8	8.5[†],***	-0.4 ± 0.5	10[#], ns	-2.8 ± 0.5	12.8[†],***	6
Pontaster tenuispinus[a]	ECH	0.8 ± 0.7	-10.1 ± 1.2	18.7[†],***	-0.9 ± 0.6	3.5[†],*	-3.2 ± 2.4	3.0[†],*	5
Porania pulvillus[b]	ECH	0.9	-7.3	NA	+0.2	NA	-2.9	NA	1
Psilaster andromeda[a]	ECH	0.8 ± 0.1	-8.7 ± 0.7	21.1[†],**	-0.3 ± 0.1	5.2[†],*	-2.9 ± 0.3	6[#], ns	3
Strongylocentrotus droebachiensis[a]	ECH	1.3 ± 0.4	-9.0 ± 0.7	27.1[†],***	-0.3 ± 0.2	10[#], ns	-5.0 ± 1.0	11.1[†],***	5
Phylum average	ECH	0.9 ± 0.5	-9.4 ± 1.6	1770[#],***	-0.5 ± 0.5	7.5[†],***	-3.6 ± 1.7	1770[#],***	59
Polycarpa pomaria[b]	CHO	0.0 ± 0.1	-0.2 ± 1.7	10[#], ns	0.0 ± 0.5	6[#], ns	0.0 ± 0.7	10[#], ns	5

[a]Arctic; [b]cold-water coral reefs; *n*: number of replicates; Carbonate proxy (CP); cnidarians (CNI); arthropods (ART); molluscs (MOL); bryozoans (BRY); echinoderms (ECH); chordata (CHO). Mean values, standard deviations (S.D), results of statistical test ([†]parametric paired *t*-test; [#]non-parametric paired Wilcoxon test) and *P* values (***$P \leq 0.001$, **$0.001 < P \leq 0.01$, *$0.01 < P \leq 0.05$, ns: no significant) are given

The highest decreases in %C were found in echinoderms (-9.4 ± 1.6, $P < 0.001$) and bryozoans (-5.0 ± 2.4, $P < 0.01$) (i.e. in organisms with carbonate proxy values higher than +0.5). In a previous study by Serrano et al. (2008) on beach arthropods the decrease in %C was attributed to the loss of both inorganic (i.e. carbonates) and organic carbon after carbonate removal. Loss of organic carbon may have also played a role in the %C decrease in species with low carbonate content and especially in the isopod *Janira maculosa* and the chiton cf *Tonicella marmorea* where the decrease in %C was comparable to that found in echinoderms (Table 1). For those species with high carbonate content (e.g. echinoderms, calcified bryozoans; average CP values +0.9 in both Phyla; Table 1) it is suggested that the significant decrease ($P < 0.01$) in %C was driven by the removal of carbonates.

As regards %N, echinoderms were the only group where a

statistically-significant decrease was found (i.e. 5 out of 19 species, average values ranging from -0.3 ± 0.1 in *Psilaster andromeda* to -1.0 ± 0.5 in *Heliometra glacialis*). Based on this, we recommend that future studies examining %N in heavily-calcified species should avoid the acid pre-treatment of samples to avoid any miscalculations of %N and C:N ratios. Non statistically-significant shifts were found in 14 out of the 19 taxa (Table 1). In the arthropods *Aristias neglectus*, *Galathea strigosa*, the mollusc *Margarites costalis* and the echinoderm *Ophiothrix fragilis* there was a non-significant increase ($P >$ 0.05) in their %N, which was probably due to the preferential removal of organic compounds depleted in nitrogen content (Bunn et al. 1995). The absence of a uniform trend regarding the effects of carbonate removal on %N is in agreement with previous studies which have revealed a decrease (Serrano et al. 2008), an increase (Bunn et al. 1995) or no significant impact (Mazumder et al. 2010).

In 67% of the species, there was a statistically-significant decrease in the C:N ratio, which shows a proportionally-higher loss of carbon than nitrogen (Table 1) as well as the differences between carbon and nitrogen as regards their response to carbonate removal using hydrochloric acid. The decrease in the C:N ratio was higher in heavily-calcified species i.e. calcified bryozoans and echinoderms (–3.6%) than lightly-calcified species i.e. cnidarians and arthropods (–0.9%). The proportionally higher loss of carbon than nitrogen in the heavily-calcified species played a major role in the formulation of their high CP values; specifically, the CP values in echinoderms and bryozoans were among the highest that have been recorded up to now in marine invertebrates (Jacob et al. 2005; Ng et al. 2007; Kolasinski et al. 2008; Serrano et al. 2008).

The mean CP values in the vast majority of species were positive; however, a small number of samples (3 out of 102) showed negative values. Interestingly in *Aristias neglectus* (CP = –0.1), *Galathea strigosa* (CP = –0.1) and *Polycarpa pomaria* (CP = –0.2) the negative CP was due to an increase in the acidified subsamples of the %C accompanied by an increase or decrease in %N. In previous studies negative CP values were attributed to a proportionally higher loss of %N than %C in the acidified subsamples (Jacob et al. 2005; Kolasinski et al. 2008; Serrano et al. 2008); however, here it is shown that negative CP can also arise through alternative pathways [i.e. increase in %C and %N in acidified subsamples through loss of molecules comparatively low in %C and %N (Bunn et al. 1995) or decrease in %N through the volatilization of organic compounds (King et al. 1998; Lohse et al. 2000;

Serrano et al. 2008)]. This indicates that the CP should be used mainly with heavily-calcified species whereas in the lightly-calcified ones it should be used with caution. While the CP may not always represent the effects of carbonate removal on carbonates, it can sometimes reveal the effects on organic carbon and nitrogen (see also Serrano et al. 2008).

Effects of carbonate removal on isotopic composition

In 10 taxa there was a statistically-significant decrease in their δ^{13}C (average values ranging from -3.6 ± 2.0 in Anthozoa indet. to -10.5 ± 1.2 in the bryozoan Candidae sp.) while non-statistically-significant shifts were found in 8 taxa (Table 2). The highest decreases in δ^{13}C were found in bryozoans (-6.7 ± 4.4, $P < 0.01$) (Fig. 1g) and echinoderms (-8.1 ± 2.8, $P < 0.001$) (Fig. 1i) i.e. in organisms with carbonate proxy values higher than +0.5 and the least decreases in cnidarians (-2.4 ± 2.1, $P <$ 0.001) (Fig. 1a; Table 2). Shifts in δ^{13}C values recorded in the present study (i.e. up to -14.7‰ in *Asperarca nodulosa*) (Fig. 1e) are -to the best of our knowledge- the highest ones that have been recorded up to now between acidified and non-acidified subsamples from marine invertebrates (Schlacher and Connolly 2014 and references therein). In *Parazoanthus anguicomus*, *Aristias neglectus* and *Chartella barleei* there were not significant differences in δ^{13}C between acidified and non-acidified subsamples (-1.4 ± 2.1, $P = 0.09$, +0.7 ± 0.5, $P = 0.25$ and -2.8 ± 1.8, $P = 0.25$, respectively) but the mean shift in their δ^{13}C exceeded the range of +0.5‰ to +1.0‰, which is regarded as the shift between consumers and their prey (Post 2002; McCutchan et al. 2003). Similarly, the asteroid *Pontaster tenuispinus* did not show significant differences between acidified and non-acidified δ^{13}C values; however, this result should be treated with caution since the P-value (0.06) was marginally higher than 0.05. Based on our findings about the absence of statistically-significant shifts in δ^{13}C values in those species mentioned above, we recommend that researchers should be careful about omitting carbonate removal in lightly-calcified species (see also Yokoyama et al. 2005; Vafeiadou et al. 2013).

As regards the effects of carbonate removal on δ^{15}N, there were statistically-significant shifts in 5 out of the 19 taxa ranging from -0.4 ± 0.3‰ in the ascidian *Polycarpa pomaria* ($P < 0.05$) to -0.8 ± 0.6‰ in the sea urchin *Strongylocentrotus droebachiensis* ($P < 0.05$). In two species (i.e. the echinoderms *Ophiacantha bidentata* and *Ophiopleura borealis*), the significant decrease in δ^{15}N was accompanied by a significant decrease in %N; it is possible that the high volume of hydrochloric

Table 2. Effects of acidification (acidified minus non-acidified) on $\delta^{13}C$, $\delta^{15}N$

| Species	Phylum	CP	$\delta^{13}C$ shift (mean ± S.D)	Paired test	$\delta^{15}N$ shift (mean ± S.D)	Paired test	n
Anthozoa indet.[a]	CNI	+0.3 ± 0.1	-3.6 ± 2.0	4.4[†],**	-0.2 ± 0.4	1.7[†], ns	6
Parazoanthus anguicomus[b]	CNI	+0.2 ± 0.1	-1.4 ± 2.1	1.9[†], ns	-0.2 ± 0.4	1.7[†], ns	8
Umbellula sp.[a]	CNI	+0.2 ± 0.0	-2.3	NA	-1.1	NA	2
Phylum average	CNI	+0.2 ± 0.1	-2.4 ± 2.1	133[♯],***	-0.3 ± 0.5	117.5[♯],*	16
Aristias neglectus[b]	ART	+0.2 ± 0.2	+0.7 ± 0.5	0[♯], ns	+0.3 ± 0.5	-0.6[†], ns	3
Galathea strigosa[b]	ART	+0.1 ± 0.2	-0.4 ± 1.5	0.5[†], ns	0.0 ± 0.4	-0.1[†], ns	4
Janira maculosa[b]	ART	+0.1	+0.6	NA	0.0	NA	1
Pagurus sp.[a]	ART	+0.1	-1.8	NA	0.0	NA	1
Scalpellum scalpellum[b]	ART	+0.4	-6.6 ± 2.0	NA	-0.8 ± 0.8	NA	2
Phylum average	ART	+0.2 ± 0.2	-1.3 ± 2.9	1.4[†], ns	-0.1 ± 0.6	0.3[†], ns	11
Asperarca nodulosa juv.[b]	MOL	+2.6	-14.7	NA	-0.3	NA	1
Margarites costalis[a]	MOL	+0.1 ± 0.0	-0.1 ± 0.2	1[†], ns	-0.7 ± 0.1	9.5[†],*	3
cf Tonicella marmorea[b]	MOL	0.0	-1.2	NA	-0.4	NA	1
Phylum average	MOL	+0.6 ± 1.1	-3.2 ± 6.4	6[♯], ns	-0.5 ± 0.2	6.5[†],**	5
Candidae sp.[b]	BRY	+1.7 ± 0.2	-10.5 ± 1.2	14.4[†],**	-0.3 ± 0.1	3.0[†], ns	3
Chartella barleei[b]	BRY	+0.2 ± 0.1	-2.8 ± 1.8	6[♯], ns	-0.3 ± 0.2	2.8[†], ns	3
Reteporella beaniana[b]	BRY	NA	NA	NA	-1.4 ± 0.8	2.8[†], ns	3
Phylum average	BRY	+0.9 ± 0.8	-6.7 ± 4.4	36[♯],**	-0.7 ± 0.7	2.8[†],*	9
Ctenodiscus crispatus[a]	ECH	+0.8 ± 0.4	-6.4 ± 1.6	9.6[†],***	-0.3 ± 0.4	1.4[†], ns	6
Gorgonocephalus sp.[a]	ECH	+1.0	-8.4 ± 0.8	NA	-0.4 ± 0.3	NA	2
Heliometra glacialis[a]	ECH	+0.5 ± 0.3	-4.6 ± 1.4	7.4[†],**	-0.3 ± 0.4	10[♯], ns	5
Ophiacantha bidentata[a]	ECH	+0.8 ± 0.2	-9.2 ± 0.6	32.5[†],***	-0.5 ± 0.2	4.6[†],*	4
Ophioctenella acies[b]	ECH	+1.1 ± 0.1	-10.2 ± 0.9	18.9[†],**	-0.1 ± 0.5	4[♯], ns	3
Ophioctenella acies juv.[b]	ECH	+1.4	-12.1	NA	-0.3	NA	1
Ophiopleura borealis[a]	ECH	+1.1 ± 0.7	-9.6 ± 2.7	13.8[†],***	-0.5 ± 0.3	7.1[†],***	15
Ophiothrix fragilis[b]	ECH	+1.0 ± 0.1	-10.7 ± 1.8	6[♯], ns	-0.6 ± 0.6	1.8[†], ns	3
Ophiura ophiura[b]	ECH	+0.7 ± 0.2	-6.6 ± 1.5	10.8[†],***	+0.3 ± 0.6	-1.1[†], ns	6
Pontaster tenuispinus[a]	ECH	+0.8 ± 0.7	-7.6 ± 2.1	15[♯], ns	-0.2 ± 0.5	1.3[†], ns	5
Porania pulvillus[b]	ECH	+0.9	-3.2	NA	-0.4	NA	1
Psilaster andromeda[a]	ECH	+0.8 ± 0.1	-4.0 ± 0.5	13.9[†],**	-0.8 ± 0.3	6[♯], ns	3
Strongylocentrotus droebachiensis[a]	ECH	+1.3 ± 0.4	-10.4 ± 1.5	15.7[†],***	-0.8 ± 0.6	2.9[†],*	5
Phylum average	ECH	+0.9 ± 0.5	-8.1 ± 2.8	1770[♯],***	-0.4 ± 0.5	1482[♯],***	59
Polycarpa pomaria[b]	CHO	+0.0 ± 0.1	-0.1 ± 0.4	0.9[†], ns	-0.4 ± 0.3	3.6[†],*	5

[a]Arctic; [b]cold- water coral reefs; *n*: number of replicates. Carbonate proxy (CP); cnidarians (CNI); arthropods (ART); molluscs (MOL); bryozoans (BRY); echinoderms (ECH); chordata (CHO). Mean values, standard deviations (S.D), results of statistical test ([†]parametric paired *t*-test; [♯]non-parametric paired Wilcoxon test) and *P* values (***$P \leq 0.001$, **$0.001 < P \leq 0.01$, *$0.01 < P \leq 0.05$, ns: no significant) are given

acid added until the cessation of the effervescence (i.e. approx. 200 μl, see Table A2 in Appendix) caused a substantial loss of organic nitrogen. On the other hand, the significant decrease in the $\delta^{15}N$ of the three others (i.e. the ascidian *Polycarpa pomaria*, the mollusc *Margarites costalis* and the sea urchin *Strongylocentrotus droebachiensis*) was not followed by a significant decrease in their %N. This is particularly interesting for *P. pomaria* and *M. costalis* since the volume of hydrochloric acid added for their carbonate removal (average values were

60 and 40 μl, respectively) was much smaller than the amount used for the carbonate removal in species whose $\delta^{15}N$ values were not significantly affected by carbonate removal (e.g. the bryozoan Candidae sp., the echinoderm *Psilaster andromeda*) (see Table A2 in Appendix for hydrochloric acid added in each species and Table 2 in main text for shifts in $\delta^{15}N$ values). It is possible that the loss of a small amount of organic nitrogen-containing compounds with enriched $\delta^{15}N$ values contributed to the findings for these two species (see also Goering et al.

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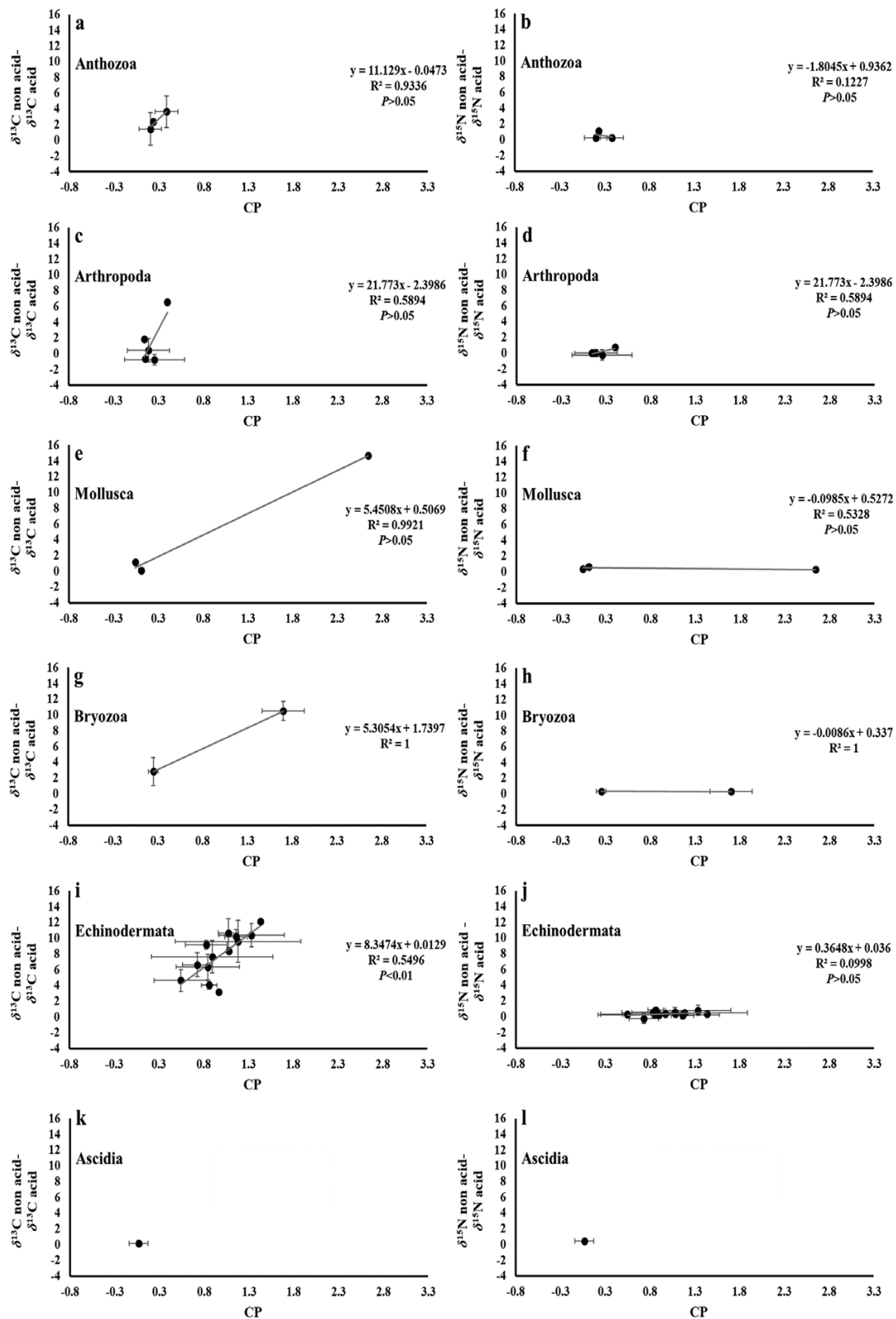
Fig. 1. (a, c, e, g, i, k) Relationship between the carbonate proxy (CP) and the shift in δ^{13}C values between acidified and non-acidified subsamples for each of the taxonomic groups; (b, d, f, h, j, l). Relationship between the carbonate proxy (CP) and the shift in δ^{15}N values between non-acidified and acidified subsamples for each of the taxonomic groups. Mean values, standard deviations, correlation coefficients (R^2) and P values are given

1990; Wolf et al. 2009).

There was no relationship between the carbonate proxy (CP) and shifts in $\delta^{15}N$ (Figs. 1b for anthozoans; 1d for arthropods; 1f for molluscs; 1j for echinoderms). These results are in agreement with previous findings about arthropods (Bosley and Wainright 1999; Fantle et al. 1999; Yokoyama et al. 2005; Carabel et al. 2006; Serrano et al. 2008; but see also Bunn et al. 1995) and various invertebrates from a tropical reef (Kolasinski et al. 2008) and from seagrass beds (Jaschinski et al. 2008; Vafeiadou et al. 2013).

Effects of carbonate removal on the interpretation of trophic structure

Studies on trophic structure of marine communities often use the $\delta^{15}N$ values of suspended or sedimented particulate organic matter as a trophic baseline (e.g. Grall et al. 2006; Iken et al. 2010; Divine et al. 2015). However, the high variability in the $\delta^{15}N$ values of primary producers introduces substantial variability in the calculations of the trophic level of species and comparisons of trophic structure between areas (Cabana and Rasmussen 1996; Vander Zanden and Rasmussen 1999; Post 2002; Vander Zanden and Fetzer 2007; Iken et al. 2010).

In the present study, the use of $\delta^{15}N$ values from acidified subsamples from primary consumers resulted in miscalculations for the number of trophic levels, distribution of species and distribution of biomass across the trophic levels. Specifically, at Mingulay reef, the use of $\delta^{15}N$ values from acidified subsamples from *Reteporella beaniana* as a baseline increased the number of trophic levels from four to five (Fig. 2a and b). This was due to the decrease of *R. beaniana's* $\delta^{15}N$ value from +8.7‰ in non-acidified subsamples ($n = 1$) to +7.3‰ in acidified subsamples ($n = 1$). The use of $\delta^{15}N$ values from acidified subsamples of *R. beaniana* resulted in miscalculations also in Logachev mound. Specifically, the use of acidified subsamples resulted in a higher number of species and higher biomass in
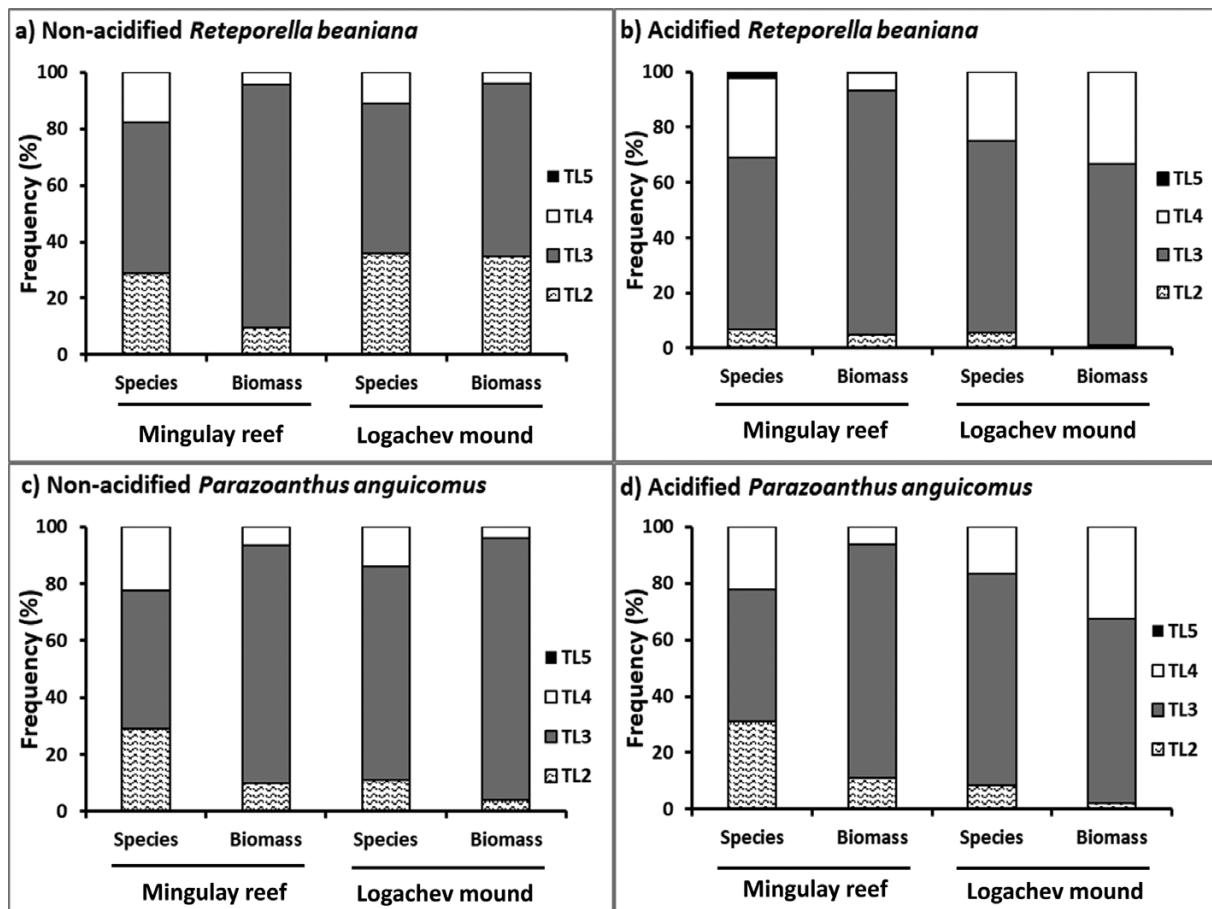
Fig. 2. Distribution (expressed as frequency %) of species and biomass across trophic levels (TL) using as a trophic baseline the $\delta^{15}N$ value of non-acidified and acidified specimens of the bryozoan *Reteporella beaniana* (a, b) and non-acidified and acidified specimens of the anthozoan *Parazoanthus anguicomus* (c, d) from Mingulay reef and Logachev mound

the third and fourth trophic levels. This was due to the decrease in the $\delta^{15}N$ from +9.1 ($n = 2$) in non-acidified subsamples to +7.8‰ in acidified subsamples ($n = 2$) (Fig. 2a and b). In contrast to findings for *R. beaniana*, the use of $\delta^{15}N$ values from acidified subsamples of *Parazoanthus anguicomus* as a baseline did not lead to miscalculations about the number of trophic levels, the distribution of species and the distribution of biomass across the trophic levels (Fig. 2c and d). This is because the acid treatment had a smaller impact on the $\delta^{15}N$ values of *P. anguicomus* than *R. beaniana* (see Table 2 for details).

The findings described above provided evidence that the use of $\delta^{15}N$ values from acidified subsamples as a trophic baseline can alter substantially our perception about the trophic structure of a community. In our case this was due to the miscalculation (underestimation) of the trophic baseline when $\delta^{15}N$ values from acidified *R. beaniana* were used. Taking these findings into account it is likely that the use of $\delta^{15}N$ values from acidified specimens in previous studies (e.g. Mintenbeck et al. 2007; Yokoyama et al. 2009; Iken et al. 2010; Feder et al. 2011; Sokolowski et al. 2014; Divine et al. 2015; Tu et al. 2015) may have introduced some bias in the assessment of community trophic structure.

Conclusions and recommendations for future work

The present study showed that the removal of carbonates through acid pre-treatment can modify the elemental and isotopic composition of both lightly- and heavily-calcified marine invertebrates. Apart from the removal of carbonates, acid pre-treatment removed also organic carbon and nitrogen. This loss of organic matter affected the carbonate proxy (CP) values, thus confounding the interpretation of species' CP. Based on our findings we recommend that this proxy should be used with heavily-calcified species.

Furthermore, the present study provided evidence that the use of $\delta^{15}N$ values from acidified samples can introduce substantial bias in our perception about the trophic structure of a community. This is the first time that the effects of acid pre-treatment are examined within such a framework and the results of our analysis should be taken seriously into account in future food-web studies.

The effects of carbonate removal varied among Phyla with heavily-calcified organisms like echinoderms and calcified bryozoans being the most affected in terms of carbon content (%C) and $\delta^{13}C$. On the other hand, we did not detect such a trend for nitrogen content (%N) and $\delta^{15}N$. The different

sensitivity to carbonate removal between elements and among taxonomic groups, and thus the difficulties arising in predicting the shifts in elemental and isotopic composition following acid pre-treatment, should be taken into account in future ecological and biogeochemical works studying the elemental (%C, %N, C:N ratio) and isotopic composition ($\delta^{13}C$, $\delta^{15}N$) of marine invertebrates. This will contribute to the optimum use of available samples and prevent bias in the interpretation of findings.

Acknowledgments

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indicate if changes were made.

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Appendix

Table A1. Locations of sample collection in the east Canadian Arctic and cold-water coral reefs in the northeast Atlantic

	Latitude	Longitude	Depth (m)
Canadian Arctic			
Labrador Sea (633)	57°36.49'N	61°53.98'W	187
Baffin Bay (176)	69°35.33'N	65°47.11'W	200
Nares Strait (253b)	79°19.26'N	71°24.76'W	201
North Water Polynya (117)	77°18.80'N	76°57.40'W	475
Lancaster Sound (301)	74°06.38'N	83°20.62'W	693
Cold-water coral reefs			
Mingulay reef Complex	56°49.3'N	7°23.6'W	122–131
Logachev mound	55°29.6'N	15°49.1'W	683–800

Table A2. Volume of 1M hydrochloric acid (HCl) (µl) used for the carbonate removal of each species

Species	Phylum	HCl added (mean ± S.D)	n	Species	Phylum	HCl added (mean ± S.D)	n
Anthozoa indet.[a]	CNI	63.0 ± 14.8	6	*Gorgonocephalus* sp.[a]	ECH	210.0 ± 0.0	2
Parazoanthus anguicomus[b]	CNI	45.00 ± 0.0	8	*Heliometra glacialis*[a]	ECH	213.0 ± 52.4	5
Umbellula sp.[a]	CNI	60.0 ± 0.0	2	*Ophiacantha bidentata*[a]	ECH	187.5 ± 26.0	4
Aristias neglectus[b]	ART	60.0 ± 0.0	3	*Ophioctenella acies*[b]	ECH	150.0 ± 0.0	3
Galathea strigosa[b]	ART	60.0 ± 0.0	4	*Ophioctenella acies* juveniles[b]	ECH	165.0	1
Janira maculosa[b]	ART	60.0	1	*Ophiopleura borealis*[a]	ECH	212.0 ± 29.4	15
Pagurus sp.[a]	ART	75.0	1	*Ophiothrix fragilis*[b]	ECH	130.0 ± 8.7	3
Scalpellum scalpellum[b]	ART	60.0 ± 0.0	2	*Ophiura ophiura*[b]	ECH	110.0 ± 12.3	6
Asperarca nodulosa juveniles[b]	MOL	300.0	1	*Pontaster tenuispinus*[a]	ECH	210.0 ± 97.2	5
Margarites costalis[a]	MOL	40.0 ± 17.3	3	*Porania pulvillus*[b]	ECH	150.0	1
Candidae sp.[b]	BRY	140.0 ± 17.3	3	*Psilaster andromeda*[a]	ECH	100.0 ± 8.7	3
Chartella barleei[b]	BRY	60.0 ± 0.0	3	*Strongylocentrotus droebachiensis*[a]	ECH	246.0 ± 13.4	5
Reteporella beaniana[b]	BRY	135.0 ± 54.1	3	*Polycarpa pomaria*[b]	ASC	60.0 ± 0.0	5
Ctenodiscus crispatus[a]	ECH	155.0 ± 40.0	6				

CNI: cnidarians; ART: arthropods; MOL: molluscs; BRY: bryozoans; ECH: echinoderms; ASC: ascidians; [a]Arctic; [b]cold-water coral reefs; Mean values and standard deviations are given. *n*: number of replicates.