The following supplement accompanies the article

Long-term decline in the trophic level of megafauna in the deep Mediterranean Sea: a stable isotopes approach

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Supplementary material

Meta-analysis methods

We searched the ISI Web of Knowledge electronic data base (1983–2014, http://portal.isiknowledge.com) for literature involving the effects of preservatives on the stable carbon and nitrogen isotopic compositions of aquatic species, using the keywords "stable isotope", "stable carbon", "stable nitrogen", "carbon-13", "nitrogen-15", "preservatives", "preservation", "fixative", "formalin", "formaldehyde", "ethanol" and "alcohol". Data available only in a graphical form were converted to a numerical form following fourfold enlargement of the graphs (estimated error = 0·05‰). For the calculation of effect sizes, only data containing means, samples sizes and standard deviations for both the experimental (treatment) and control groups, or data presented as two x two contingency tables, or finally data whose summary statistics can be transformed and represented as a correlation coefficient, were used. Thus, we excluded studies in which the numbers of replicates and/or variances were unavailable and could not be calculated because these parameters are needed for the meta-analyses.

To test whether estimates of $\delta^{15}N$ or $\delta^{13}C$ varied consistently according to different sources of variation, a random-effects meta-analysis model was used (Gurevitch and Hedges 1993; 1999). Under a random-effects model we allow that the

true magnitude of an effect could vary from study to study: that is, there is not a uniquely true effect that is shared by all the included studies as in a fixed-effect model (e.g. the effect might be a little greater if the study used a longer time of preservation, or if the effect were measured more reliably, etc.). The studies included in the meta-analysis are assumed to be a random sample of the relevant distribution of effects, and the combined effect estimates the mean of this distribution (Borenstein et al. 2007).

For isotopic ratios, the effect of each of the pre-defined categories ("taxon" meaning fishes and other invertebrates including decapods and "habitat" considering only marine organisms) was considered fixed, with observations (i.e., the $\delta^{15}N$ or the $\delta^{13}C$ values) within each category considered random. The resulting effect sizes for $\delta^{15}N$ and $\delta^{13}C$ were used to correct the stable isotope values of *'80s period* specimens. Meta-analyses were run with the R library *meta* (Chen and Peace 2013) in R 3.1.1.

Results

Meta-analyses carried out on literature data concerning formalin preservation found no statistically significant effect of preservation on $\delta^{15}N$, whereas some effect was significantly great for $\delta^{13}C$ (Table S1). On the other hand, the meta-analysis for the effect of ethanol on the isotopic composition of preserved samples showed a significant effect on $\delta^{15}N$ (though small) and no significant effect for $\delta^{13}C$ (Table S1). Thus we are confident that the effect magnitude used to correct the *'80s period* isotopic data (preserved specimens) gives "corrected" values that can be compared with *current period* data (frozen specimens). Nevertheless, the significant effect found for $\delta^{13}C$ values using normalized data (i.e. values corrected with the effect level returned by the meta-analysis) should be interpreted with caution, due to the species-specific variations caused by formalin preservation reported in literature (see the above cited references). The correction factors we used for the *'80s period* isotopic data were applied with the

effect levels resulting from the separate meta-analyses carried out on fish and invertebrates The factors selected were the most conservative among those derived (including both freshwater and marine species, see Table S1).

Discussion

Preservation techniques differ depending on the original intent of tissue archival, and generally encompass immersion in formalin and/or ethanol, as well as freezing. Correctly predicting effects of solvents on chemical tracers would allow for the use of archived samples in long-term studies of trophic ecology of marine and aquatic species.

The results of our meta-analysis showed a significant effect of formalin preservation on $\delta^{13}C$, indicating ^{13}C-depletion in preserved specimens with respect to controls, while a small, non-significant, change was observed for $\delta^{15}N$ values (preserved specimens showing a slight ^{15}N-enrichment). Ethanol only marginally affected the isotopic composition of species, and its mean effect size was found to be quite small both for fish (effect size – ES – for $\delta^{15}N$=0.46; ES for $\delta^{13}C$=0.20) and invertebrates (ES for $\delta^{15}N$=0.31; ES for $\delta^{13}C$=0.15), confirming the general trend reported in the literature (see Fanelli et al. 2010 and references cited therein).

The effect levels provided by meta-analyses were used to correct both $\delta^{13}C$ and $\delta^{15}N$ values. However, considering the results of meta-analyses, and that several authors (Mullin et al. 1984; Sarakinos et al. 2002; Feuchtmayr and Grey 2003; Fanelli et al. 2010; Syväranta et al. 2008, 2012; de Lecea et al. 2011; Rennie et al. 2012, Correa 2012) suggested species-specific effects of formalin-preservation on $\delta^{13}C$, we interpret results on $\delta^{13}C$ changes observed in benthopelagic fishes with caution, mainly focusing on the difference for this group in $\delta^{15}N$ between the two periods.

Consistent with our meta-analysis results and also with previous findings (see Fanelli et al. 2010; Rennie et al. 2012; Correa 2012 and references therein cited), the

magnitude of isotopic shifts in $\delta^{15}N$ appears to be small (for both formalin and ethanol preservation) compared to natural fractionation processes that occur in ecological communities. Our results agree with other studies (Edwards et al. 2002; Arrington and Winemiller, 2002) in suggesting that it may be appropriate to simply subtract 0.2-0.5‰ from $\delta^{15}N$ values of preserved material. However, even if values remain uncorrected, a 0.5‰ enrichment is minor compared to the average shift between trophic levels of 2.9-3.4‰ (Minagawa and Wada 1984; Vander Zanden and Rasmussen 1999; McCutchan et al. 2003). Even so, we included a correction factor for $\delta^{15}N$ in order to prevent for any bias incurred by preservation. Thus the ^{15}N-enrichment of preserved specimens here observed cannot be attributed to effects of the preservation method.

On the other hand, the exceptional depletion observed in ^{13}C of fishes from the older period cannot be considered consistent with the environmental trends and their consequences discussed in the paper. This could be the result of an inappropriate ^{13}C correction, since all studies examined explored samples preserved no longer than 15 years. The correction here applied is the result of a meta-analysis and other studies have estimated similar correction factors, even when they are derived from a mean of preservation effects (Vander Zanden et al. 2003). However, it could be that the effect levels increase with time in deep-sea fish, as observed for other marine and freshwater species (Ogawa et al. 2001; Ponsard and Amlou 1999; Sweeting et al. 2004) and that a greater effect size should have been considered. Indeed a 2‰ depletion was observed in *Hoplostethus mediterraneus* and *Hymenocephalus italicus* ^{13}C values (Fanelli and Cartes 2010) after 24 months of preservation and the authors suggested for them a relationship between species behavior (i.e. increasing depletion with increasingly pelagic diet and habits) and effects of preservative on $\delta^{13}C$. That interaction appeared to

be related to the lipid content of the species (the greater the lipid content the greater the ^{13}C-depletion).

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Table S1. Results of meta-analysis carried out on literature data and considering the effect of formalin (a) and ethanol preservation (b) on the $\delta^{15}N$ and $\delta^{13}C$ values of fishes and aquatic invertebrates (including both freshwater and marine taxa). τ^2 is the estimated amount of total heterogeneity; τ is the square root of estimated τ^2; H^2 is the ratio between total heterogeneity and total variability; I^2 is the ratio between total variability and sampling variability; N is the number of studies combined. Values in bold are the effect sizes (ES) used as correction factor for $\delta^{15}N$ and $\delta^{13}C$ values of *'80s period* specimens.

a)

	$\delta^{15}N$						**$\delta^{13}C$**				
						Fish					
AIC	τ^2	τ	I^2	H^2	**N=22**	AIC	τ^2	τ	I^2	H^2	**N=18**
36.67	0.18 (0.09)	0.43	72%	3.57		34.95	0.20(0.11)	0.44	95%	0.01	
Model	Results:					Model	Results:				
ES	se	zval	p	ci.lb	ci.ub	**ES**	se	zval	p	ci.lb	ci.ub
0.43	0.11	3.81	0.05	0.21	0.65	**1.18**	0.13	8.82	<0.001	0.92	1.45
						Invertebrates					
AIC	τ^2	τ	I^2	H^2	**N=39**	AIC	τ^2	τ	I^2	H^2	**N=38**
95.01	0.36(0.11)	0.6	87%	7.42		197.87	9.89(2.35)	3.15	99%	153.63	
Model	Results:					Model	Results:				
ES	se	zval	p	ci.lb	ci.ub	**ES**	se	zval	p	ci.lb	ci.ub
0.23	0.11	2.11	0.06	0.02	0.45	**1.45**	0.52	2.81	<0.01	0.44	2.46

b)

	$\delta^{15}N$						**$\delta^{13}C$**				
						Fish					
AIC	τ^2	τ	I^2	H^2	**N=23**	AIC	τ^2	τ	I^2	H^2	**N=19**
39.23	0.20 (0.08)	0.45	84%	6.45		43.58	0.39(0.16)	0.44	91%	11.55	
Model	Results:					Model	Results:				
ES	se	zval	p	ci.lb	ci.ub	**ES**	se	zval	p	ci.lb	ci.ub
0.46	0.11	4.28	<0.001	0.25	0.68	**0.20**	0.163	1.29	0.20	-0.11	0.51
						Invertebrates					
AIC	τ^2	τ	I^2	H^2	**N=34**	AIC	τ^2	τ	I^2	H^2	**N=34**
46.78	0.11(0.05)	0.34	70%	3.36		113.91	1.43(0.39)	1.19	96%	23.99	
Model	Results:					Model	Results:				
ES	se	zval	p	ci.lb	ci.ub	**ES**	se	zval	p	ci.lb	ci.ub
0.31	0.07	3.96	<0.001	0.16	0.47	**0.15**	0.21	0.70	0.48	-0.27	0.58