

## Article

# Use of Formalin-Preserved Collections to Infer Trophic Indicators of Marine Zooplankton from Stable Isotopes

Antonio Bode <sup>\*</sup> , Jaime Otero, Ángel F. Lamas  and Carmen Mompeán

Instituto Español de Oceanografía, IEO-CSIC, Centro Oceanográfico de A Coruña, 15001 A Coruña, Spain

<sup>\*</sup> Correspondence: antonio.bode@ieo.csic.es; Tel.: +34-981218151

**Abstract:** Formalin preservation affects the stable isotope composition of zooplankton samples, thus limiting the analysis of valuable collections covering large time intervals. Here, we compare different procedures for correcting the bias caused by formalin in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of zooplankton community samples. Zooplankton samples representative of seasonal variations in the period 2000–2009 were collected off A Coruña (NW Spain). Part of the sample was immediately dried and analysed for  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and elemental composition within 3 years of collection. These values were used as the unpreserved reference. The remaining sample was preserved in 4% formaldehyde and aliquots obtained after a period ranging from 3 years to more than 10 years of storage were analysed as the originally dried samples. Additionally, the copepod fraction of total biomass was determined in the preserved samples. Corrections of formalin effects based on ordinary least squares regression had large uncertainties, while mass balance corrections based on the change in C:N ratio (only possible for  $\delta^{13}\text{C}$ ) overestimated reference values. However, either simple corrections based on the mean difference between values in dry and preserved samples or more complex generalised additive models considering seasonality, copepod biomass, and time of sample storage, produced estimations with relatively low uncertainty. Our results highlight the importance of determining specific correction solutions for each preserved collection before reconstructing stable isotope time series. Furthermore, the uncertainties associated with the estimates can be used in sensitivity analysis to assess their potential impact on the interpretation of the series.



**Citation:** Bode, A.; Otero, J.; Lamas, Á.F.; Mompeán, C. Use of Formalin-Preserved Collections to Infer Trophic Indicators of Marine Zooplankton from Stable Isotopes. *Diversity* **2023**, *15*, 459. <https://doi.org/10.3390/d15030459>

Academic Editors: Joana Cruz, Alexandra Teodósio, Cátia Bartilotti and Michael Wink

Received: 29 November 2022

Revised: 15 February 2023

Accepted: 17 March 2023

Published: 20 March 2023



**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Keywords:** zooplankton; stable isotopes; formalin; food web; time series

## 1. Introduction

Marine ecosystems are in a permanent change because of external and internal drivers. Climate and its present interaction with anthropogenic factors is one of the main external forcing mechanisms [1,2]. In turn, internal drivers, generally identified with changes and adjustments in the species composition, affect ecological interactions and succession patterns [3]. Recent changes in global and regional ocean biogeochemistry and productivity [4] have altered species distribution and phenology [5]. Most of these changes are reflected in the structure and functioning of food webs, modifying their length (i.e., the number of trophic steps) or the trophic position of some species [6–8].

Changes in marine food web structure can be investigated by the stable isotope analysis of carbon and nitrogen. For instance, the time series of natural isotope has been successfully employed to detect long-term changes in the planktonic food web structure in the ocean. Temporal variations in nitrogen isotopes have been associated with changes in the source of nutrients [8,9] but also on the trophic position of some species [10]. However, determining long-term changes in planktonic food web structure from stable isotopes is limited by the availability of samples covering large periods. One way to overcome this problem is the use of sample collections initially intended for biodiversity analyses by microscopical examination [8,11]. Studies on preserved marine zooplankton samples revealed a variety of effects on stable isotope composition depending on the type of

organisms considered, the chemical nature of the preservative, and the conditions of the preservation. For instance, a significant decrease in the abundance of heavy isotopes of carbon ( $\delta^{13}\text{C}$ ) and small changes in those of nitrogen ( $\delta^{15}\text{N}$ ) were generally reported for species preserved in formaldehyde solutions (formalin), mainly copepods [8,11–15] but also from other groups such as chaetognaths [12] or euphausiids [13]. However, the magnitude of the changes in the isotopic composition varied not only with the species but also with the time of storage of the specimens in the preservative solution before analysis [11,13], thus requiring specific studies for each collection of samples and selected species or taxonomic groups. In contrast with the results obtained with freshwater zooplankton, [16], the changes in the C:N ratio caused by the preservative in marine species did not allow for a prediction of the effects beyond relatively small storage periods [11], while the magnitude of the changes in isotopic composition was generally higher in freshwater species [16,17].

An alternative to analyse only selected species would be using samples of zooplankton assemblages including all the species in a given size fraction but, to our knowledge, there were no studies considering the effect of the preservative on isotopes analysed in samples composed of a mixture of species. Therefore, there is a need for determining feasible methodological corrections of isotopic results obtained from collections of formalin-preserved samples of zooplankton species assemblages to infer changes in food web structure during long time periods. One application of these corrections would be the investigation of the food web structure in the different regimes identified in plankton communities of the North Atlantic and other ocean basins in recent decades [18–20].

The objective of this study is to develop a procedure for correcting the bias observed in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of samples of zooplankton species assemblages stored for long periods in a formaldehyde solution. For this purpose, we compare several models of correction, including linear regression, direct subtraction of mean differences, and more complex generalised additive models considering the effect of seasonality, storage time, and copepod dominance.

## 2. Materials and Methods

### 2.1. Zooplankton Sampling

The zooplankton samples were collected at stations E2CO (43°25.32 N, 08°26.22 W, 77 m depth) and E4CO (43°21.78 N, 08°22.20 W, 22 m depth) off A Coruña (NW Spain) with double-oblique tows of Juday-Bogorov or Bongo plankton nets (200  $\mu\text{m}$  mesh size, 50 cm diameter). Samples were obtained at approximate monthly time intervals as part of a long-term study of oceanographic and planktonic variables [21]. This ecosystem is under the influence of a seasonal upwelling and is characterised by large variations in zooplankton diversity and biomass, as described in previous studies [20–22]. In the present study, we used samples collected at station E2CO during the period 2000–2009 and at station E4CO during 2006. In all cases, visible gelatinous organisms (e.g., jellyfish or salps) were removed and subsamples were obtained using a Folsom splitter. One half of the sample was immediately dried (60 °C, 48 h) to measure the dry weight biomass ( $\pm 0.01$  mg) and subsequently analysed for C and N elemental and stable isotope composition within 3 years of collection (dry samples). The remaining sample was preserved in borax-buffered formalin solution (PANREAC 37–38% formaldehyde solution stabilised with methanol, analytical grade) diluted with seawater to a final concentration of ca. 4% formaldehyde. Preserved samples were stored until analysis of stable isotope composition by first rinsing the sample with filtered seawater using a 40  $\mu\text{m}$  mesh sieve before drying in an oven as with the unpreserved samples. Previously, species composition was analysed in the preserved samples by microscopical examination [20–22]. Percent contribution of copepods to total zooplankton biomass (i.e., total dry weight of the sample) was estimated from species abundance and body size values. The mean length (L, mm) for each species was obtained from the Marine Planktonic Copepods Database from the Oceanological Observatory of Banyuls sur Mer [23]. Length and individual biomass in wet weight (WW, mg) were

related by an allometric relationship using the WW values provided by Acuña et al. [24] for some species:

$$\log WW = 2.949 \log L - 1.463 \quad (1)$$

This equation was then applied to compute the WW for the remaining species. Biomass data in WW were converted into DW using the regression equation proposed by Wiebe [25]:

$$\log DW = \log [(0.001 WW) + 2.002]/0.950 \quad (2)$$

Finally, the biomass of the copepod species in each sample was calculated (as  $\text{mg m}^{-3}$ ) by multiplying their abundance (individuals  $\text{m}^{-3}$ ) by the corresponding species DW. The dominance of copepods in the zooplankton biomass (% copepod biomass) was computed as:

$$\% \text{ copepod biomass} = 100 \sum DW_i / DW_s \quad (3)$$

where  $DW_i$  and  $DW_s$  are the biomass of individual species and of the whole zooplankton sample, respectively.

## 2.2. Stable Isotope Analysis

The elemental and stable isotope composition of carbon and nitrogen were determined in aliquots of dry zooplankton material, homogenised using a mortar and pestle and encapsulated in tin boats that were subsequently fed into an elemental analyser (Carlo Erba CHNSO 1108) coupled to an isotope-ratio mass spectrometer (Thermo Finnigan Mat Delta Plus). The system was calibrated with IAEA isotope standards (USGS40 and L-alanine) obtaining offsets from certified  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values  $<0.1\%$ . The standard error of triplicate determinations of internal acetanilide standards and samples was  $<0.1\%$  and  $<0.3\%$  for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively. Isotopic determinations were made at the Servicios de Apoyo a Investigación of the Universidad da Coruña (Spain). Raw results including sampling dates, copepod biomass fraction, and stable isotopes in preserved and unpreserved samples, are available through the PANGAEA repository [26].

## 2.3. Statistical Analysis

The differences between mean values of  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and the C:N ratio determined in the formalin-preserved and dry samples were first assessed using Student's *t*-tests for paired observations. Thereafter, up to four models for correction of the bias in stable isotope composition caused by the formalin preservation were employed. First, values measured in formalin-preserved samples were linearly related with those measured in dry samples by ordinary least squares regression (OLS), as the resulting equations were later employed for estimating values in unpreserved samples [27]. Second, the difference between the formalin-preserved and dry sample values was modeled by OLS with the relative change in the C:N ratio [16,28]:

$$\delta_{\text{for}} - \delta_{\text{dry}} = D (C:N_{\text{dry}} - C:N_{\text{for}}) / C:N_{\text{dry}} \quad (4)$$

where  $\delta_{\text{for}}$  and  $\delta_{\text{dry}}$  are the isotopic values measured in the formalin and dry samples, respectively, and  $C:N_{\text{for}}$  and  $C:N_{\text{dry}}$  the corresponding C:N values. *D* is a constant indicating the correction factor.

Third, the values obtained from formalin-preserved samples were corrected by subtracting the mean value of the difference between the preserved and dry samples. Finally, generalised additive models (GAM) [25] assuming a Gaussian distribution were constructed to predict values in unpreserved samples. Predictors in the GAMs included the sampling date measured as the day of the year (DoY) as an index of seasonality in nutrient sources, the storage time in formalin (number of days) before isotopic analysis, and the fraction of copepods represented in total biomass, as an index of variability in taxonomic composition. The non-parametric smoothing functions in the GAMs were fit by penalised cyclic and cubic regression splines with a maximum of six and three knots for the day of the year, storage

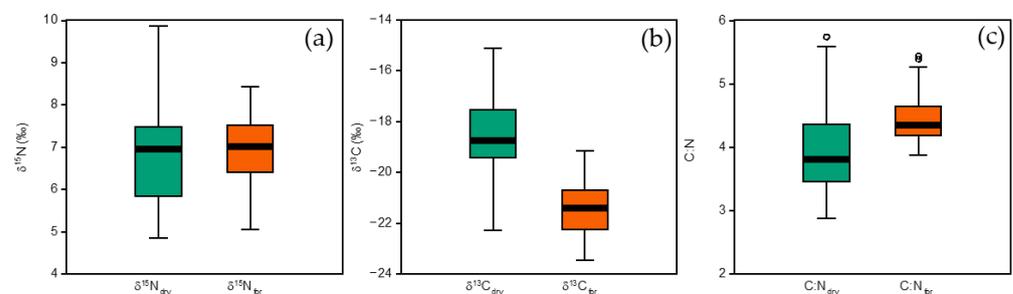
time, and fraction of copepods. Models were fit using the ‘mgcv 1.8–40’ package [29] in R (version 4.2.) [30].

The final assessment of the performance of the different models when correcting for the bias in formalin samples was carried out by comparing the values of the coefficient of determination ( $r^2$ ) of the regression models and the 95% confidence interval of the mean offset between the estimated and observed values in dry zooplankton samples.

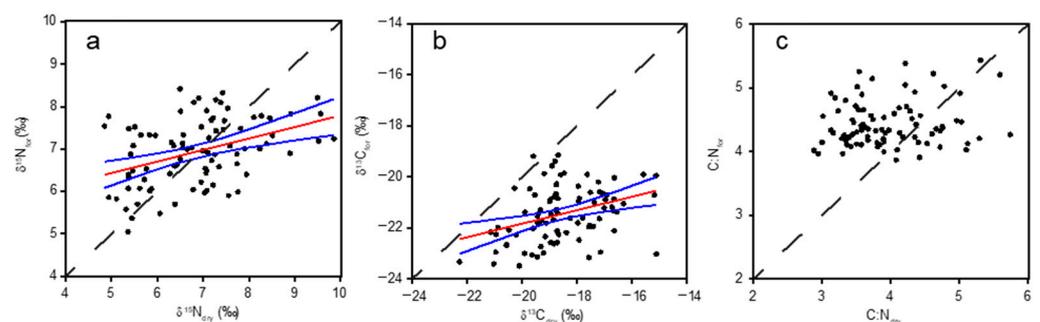
### 3. Results

#### 3.1. Comparison of Formalin vs. Dry Sample Measurements

Measurements in formalin preserved samples differed from those in dry samples (Figure 1). These differences were not significant for  $\delta^{15}\text{N}$  (Student's  $t = -0.707$ ,  $p > 0.05$ ,  $n = 87$ ) but highly significant for  $\delta^{13}\text{C}$  (Student's  $t = 18.659$ ,  $p < 0.001$ ) and C:N ratio (Student's  $t = -6.570$ ,  $p < 0.001$ ). Mean ( $\pm$ se) differences between formalin and dry values were  $0.08 \pm 0.12\text{‰}$ ,  $-2.86 \pm 0.15\text{‰}$ , and  $0.48 \pm 0.07$  for  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ , and C:N, respectively. Values measured in both types of samples showed significant positive correlations, except in the case of C:N (Figure 2).

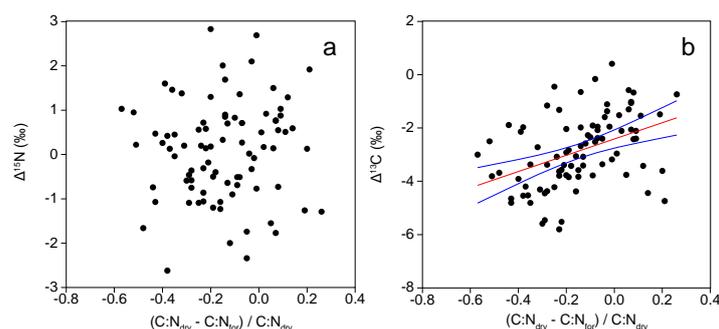


**Figure 1.** Box plot of (a)  $\delta^{15}\text{N}$ , (b)  $\delta^{13}\text{C}$ , and (c) C:N of mesozooplankton samples immediately dried after sampling (dry) or preserved in formalin (for). Box: 25 and 75% quartiles; whiskers:  $1.5 \times$  the interquartile range; horizontal line: median; circles: outliers ( $>1.5 \times$  the interquartile range).



**Figure 2.** Relationships between measurements of (a)  $\delta^{15}\text{N}$ , (b)  $\delta^{13}\text{C}$ , or (c) C:N in mesozooplankton samples immediately dried after sampling (dry) or preserved in formalin (for). Significant ordinary least squares regression lines are indicated in red and the corresponding 95% confidence limits are in blue. The dashed line indicates the 1:1 relationship.

In turn, the differences between  $\delta^{13}\text{C}$  (but not  $\delta^{15}\text{N}$ ) values in formalin and dry samples were significantly correlated with the relative shift observed in C:N (Figure 3). Therefore, a correction of  $\delta^{13}\text{C}_{\text{for}}$  based in the C:N shift described by Equation (1) was feasible.



**Figure 3.** Relationships between the difference in (a)  $\delta^{15}\text{N}$  ( $\Delta^{15}\text{N} = \delta^{15}\text{N}_{\text{for}} - \delta^{15}\text{N}_{\text{dry}}$ ) or (b)  $\delta^{13}\text{C}$  ( $\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{for}} - \delta^{13}\text{C}_{\text{dry}}$ ) and the relative change in C:N ratio between formalin-preserved and dried zooplankton samples ( $[\text{C:N}_{\text{dry}} - \text{C:N}_{\text{for}}] / \text{C:N}_{\text{dry}}$ ). The significant ordinary least squares regression line for  $\Delta^{13}\text{C}$  is indicated by red line and the corresponding 95% confidence limits in blue.

### 3.2. Models for Correction of Measurements in Formalin

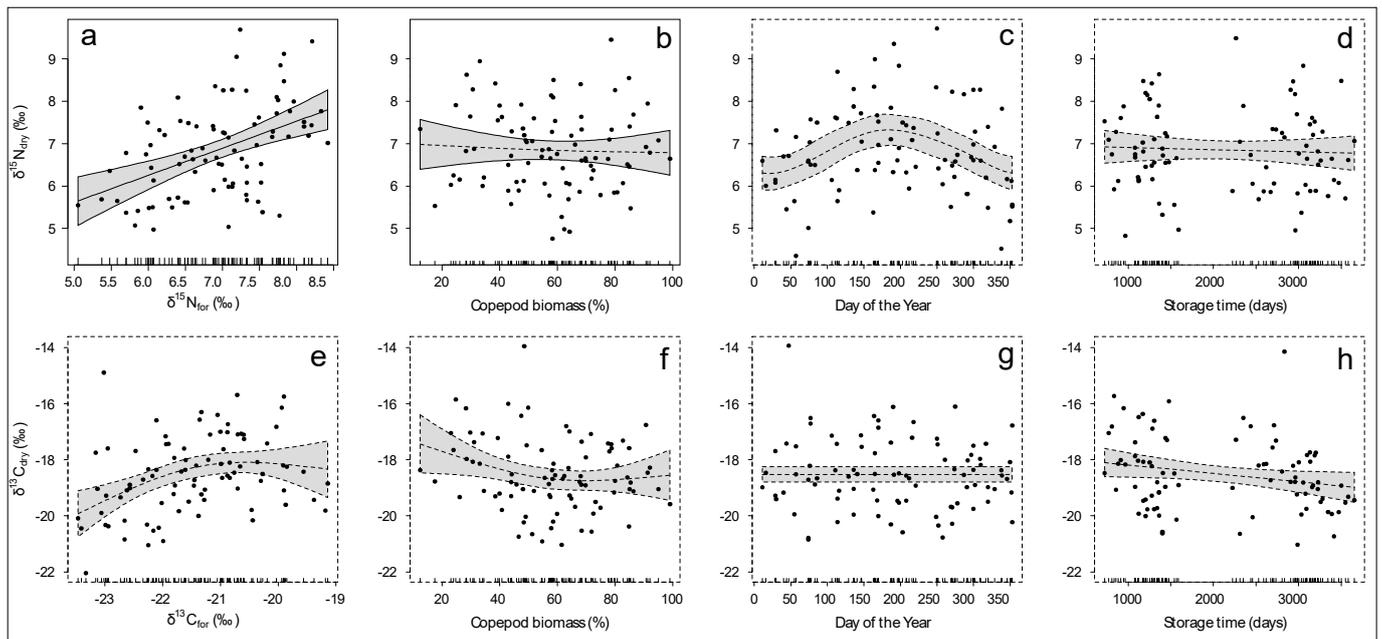
The slope of the regression lines between formalin and dry sample measurements for both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  was  $\ll 1$ , indicating that the values measured in formalin increased less than the corresponding values measured in dry samples (Table 1). In addition, the coefficients of determination of these linear regression models were low, as a large part of the variability was not accounted for the regression fits, thus limiting their predictive power.

**Table 1.** Parameters of the different regression models relating values measured in dry and formalin-preserved aliquots of zooplankton samples. The subindex  $_{\text{dry}}$  or  $_{\text{for}}$  indicates if dry or formalin-preserved aliquots were used, respectively.  $\Delta^{15}\text{N} = \delta^{15}\text{N}_{\text{for}} - \delta^{15}\text{N}_{\text{dry}}$ ;  $\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{for}} - \delta^{13}\text{C}_{\text{dry}}$ . Regressions are of the form:  $y = a + b \cdot x$ ;  $x$ : independent variable;  $y$ : dependent variable;  $a$ : constant;  $b$ : slope;  $r$ : correlation;  $r^2$ : coefficient of determination;  $p$ : significance value;  $se_a$ ,  $se_b$ : standard error of the  $a$  or  $b$  estimates, respectively. The number of data pairs was  $n = 87$  for all models.

x	y	a	se <sub>a</sub>	b	se <sub>b</sub>	r	r <sup>2</sup>	p
$\delta^{15}\text{N}_{\text{dry}}$	$\delta^{15}\text{N}_{\text{for}}$	5.075	0.463	0.272	0.067	0.404	0.164	0.000
$\delta^{13}\text{C}_{\text{dry}}$	$\delta^{13}\text{C}_{\text{for}}$	-16.575	1.396	0.263	0.075	0.355	0.126	0.001
$\text{C:N}_{\text{dry}}$	$\text{C:N}_{\text{for}}$	—	—	—	—	0.168	0.028	0.121
$\Delta^{15}\text{N}$	$(\text{C:N}_{\text{dry}} - \text{C:N}_{\text{for}}) / \text{C:N}_{\text{dry}}$	—	—	—	—	0.069	0.005	0.528
$\Delta^{13}\text{C}$	$(\text{C:N}_{\text{dry}} - \text{C:N}_{\text{for}}) / \text{C:N}_{\text{dry}}$	-2.443	0.184	3.216	0.780	0.408	0.167	0.000

In the case of the corrections based on the C:N shift, the slope of the regression line between the difference  $\delta^{13}\text{C}_{\text{for}} - \delta^{13}\text{C}_{\text{dry}}$  was used as an estimate of  $D$  in Equation (1) but, as in the previous regression models, the coefficient of determination was low (Table 1).

Because of the low predictive power of OLS models, corrections using alternative GAMs examined the potential of several explanatory variables (Figure 4). These models slightly improved  $r^2$  values (Table 2) compared to those of the OLS regressions (Table 1). In the case of  $\delta^{15}\text{N}_{\text{dry}}$ , the model confirmed the linear increase with  $\delta^{15}\text{N}_{\text{for}}$  already indicated by the OLS model, but also a seasonal increase with maximum values in summer and minimum values in winter. However, the individual contributions of other variables, as the fraction of total biomass accounted for by copepods or the storage time in formalin, were not statistically significant (Figure 4a–d and Table 2). The GAM for  $\delta^{13}\text{C}_{\text{dry}}$  also revealed a significant relationship with  $\delta^{13}\text{C}_{\text{for}}$ , but in this case in the form of a non-linear increase up to a maximum  $\delta^{13}\text{C}_{\text{for}}$  value of ca.  $-21.5\%$ , reaching a constant value thereafter. Seasonality or the fraction of copepods were not significant predictors of  $\delta^{13}\text{C}_{\text{dry}}$ . Finally, the GAM indicated that the storage time was negatively correlated with  $\delta^{13}\text{C}_{\text{dry}}$  (Table 2).

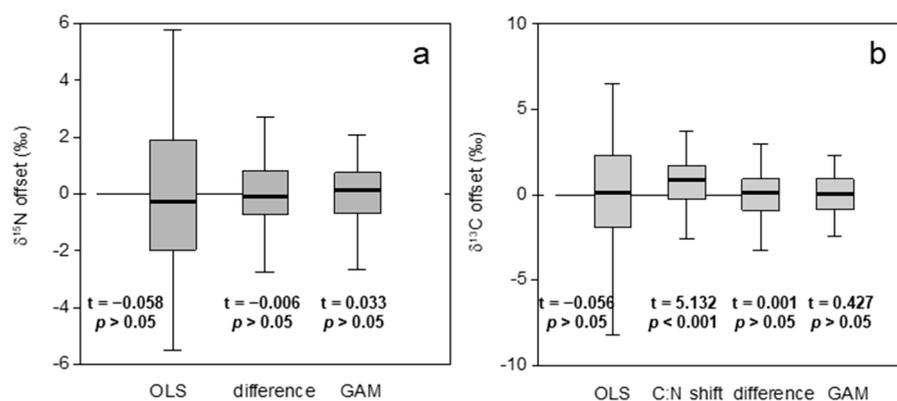


**Figure 4.** Partial effects plots obtained from fitting a GAM to (a–d)  $\delta^{15}\text{N}_{\text{dry}}$  or (e–h)  $\delta^{13}\text{C}_{\text{dry}}$  data. Shown are the effects for (a,e) formalin, (b,f) copepod contribution to total biomass (%), (c,g) seasonality (day of the year), and (d,h) storage time in formalin. Significant (non-significant) trends are indicated by continuous (dashed) lines. Bands indicate 95% confidence intervals and the rugs along the x-axes display the distribution of the data.

**Table 2.** Results of the generalised additive models (GAMs) fitted to the isotope measures. SE: standard error; EDF: estimated degrees of freedom;  $p$ : significance; N: number of data;  $r^2_{\text{adj}}$ : adjusted determination coefficient.

Isotope	Parameter	Estimate ( $\pm$ SE)	EDF	$t$ -Value	F-Value	$p$
$\delta^{15}\text{N}_{\text{dry}}$ N = 87 $r^2_{\text{adj}} = 0.27$	Intercept	6.85 ( $\pm$ 0.11)		64.91		<0.001
	$\delta^{15}\text{N}_{\text{for}}$		1.00		20.76	<0.001
	Copepod biomass		1.08		0.10	0.779
	Day of the Year		2.10		3.40	0.001
	Storage time		1.00		0.20	0.659
$\delta^{13}\text{C}_{\text{dry}}$ N = 87 $r^2_{\text{adj}} = 0.20$	Intercept	−18.52 ( $\pm$ 0.14)		−133.90		<0.001
	$\delta^{13}\text{C}_{\text{for}}$		1.81		7.81	0.003
	Copepod biomass		1.69		1.90	0.108
	Day of the Year		0.00		0.00	0.746
	Storage time		1.00		4.04	0.048

Corrections of  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  values measured in formalin based on OLS, C:N, shift or GAM, along with the simple correction based on subtracting the mean value of the difference observed between values in formalin and dry samples, were compared with the dry sample values (Figure 5). The corresponding offsets between estimated and measured values indicated a good agreement (i.e., mean offsets non-significantly different from 0, Student's  $t$ ,  $p > 0.05$ ) in most cases with the exception of the estimates based on the C:N shift (Student's  $t$ ,  $p < 0.001$ ). The latter overestimated  $\delta^{13}\text{C}$  values measured in dry samples by  $0.76 \pm 0.15\text{‰}$  (mean  $\pm$  se).



**Figure 5.** Box plot of the offset in (a)  $\delta^{15}\text{N}$  or (b)  $\delta^{13}\text{C}$  between measured values in dried samples and values estimated by ordinary least squares regression (OLS), the relative shift in C:N ratio (C:N shift), subtraction of the mean difference between formalin-preserved and dried samples (difference), or by generalised additive models (GAM). Box: 25 and 75% quartiles; whiskers:  $1.5 \times$  the interquartile range; horizontal line: median. The values of the Student's *t* statistic (*t*) and the significance (*p*) of differences between the mean offset and zero were indicated in each case.

However, most models showed large variability in the estimations, with interquartile ranges exceeding 2‰ (Table 3). Only the corrections based on the subtraction and GAM models had interquartile ranges  $< 2\%$  and the 95% confidence intervals for the mean offset were  $< 0.5\%$  and  $< 0.6\%$ , for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , respectively.

**Table 3.** Values (‰) of the interquartile range (25–75% quartiles) and the 95% confidence interval of the mean offset between estimated and observed values in dry zooplankton samples using different types of correction models. OLS: ordinary least squares regression; difference: mean difference between formalin and dry sample values; GAM: generalised additive models; C:N shift: regression between difference values and the relative change in C:N caused by formalin.

Variable	Type of Correction	Interquartile Range	95% Interval
$\delta^{15}\text{N}$	OLS	3.87	1.12
	difference	1.52	0.47
	GAM	1.43	0.41
$\delta^{13}\text{C}$	OLS	4.21	1.32
	C:N shift	1.99	0.59
	difference	1.85	0.58
	GAM	1.80	0.50

#### 4. Discussion

Our results confirm the significant depletion in heavy carbon isotopes and the low effect on nitrogen isotopes of formalin in samples of marine zooplankton assemblages preserved for long periods ( $> 1$  year). These findings are, to our knowledge, the first reported for samples representative of plankton assemblages, as previous studies examined these effects in individual species, mostly copepods [8,12]. The described changes showed lower variability than those considering smaller sampling storage periods [11,14,31], likely because of the relatively long equilibration time required for the preservative to interact with the zooplankton tissues. In fact, we did not detect a significant effect on  $\delta^{15}\text{N}$ , and only a slight linear decrease on  $\delta^{13}\text{C}$ , when comparing samples stored for periods between 3 and 10 years. It must be noted that through our study, we used formalin from the same provider, thus reducing the probability of differences caused by the composition of the preservative [11].

Particularly in the case of carbon isotopes, the decrease in  $\delta^{13}\text{C}$  has been attributed to the hydrolysis of  $^{13}\text{C}$  enriched molecules, as proteins, caused by the formaldehyde while

less enriched molecules, as lipids, are conserved [32,33]. In addition, the loss of heavy isotopes may be compensated by the incorporation of light isotopes from the formalin solution [11,32–34]. In any case, both effects would result in an increase in the C:N ratio, as observed in our study. In contrast, the effects on  $\delta^{15}\text{N}$  were small, likely because the formalin solution does not contain nitrogen and the only effect would be the leaching of light nitrogen atoms after hydrolysis of the zooplankton tissues [11]. Our study showed that the difference in  $\delta^{15}\text{N}$  between formalin and dry samples was non-significant, in agreement with published studies with marine copepoda [8,11–13] where mean differences were up to 0.3‰, and thus within the limits of the analytical error of  $\delta^{15}\text{N}$  determinations. This result suggests that, in our series, no corrections would be necessary when comparing  $\delta^{15}\text{N}$  values measured in zooplankton samples stored in formalin, as assumed in other studies [8,12,13]. Indeed, both the OLS and GAM indicated a significant linear relationship between values measured in formalin and those in dry samples, but this relationship accounted for only <30% of the variability in  $\delta^{15}\text{N}_{\text{dry}}$ . Therefore, the correction of  $\delta^{15}\text{N}_{\text{for}}$  using either OLS or GAM would not substantially improve the estimation of  $\delta^{15}\text{N}_{\text{dry}}$ .

The correction of formalin effects on  $\delta^{13}\text{C}$  was generally not recommended for marine zooplankton because of the large variety of effects described for various combinations of species, preservation time, and fixative agents [11,14,31]. Since we used samples composed of several species, variations in the proportions of species as a consequence of seasonal succession [35] could be one of the causes of this variability. However, as shown in the GAM analysis, the dominance of copepods or the seasonality were not significantly related to the variations in  $\delta^{13}\text{C}_{\text{dry}}$ . Furthermore, the average effect of formalin on  $\delta^{13}\text{C}$  in our case is within the values reported for marine copepods stored for similar periods [8,12], and has an associated error of similar magnitude to the analytical error, thus suggesting that the simple subtraction of this value from  $\delta^{13}\text{C}_{\text{for}}$  can be a robust estimate of  $\delta^{13}\text{C}_{\text{dry}}$ . Estimations based on the OLS regression between  $\delta^{13}\text{C}_{\text{for}}$  and  $\delta^{13}\text{C}_{\text{dry}}$  had large uncertainty because of the low fraction of variance accounted for by the model ( $r^2 = 0.13$ ). The mass balance correction based on the C:N shift slightly improved the prediction ( $r^2 = 0.17$ ); however, in contrast to the findings reported for freshwater zooplankton species [15], it still resulted in being a poor predictor of  $\delta^{13}\text{C}_{\text{dry}}$ . Finally, the estimations using the GAM model increased the  $r^2$  value (0.20), though this model produced similar variability in  $\delta^{13}\text{C}_{\text{dry}}$  estimates when compared to the subtraction method, the later requiring fewer complex calculations.

This study provides feasible procedures for estimating  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values in dry (unaltered) samples of zooplankton assemblages from measurements in samples preserved in formalin for more than 3 years. These procedures can be used to reconstruct the long time series of isotopic data from stored zooplankton collections, as implemented for other aquatic organisms [36–39]. However, it must be noted that the estimated values, particularly in the case of  $\delta^{13}\text{C}$ , have an associated error that can confound the interpretation of the results. For instance, the reconstruction of isotopic signatures for Bayesian stable isotope mixing models can be compromised by these errors [40]. The use of corrections could not be required in the case of time series studies, as long as all the data were obtained from samples preserved or analysed in the same way (e.g., dry or formalin preserved). Alternatively, the effect of the variability in the estimates on the interpretation of trends in isotope signatures can be examined through sensitivity analysis based on the error intervals obtained. For instance, the correction by subtraction of the mean difference between formalin and dry samples can be modeled for various estimates within the 95% interval of the mean determined in this study.

The use of dry or frozen samples of single species is always preferable to obtain accurate measurements of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of marine zooplankton because any other preservation method can affect the isotopic determinations. However, the preservation and availability of samples in adequate quantities for isotopic analysis is often compromised and alternative determinations can be performed using preserved collections. The interpretation of isotopic measurements on preserved samples requires the critical application of corrections to estimate the original values in unpreserved samples. Furthermore, non-living particles,

as microplastics, are often collected by plankton nets [41] but its effect on the isotopic determinations of plankton samples is not known. Our study illustrates the search for feasible correction methods applied to a specific sample collection. Here, we have proposed the simple subtraction of the mean difference between isotopic values measured in formalin samples and those in dry samples determined in this study as a simple method to extend the time series of zooplankton to periods when dry samples are not available but there are samples preserved in formalin. However, we stress that any correction procedure may be not of general application to all collections of plankton samples, thus requiring the development of specific protocols in each particular case. The resulting time series and the associated estimation errors would allow performing sensitivity analyses in order to assess the reliability of specific results, as those related to the patterns of temporal variability.

**Author Contributions:** Conceptualisation, A.B. and C.M.; methodology, C.M. and Á.F.L.; formal analysis, A.B. and J.O.; investigation, A.B. and C.M.; resources, A.B. and J.O.; data curation, A.B.; writing—original draft preparation, A.B. and C.M.; writing—review and editing, A.B., J.O., Á.F.L. and C.M.; visualization, A.B. and J.O.; funding acquisition, A.B. and J.O. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by projects QLOCKS (PID2020-115620RB-100, MCIN/AEI/10.13039/501100011033) and RADIALES (Instituto Español de Oceanografía, Spain). C.M. was supported by a FPI fellowship from the Instituto Español de Oceanografía (Spain).

**Data Availability Statement:** Isotopic data are available through the PANGAEA repository (<https://doi.pangaea.de/10.1594/PANGAEA.932538>, accessed on 2 November 2022).

**Acknowledgments:** We acknowledge the collaboration of the crew of the R/V Lura during sampling. Isotopic analysis was carried out by M. Lema at the Servicio de Apoyo a Investigación (Universidade da Coruña, Spain).

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## References

1. Jackson, J.B.C.; Kirby, M.X.; Berger, W.H.; Bjorndal, K.A.; Botsford, L.W.; Bourque, B.J.; Bradbury, R.H.; Cooke, R.; Erlandson, J.; Estes, J.A.; et al. Historical Overfishing and the Recent Collapse of Coastal Ecosystems. *Science* **2001**, *293*, 629–637. [[CrossRef](#)] [[PubMed](#)]
2. Ruiz-Cooley, R.I.; Gerrodette, T.; Fiedler, P.C.; Chivers, S.J.; Danil, K.; Ballance, L.T. Temporal variation in pelagic food chain length in response to environmental change. *Sci. Adv.* **2017**, *3*, e1701140. [[CrossRef](#)] [[PubMed](#)]
3. Barton, A.D.; Finkel, Z.V.; Ward, B.A.; Johns, D.G.; Follows, M.J. On the roles of cell size and trophic strategy in North Atlantic diatom and dinoflagellate communities. *Limnol. Oceanogr.* **2013**, *58*, 254–266. [[CrossRef](#)]
4. Malone, T.C.; Azzaro, M.; Bode, A.; Brown, E.; Duce, R.; Kamykowski, D.; Kang, S.H.; Kedong, Y.; Thorndyke, M.; Wang, J.; et al. Primary production, cycling of nutrients, surface layer and plankton. In *The First Global Integrated Marine Assessment: World Ocean*; Cambridge University Press: Cambridge, UK, 2017; pp. 119–148.
5. Walther, G.-R.; Post, E.; Convey, P.; Menzel, A.; Parmesan, C.; Beebee, T.J.C.; Fromentin, J.M.; Hoegh-Guldberg, O.; Bairlein, F. Ecological responses to recent climate change. *Nature* **2002**, *416*, 389–395. [[CrossRef](#)] [[PubMed](#)]
6. Reum, J.C.P.; Jennings, S.; Hunsicker, M.E. Implications of scaled  $\delta^{15}\text{N}$  fractionation for community predator-prey body mass ratio estimates in size-structured food webs. *J. Anim. Ecol.* **2015**, *84*, 1618–1627. [[CrossRef](#)]
7. Ruiz-Cooley, R.I.; Koch, P.L.; Fiedler, P.C.; McCarthy, M.D. Carbon and Nitrogen Isotopes from Top Predator Amino Acids Reveal Rapidly Shifting Ocean Biochemistry in the Outer California Current. *PLoS ONE* **2014**, *9*, e110355. [[CrossRef](#)]
8. Rau, G.H.; Ohman, M.D.; Pierrot-Bults, A. Linking nitrogen dynamics to climate variability off central California: A 51 year based on  $^{15}\text{N}/^{14}\text{N}$  in CalCOFI zooplankton. *Deep Sea Res. II* **2003**, *50*, 2431–2447. [[CrossRef](#)]
9. El-Sabaawi, R.W.; Trudel, M.; Mackas, D.L.; Dower, J.F.; Mazumder, A. Interannual variability in bottom-up processes in the upstream range of the California Current system: An isotopic approach. *Prog. Oceanogr.* **2012**, *106*, 16–27. [[CrossRef](#)]
10. Chiba, S.; Sugisaki, H.; Kuwata, A.; Tadokoro, K.; Kobari, T.; Yamaguchi, A.; Mackas, D.L. Pan-NorthPacific comparison of long-term variation in *Neocalanus* copepods based on stable isotope analysis. *Prog. Oceanogr.* **2012**, *97*, 63–75. [[CrossRef](#)]
11. Bicknell, A.W.J.; Campbell, M.; Knight, M.E.; Bilton, D.T.; Newton, J.; Votier, S.C. Effects of formalin preservation on stable carbon and nitrogen isotope signatures in Calanoid copepods: Implications for the use of Continuous Plankton Recorder Survey samples in stable isotope analyses. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1794–1800. [[CrossRef](#)]

12. Mullin, M.M.; Rau, G.H.; Eppley, R.W. Stable nitrogen isotopes in zooplankton: Some geographic and temporal variations in the North Pacific. *Limnol. Oceanogr.* **1984**, *29*, 1267–1273. [[CrossRef](#)]
13. Hannides, C.C.S.; Popp, B.N.; Landry, M.R.; Graham, B.S. Quantification of zooplankton trophic position in the North Pacific Subtropical Gyre using stable nitrogen isotopes. *Limnol. Oceanogr.* **2009**, *54*, 50–61. [[CrossRef](#)]
14. De Lecea, A.M.; Cooper, R.; Omarjee, A.; Smit, A.J. The effects of preservation methods, dyes and acidification on the isotopic values ( $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) of two zooplankton species from the KwaZulu-Natal Bight, South Africa. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1853–1861. [[CrossRef](#)]
15. Hetherington, E.D.; Kurle, C.M.; Ohman, M.D.; Popp, B.N. Effects of chemical preservation on bulk and amino acid isotope ratios of zooplankton, fish, and squid tissues. *Rapid Commun. Mass Spectrom.* **2019**, *33*, 935–945. [[CrossRef](#)]
16. Ventura, M.; Jeppesen, E. Effects of fixation on freshwater invertebrate carbon and nitrogen isotope composition and its arithmetic correction. *Hydrobiologia* **2009**, *632*, 297–308. [[CrossRef](#)]
17. Feuchtmayr, H.; Grey, J. Effect of preparation and preservation procedures on carbon and nitrogen stable isotope determinations from zooplankton. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2605–2610. [[CrossRef](#)]
18. Beaugrand, G.; Reid, P.C.; Ibañez, F.; Lindley, J.A.; Edwards, M. Reorganization of North Atlantic Marine Copepod Biodiversity and Climate. *Science* **2002**, *296*, 1692–1694. [[CrossRef](#)]
19. Reid, P.C.; Hari, R.E.; Beaugrand, G.; Livingstone, D.M.; Marty, C.; Straile, D.; Barichivich, J.; Goberville, E.; Adrian, R.; Aono, Y.; et al. Global impacts of the 1980s regime shift. *Glob. Change Biol.* **2016**, *22*, 682–703. [[CrossRef](#)]
20. Bode, A.; Álvarez, M.; García García, L.M.; Louro, M.A.; Nieto-Cid, M.; Ruíz-Villarreal, M.; Varela, M.M. Climate and local hydrography underlie recent regime shifts in plankton communities off Galicia (NW Spain). *Oceans* **2020**, *1*, 181–197. [[CrossRef](#)]
21. Valdés, L.; Bode, A.; Latasa, M.; Nogueira, E.; Somavilla, R.; Varela, M.M.; González-Pola, C.; Casas, G. Three decades of continuous ocean observations in North Atlantic Spanish waters: The RADIALES time series project, context, achievements and challenges. *Prog. Oceanogr.* **2021**, *198*, 102671. [[CrossRef](#)]
22. Bode, A.; Alvarez-Ossorio, M.T.; Miranda, A.; López-Urrutia, A.; Valdés, L. Comparing copepod time-series in the north of Spain: Spatial autocorrelation of community composition. *Prog. Oceanogr.* **2012**, *97–100*, 108–119. [[CrossRef](#)]
23. Razouls, C.; Desreumaux, N.; Kouwenberg, J.; de Bovée, F. Biodiversity of Marine Planktonic Copepods (Morphology, Geographical Distribution and Biological Data). 2005–2023. Available online: <http://copepodes.obs-banyuls.fr/en> (accessed on 30 June 2022).
24. Acuña, J.L.; Lopez-Urrutia, A.; Colin, S. Data compilation of marine pelagic organism oxygen respiration rates and biomasses. *Pangaea* **2014**. [[CrossRef](#)]
25. Wiebe, P. Functional regression equations for zooplankton displacement volume, wet weight, dry weight, and carbon: A correction. *Fish. Bull.* **1988**, *86*, 833–835. Available online: <https://spo.nmfs.noaa.gov/node/14372> (accessed on 30 June 2022).
26. Bode, A.; Mompeán, C.; Lamas, Á.F. Stable C and N isotope data for mesozooplankton samples unpreserved and preserved in formalin. *Pangaea* **2021**. [[CrossRef](#)]
27. Sokal, R.R.; Rohlf, F.J. *Biometry*, 2nd ed.; W.H. Freeman: San Francisco, CA, USA, 1981; p. 858.
28. Smyntek, P.M.; Teece, M.A.; Schulz, K.L.; Thackeray, S.J. A standard protocol for stable isotope analysis of zooplankton in aquatic food web research using mass balance correction models. *Limnol. Oceanogr.* **2007**, *52*, 2135–2146. [[CrossRef](#)]
29. Wood, S. *Generalized Additive Models: An Introduction with R*; Chapman & Hall/CRC Press: Boca Raton, FL, USA, 2006; p. 384.
30. R Core Team. *R: A language and environment for statistical computing*. In *R Foundation for Statistical Computing*; The R Foundation: Vienna, Austria, 2022; ISBN 3-900051-07-0. Available online: <http://R-project.org> (accessed on 30 June 2022).
31. Sano, M.; Makabe, R.; Kurosawa, N.; Moteki, M.; Odate, T. Effects of Lugol's iodine on long-term preservation of marine plankton samples for molecular and stable carbon and nitrogen isotope analyses. *Limnol. Oceanogr. Methods* **2020**, *18*, 635–643. [[CrossRef](#)]
32. Hobson, K.A.; Gibbs, H.L.; Gloutney, M.L. Preservation of blood and tissue samples for stable-carbon and stable-nitrogen isotope analysis. *Can. J. Zool.-Rev. Can. Zool.* **1997**, *75*, 1720–1723. [[CrossRef](#)]
33. Bosley, K.L.; Wainright, S.C. Effects of preservatives and acidification on the stable isotope ratios ( $^{15}\text{N}:^{14}\text{N}$ ,  $^{13}\text{C}:^{12}\text{C}$ ) of two species of marine animals. *Can. J. Fish. Aquat. Sci.* **1999**, *56*, 2181–2185. [[CrossRef](#)]
34. Sarakinos, H.C.; Johnson, M.L.; Vander Zanden, M.J. A synthesis of tissue-preservation effects on carbon and nitrogen stable isotope signatures. *Can. J. Zool.* **2002**, *80*, 381–387. [[CrossRef](#)]
35. Valdés, L.; López-Urrutia, A.; Beaugrand, G.; Harris, R.P.; Irigoien, X. Seasonality and interannual variability of copepods in the Western English Channel, Celtic Sea, Bay of Biscay, and Cantabrian Sea with a special emphasis to *Calanus helgolandicus* and *Acartia clausi*. *ICES J. Mar. Sci.* **2022**, *79*, 727–740. [[CrossRef](#)]
36. Le Bourg, B.; Lepoint, G.; Michel, L.C.N. Effects of preservation methodology on stable isotope compositions of sea stars. *Rapid Commun. Mass Spectrom.* **2020**, *34*, e8589. [[CrossRef](#)]
37. Durante, L.M.; Sabadel, A.J.M.; Frew, R.D.; Ingram, T.; Wing, S.R. Effects of fixatives on stable isotopes of fish muscle tissue: Implications for trophic studies on preserved specimens. *Ecol. Appl.* **2020**, *30*, e02080. [[CrossRef](#)]
38. Willert, M.S.; France, C.A.M.; Brooks, B.L.; Baldwin, C.C.; Hay, M.E. Effects of formalin preservation on carbon and nitrogen stable isotopes of seaweeds: A foundation for looking back in time. *Limnol. Oceanogr. Methods* **2020**, *18*, 717–724. [[CrossRef](#)]
39. Welicky, R.L.; Rolfe, T.; Leazer, K.; Maslenikov, K.P.; Tornabene, L.; Holtgrieve, G.W.; Wood, C.L. Fluid-preserved fishes are one solution for assessing historical change in fish trophic level. *Ecol. Evol.* **2021**, *11*, 415–426. [[CrossRef](#)]

40. Silberberger, M.J.; Koziarowska-Makuch, K.; Kulinski, K.; Kedra, M. Stable isotope mixing models are biased by the choice of sample preservation and pre-treatment: Implications for studies of aquatic food webs. *Front. Mar. Sci.* **2021**, *7*, 621978. [[CrossRef](#)]
41. Carretero, O.; Gago, J.; Filgueiras, A.V.; Viñas, L. The seasonal cycle of micro and meso-plastics in surface waters in a coastal environment (Ría de Vigo, NW Spain). *Sci. Total Environ.* **2022**, *803*, 150021. [[CrossRef](#)]

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.