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RESEARCH ARTICLE

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Mathematical lipid correction of $\delta^{13}C$ and effect of lipid extraction on $\delta^{15}N$ of European eel (*Anguilla anguilla*) muscle

Raphaël Lagarde[1,2] | Christophe Menniti[1,2] | Nils Teichert[3,4] | Elsa Amilhat[1,2] | Elisabeth Faliex[1,2] | Sarah Nahon[5]

[1]Centre de Formation et de Recherche sur les Environnements Méditerranéens, Université de Perpignan Via Domitia, Perpignan, France

[2]Centre de Formation et de Recherche sur les Environnements Méditerranéens, CNRS, Perpignan, France

[3]UMR 7208 BOREA (MNHN, CNRS, IRD, SU, UCN, UA), Laboratoire de Biologie des Organismes et Ecosystèmes Aquatiques, Paris, France

[4]Station Marine de Dinard, CRESCO, MNHN, Dinard, France

[5]MARBEC, Université de Montpellier, CNRS, Ifremer, IRD, INRAE, Sète, France

Correspondence
Raphaël Lagarde, Centre de Formation et de Recherche sur les Environnements Méditerranéens, UMR 5110, CNRS, F 66860, Perpignan, France.
Email: raphael.lagarde@univ-perp.fr

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Abstract

Rationale: Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope analysis is a powerful tool to investigate diverse questions in fish ecology, such as their trophic position or migration strategies. These questions appear particularly important to protect endangered European eel. However, elevated lipid content in eel muscle can bias $\delta^{13}C$ values, as lipids are ^{13}C-depleted compared to proteins and carbohydrates.

Methods: We measured $\delta^{13}C$ and $\delta^{15}N$ values of bulk and lipid-free samples of eel muscle. Lipid-free samples were obtained after the extraction of lipids with cyclohexane. Lipid-corrected $\delta^{13}C$ values, using five different mathematical equations based on bulk $\delta^{13}C$ values, were compared to lipid-free $\delta^{13}C$ values. We also evaluated the effect of lipid extraction on $\delta^{15}N$ values. The analyses were based on linear regression performed on 333 individuals captured in nine lagoons and four rivers.

Results: Independently to the capture site or habitat (river or lagoon), the predicted lipid-corrected $\delta^{13}C$ values were highly consistent with the measured lipid-free $\delta^{13}C$ values ($R^2 > 0.90$). The application of specific equations for each habitat or capture site only slightly increases these R^2 (1.5% or less). The lipid extraction treatment significantly decreased by 0.2‰ the $\delta^{15}N$ values compared to bulk samples.

Conclusions: Given the excellent prediction of mathematical equations and the small decrease of $\delta^{15}N$ values after lipids extraction, we propose to use mathematical correction to estimate $\delta^{13}C$ values of eel muscle. As the habitats or sites did not strongly influence the results, the coefficients from our study can be applied to other studies on European eel.

1 | INTRODUCTION

Carbone ($\delta^{13}C$) and azote ($\delta^{15}N$) stable isotope analysis is a common and powerful method to investigate diverse ecological questions in fish ecology, such as their trophic position in food webs,[1] the intra-specific variability of the trophic niche,[2,3] or habitat use and migration strategies.[4–6] These question are of pivotal importance in fish populations dynamic as they can be related to individuals growth and survival,[7,8] to their sensitivity of contamination by several anthropogenic pollutants[9] and to their parasites infestation.[10] However, in species with an elevated lipid content in the studied tissues, the measures of $\delta^{13}C$ can be biased as lipids are naturally ^{13}C

depleted compared to proteins and carbohydrates.[11] Individuals with a high lipid content will have lower $\delta^{13}C$ values, regardless of their trophic behavior, leading to erroneous interpretation of stable isotope data. Two methods are currently used to remove the effect of lipid content on $\delta^{13}C$ values. The first method consists to chemically extract lipids from the sample prior to measuring lipid-free $\delta^{13}C$ values.[12–14] However, many different protocols including several solvents are currently used with a diverse degree of lipid extraction that impact $\delta^{13}C$ values. Moreover, this method is costly and time-consuming due to the pretreatment associated with lipid extraction. Additionally, lipid extraction can modify the $\delta^{15}N$ values by removing protein compounds linked to lipids.[15] An alternative method to chemical extraction is mathematical correction to predict lipid-corrected $\delta^{13}C$ values. Many species- and tissue-specific models have been developed in literature to accurately estimate $\delta^{13}C$ values from various environments.[16] All models are based on empirical relationship between bulk and lipid-free $\delta^{13}C$ values to predict corrected $\delta^{13}C$ values.[16,17]

European eel (*Anguilla anguilla*, referred to as eel hereafter) is a facultative catadromous species[18] which migrate from its growing habitat in the coastal and continental waters of Europe and North Africa to its breeding ground in the Sargasso sea.[19,20] Eel complex life cycle exposes it to a variety of anthropogenic impacts,[21] which led to a drastic decrease of its population[22] and its classification as critically endangered on the IUCN red list.[23,24] Several ecological questions are yet to be disentangle to help the implementation of efficient conservation and restauration measures[25] such as the description of eel trophic ecology and movements between habitat[26,27] which can be assessed using $\delta^{13}C$ and $\delta^{15}N$ analyses. In eels, the use of stable isotopes implicates to deal with the high content of lipid in muscle tissues. Indeed, the main energetic fuel used by the future genitors (silver eels) to migrate from their growing habitats to the Sargasso sea is the lipids stored in their muscle.[28] As a consequence, the percentage of lipids in eel muscle can frequently reach 20% to 30%[29,30] and consequently C:N ratios are higher than 3.5[31] which is the threshold above which the effect of lipid content on $\delta^{13}C$ values has to be removed.[16] In eel tissues, the use of mathematical correction needs to be further investigated as the best models, including the equation and/or the parameters used, have not been calibrated for this species.[32] Moreover, mathematical correction of eel $\delta^{13}C$ values can be further complicated by the variety of growing stages as well as habitat inhabited by eels such as coastal waters, estuaries, rivers, lakes, or lagoons.[33] This variety of habitat is related to extended $\delta^{13}C$ values[8,34] and lipid content in eel muscle tissues.[30]

In this context, our study aims at (1) evaluating the performances of commonly used mathematical equations to predict the lipid-corrected $\delta^{13}C$ values from the bulk $\delta^{13}C$ values, (2) determining the equations parameters specific to eel and investigating the necessity to use different equations and/or parameters depending on eel growing habitat or capture site, (3) evaluating the effect of lipid extraction on $\delta^{15}N$ values, and (4) recommending the best method to measure $\delta^{13}C$ and $\delta^{15}N$ in eel muscle tissues.

2 | MATERIALS AND METHODS

2.1 | Fish collection

A total of 333 silver eels were sampled between 2016 and 2023 in four rivers from the French Atlantic coast and nine coastal French Mediterranean lagoons (Table S1). The number of eels sampled per site ranged from five in the Canet lagoon to 64 in the Sélune river (Table S1). All captured eels were euthanized with an overdose of anesthetic (iso-eugenol in lagoons and benzocaine in rivers) in accordance with the European Union regulations concerning the protection and welfare of experimental animals (European directive 91/492/CCE). The total length (TL, mm) and body weight (BW, g) of eels were measured (Table S1). Eels were then frozen at −20°C until dissection at the laboratory.

For each collected eel, a sample of dorsal muscle tissue was dissected, freeze-dried, and manually grounded. Prior to stable isotope analyses, the samples were separated into two aliquots of approximately 10 mg. The first aliquot was kept untreated for bulk analyses of stable isotopes. The second aliquot was treated with cyclohexane[13] to remove lipids (lipid-free sample). The cyclohexane method was preferred to other commonly used methods such as the chloroform–methanol or dichloromethane–methanol methods because cyclohexane did not affect the $\delta^{15}N$ values compared to bulk samples in a previous study.[35] The 10-mg aliquots were placed in a glass vial and submerged with 4 mL of cyclohexane during 1 h. The samples were centrifuged (1200 *g*, 10 min, 10°C), and the supernatant was discarded before repeating the procedure another time. The samples were then dried in a sand bath at 45°C during at least 2 h.

For stable isotope analyses, approximately 0.3 mg of each aliquot was weighted and packed into a tin capsule for simultaneous analysis of carbon and nitrogen stable isotopes. $\delta^{13}C$ and $\delta^{15}N$ values were analyzed by a Euro EA3000 (Pavia, Italia) elemental analyzer coupled with a GVI Isoprime (Manchester, England) isotope ratio mass spectrometer used in continuous-flow mode. The $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios are expressed in conventional delta notation in per mil (‰) relative to the levels of ^{13}C in Vienna Pee Dee Belemnite and ^{15}N in atmospheric air. Repeated measurements on alanine exhibited a precision of ± 0.11‰ and ± 0.12‰ for $\delta^{13}C$ and $\delta^{15}N$ values, respectively. Commercial standards, including alanine, wheat flour, and corn flour from IsoAnalytical Lab (Crew, UK), as well as IAEA-N-1, IAEA-N-2, IAEA-CH3 cellulose and USGS24 graphite from National Institute of Standard and Technology (Gaithersburg, USA), were used for a multipoint calibration.

2.2 | Mathematical corrections of $\delta^{13}C$ values

Five different mathematical equations, previously published, were used to estimate $\delta^{13}C$ ($\delta^{13}C_{corrected}$) from the $\delta^{13}C$ measured in bulk samples ($\delta^{13}C_{bulk}$). The first equation (Equation 1) was this described in Kiljunen et al.[36]:

$$\delta^{13}C_{corrected=}\delta^{13}C_{bulk}+D\left[\frac{I+3.90}{\left(1+\left(\frac{287}{L}\right)\right)}\right] \quad (1)$$

where D is the isotopic difference between proteins and lipids, here estimated to 7.018[36], I is a constant (0.048[36]), and L is the percentage of lipid in the sample estimated from Equation (1′):

$$L=\frac{93}{1+(0.246\times(C:N_{bulk})-0.775)^{-1}} \quad (1)$$

where $C:N_{bulk}$ is the C:N ratio of the bulk sample.

The second equation (Equation 2) was an equation for aquatic organisms from Post et al.[16]:

$$\delta^{13}C_{corrected}=\delta^{13}C_{bulk}-3.32+0.99\times C:N_{bulk} \quad (2)$$

The third equation (Equation 3) was described by Shipley et al.[37]:

$$\delta^{13}C_{corrected=}\alpha+\beta\times\delta^{13}C_{bulk} \quad (3)$$

where α is the intercept and β the slope of the linear regression between $\delta^{13}C_{corrected}$ and $\delta^{13}C_{bulk}$ estimated from our data.

Finally, the fourth (Equation 4) and fifth (Equation 5) equations were described by Logan et al[17] with Equation (4) and Equation (5):

$$\delta^{13}C_{corrected}=\delta^{13}C_{bulk}+\gamma+\varepsilon\times\ln(C:N_{bulk}) \quad (4)$$

where γ is the intercept and ε the slope of the linear regression between $\delta^{13}C_{corrected}-\delta^{13}C_{bulk}$ and $C:N_{bulk}$ estimated from our data.

$$\delta^{13}C_{corrected}=\delta^{13}C_{bulk}+\frac{a\times C:N_{bulk}+b}{C:N_{bulk}+c} \quad (5)$$

where a is the difference in carbon isotopic composition between proteins and lipids, estimated to 6 by Logan et al.,[17] and b and c are two constants estimated from our data as described in Logan et al.[17]

2.3 | Statistical analyses

First, the effect of the lipid extraction treatment on $\delta^{13}C$, $\delta^{15}N$, and C:N values was evaluated by comparing the lipid-free values to the bulk values using a paired t-test of student. When a significant difference between $\delta^{13}C$, $\delta^{15}N$, and C:N values of the lipid-free and bulk samples was observed, the lipid extraction treatment was considered to affect the $\delta^{13}C$, $\delta^{15}N$, and C:N values.

The effectiveness of each mathematical equation was evaluated using linear models between the $\delta^{13}C$ measured after lipids extraction ($\delta^{13}C_{lipid\text{-}free}$) and the five different $\delta^{13}C_{corrected}$ estimated from the $\delta^{13}C_{bulk}$ with mathematical equations. Two criteria were used to evaluate the effectiveness of each mathematical equations. First, the percentage of deviance explained by the linear model (R^2) with values closest to 1 reflecting highest concordance between $\delta^{13}C_{lipid\text{-}free}$ and $\delta^{13}C_{corrected}$. Second, when the values of the slope and intercept of the linear models did not significantly differ from 1 and 0 respectively, the $\delta^{13}C_{corrected}$ was considered robust as it did not systematically under- or over-estimate the $\delta^{13}C_{corrected}$ compared to $\delta^{13}C_{lipid\text{-}free}$ in the whole range of variation of $\delta^{13}C_{corrected}$. To evaluate the potential effect of habitat (river versus lagoon) and capture site on the estimation of $\delta^{13}C_{corrected}$, these two variables were added in the model as covariates.

Two different approaches were developed for the different mathematical equations. For Equations (1) and (2), the equation parameters were constants and estimated from the literature. In this case, $\delta^{13}C_{corrected}$ was calculated using these constants and $C:N_{bulk}$ ratio for every samples and then compared to $\delta^{13}C_{lipid\text{-}free}$ as explained above. For Equations (3), (4), and (5), the equations parameters were estimated from our data. This estimation was performed on a subset of our data representing 70% of our total data. The subset was selected using a stratified sampling strategy by sampling site. Finally, the $\delta^{13}C_{corrected}$ was estimated from the estimated parameters and compared to the $\delta^{13}C_{lipid\text{-}free}$ on the remaining 30% of our data.

All statistical analyses were performed in the R environment (version 4.1.2).[38]

3 | RESULTS

3.1 | Effects of lipid extraction

For all habitats considered together, lipid extraction resulted in significantly higher $\delta^{13}C$ values than those obtained from bulk samples (paired t-test; $t_{332}=-69.6$; $p<0.001$; Figure S4; Table S2). The mean increase of $\delta^{13}C$ values was 3.3‰. Similarly, the C:N ratios of the bulk samples were significantly higher than those of lipid-free samples (paired t-test; $t_{332}=36.9$; $p<0.001$); the mean C:N ratio of the bulk samples was 7.8 compared to 3.4 for the lipid-free sample. Finally, lipid extraction also had a small, yet significant, increase of $\delta^{15}N$ values between the bulk and the lipid-free samples (paired t-test; $t_{332}=-14.1$; $p<0.001$; Figure 1A). The mean increase of $\delta^{15}N$ values was 0.23‰ (Table S2; Figure 1A).

3.2 | Evaluation of mathematical corrections

The five mathematical models used to estimate $\delta^{13}C_{corrected}$ values were very accurate (Figure 1B–F; Table S3) with R^2 of the linear regression between $\delta^{13}C_{corrected}$ and $\delta^{13}C_{lipid\text{-}free}$ values ranging from 0.92 for Equation (2) (Figure 1C) to 0.98–0.99 for the four others. The habitat type (lagoon or river) significantly influenced the $\delta^{13}C_{corrected}$ values in Equation (2) and Equation (4) ($p \le 0.03$), while its effect remained unsignificant for the other three equations ($p \ge 0.11$). Conversely, the site-specific effect was significant on $\delta^{13}C_{corrected}$ for all equations ($p \le 0.03$), but only slightly improved the models with a

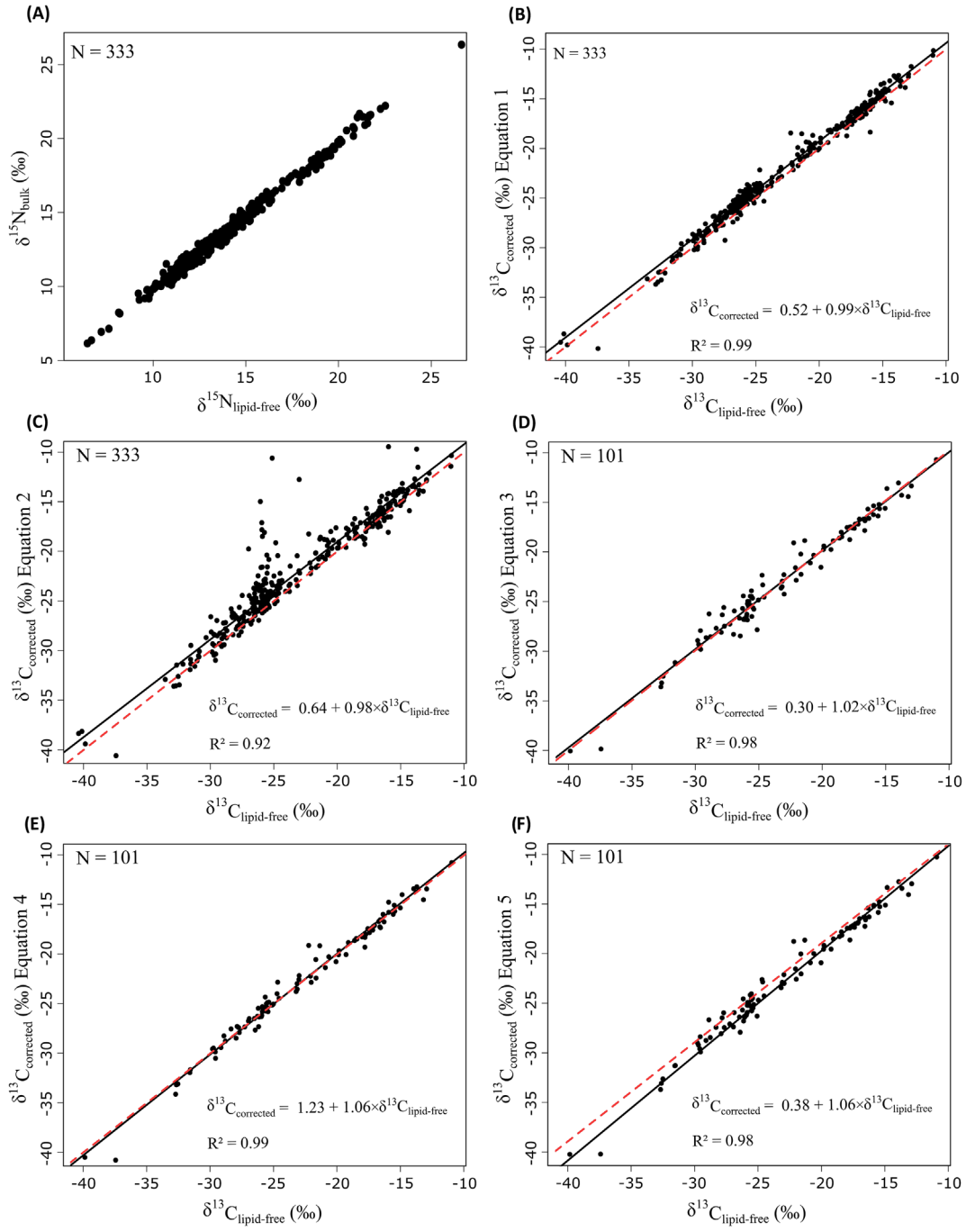

FIGURE 1 $\delta^{15}N$ values of the lipid-free samples ($\delta^{15}N_{lipid\text{-}free}$) compared to the bulk samples ($\delta^{15}N_{bulk}$) (A) and lipid-corrected $\delta^{13}C$ values ($\delta^{13}C_{corrected}$) estimated with the mathematical Equations (1) (B), (2) (C), (3) (D), (4) (E) and (5) (F) compared to the lipid-free $\delta^{13}C$ values ($\delta^{13}C_{lipid\text{-}free}$). The black lines represent the estimate of the linear models between $\delta^{13}C_{corrected}$ and $\delta^{13}C_{lipid\text{-}free}$ for both habitats, and including all sites, the intercept slope and R^2 of each regression are specified in the panels. The dashed red lines represent the 1:1 line. (N) is the number of eels integrated in the linear model and/or the panel. [Color figure can be viewed at wileyonlinelibrary.com]

percentage of deviance explained by the site effect ranging from 0.3% to 1.5%. For all linear regressions, the slope of the linear models did not significantly differ from 1 ($p \geq 0.09$; Table S3). The intercept of the linear models was significantly different from 0 in Equation (1) and Equation (4) ($p \leq 0.04$) but not in other equations ($p \geq 0.33$). Finally, the values of the slope and intercept of the linear models did not significantly differ from 1 and 0 respectively only for Equations (3) and (5). These two equations had an R^2 of 0.98 which was very close to the maximum R^2 of 0.99 for Equations (1) and (4). With the values estimated from our test dataset, Equation (3) was:

$$\delta^{13}C_{corrected} = 2.5 + 0.97 \times \delta^{13}C_{bulk.}$$

and Equation (5) was:

$$\delta^{13}C_{corrected} = \delta^{13}C_{bulk} + \frac{7.018 \times C:N_{bulk} + 35.3}{C:N_{bulk} + 26.9}$$

4 | DISCUSSION

4.1 | Lipid extraction method and effect on $\delta^{15}N$

In our study, cyclohexane was chosen to extract lipids from samples because of its lower toxicity compared to other commonly used solvents such as chloroform–methanol or dichloromethane–methanol. Lipid extraction with cyclohexane was also supposed to prevent the impact on $\delta^{15}N$ values observed with other mixtures.[13,35] C:N ratio is a good proxy of lipid content[16,39] stored in eel muscle. Our results support the efficiency of lipids extraction with cyclohexane because an important and significant decrease of C:N ratio of 4.4 was observed between the bulk and the lipid-free samples. Additionally, the mean C:N value of our samples after the lipid extraction treatment (3.4) was below the 3.5 threshold above which lipid content in tissues do not influence $\delta^{13}C$ values.[16] However, the hypothesis that lipid extraction using cyclohexane does not affect $\delta^{15}N$ values was not confirmed with our data, as a significant, although small, decrease of 0.2‰ was observed in $\delta^{15}N$ values between the bulk and the lipid-free samples. This impact on $\delta^{15}N$ values may be explained by a small loss of proteins during lipids extraction even if apolar solvents such as cyclohexane are not supposed to alter proteins.[40] This increase is slightly superior to the analytical precision estimated to 0.12‰ with repeated measurements on internal standard. The increase of $\delta^{15}N$ values after cyclohexane lipid extraction is probably negligeable to interpret ecological processes. Trophic enrichment factor between a consumer and its food sources is usually 3.4‰,[41,42] and in this study, $\delta^{15}N$ values ranged from 6.2‰ to 26.6‰. To conclude, the $\delta^{15}N$ values are slightly biased by cyclohexane lipid extraction and, even if this bias could be acceptable, a rigorous estimate of $\delta^{15}N$ should be measured on bulk samples.[43]

4.2 | Mathematical lipid correction of $\delta^{13}C$ values

The five tested mathematical equations to estimate lipid-corrected $\delta^{13}C$ values performed very well. The R^2 of the linear models between lipid-corrected $\delta^{13}C$ and lipid-free $\delta^{13}C$ was higher than 0.92. The habitat type (lagoon or river) had little if any effect on the precision of the mathematical corrections. Conversely, the different sampling sites significantly improved the precision of the mathematical corrections with all tested equations. This result support the conclusion of Van Der Merwe et al.,[44] who suggested a site-specific calibration of the mathematical equations to estimate lipid-corrected $\delta^{13}C$ values. However, site-specific calibration had only improved $\delta^{13}C$ values of 1% or less for most of the equation. This improvement is within the analytical precision (0.11‰) of our mass spectrometer.

Finally, two mathematical corrections fulfilled the two criterion used to evaluate the effectiveness of the mathematical correction: Equations (3)[37] and (5).[17] The linear models between the $\delta^{13}C_{corrected}$ and $\delta^{13}C_{bulk}$ for the two equations had the second highest values of R^2 (0.98) and their slopes and intercepts did not differ from 1 and 0, respectively. These two equations can be used to estimate $\delta^{13}C$ from the bulk samples. However, Equation (3) appears to be more robust as there are only two parameters estimated from the data compared to two parameters estimated from the data plus one theoretical constant for Equation (5). The greater number of parameters in Equation (5) probably reduce the robustness of this equation as the potential effect of measurements error on $\delta^{13}C$ estimates are higher.

4.3 | Recommendations

To conclude, we recommend the use of Equation (3) with the following parameters to estimate the values of $\delta^{13}C$ from the bulk samples in European eel:

$$\delta^{13}C_{corrected} = 2.5 + 0.97 \times \delta^{13}C_{bulk}$$

The $\delta^{15}N$ values and C:N ratios can also be measured from the bulk samples and remain unbiased. These estimates are very robust among the different study sites in our study. Consequently, Equation (3) can be used in others study sites within the geographical repartition of European eel not included in our study with a high degree of confidence. In addition to eels, this equation could be widely applied to other species, even if calibration studies similar to ours would be necessary to confirm this hypothesis. Further methodological studies on European eel should focus on the development of non-lethal methods to estimate $\delta^{13}C$ and $\delta^{15}N$. The reduction of scientific sampling on wild population of critically endangered species is today essential. The sampling of non-lethal tissues could include muscle biopsies, blood, fins, or mucus.[45] However, the use of alternative tissues in isotopic ecology requires more laboratory experiments to understand the dynamics of isotopic incorporation, trophic discrimination factors, and routing in eels.

AUTHOR CONTRIBUTIONS

Raphaël Lagarde: Conceptualization; methodology; software; investigation; formal analysis; funding acquisition; writing – original draft. **Christophe Menniti:** Methodology; data curation; investigation; writing – review and editing. **Nils Teichert:** Conceptualization; methodology; investigation; writing – review and editing. **Elsa Amilhat:** Investigation; funding acquisition; writing – review and editing. **Elisabeth Faliex:** Investigation; funding acquisition; writing – review and editing. **Sarah Nahon:** Conceptualization; investigation; validation; data curation; writing – review and editing.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article (Table S5).

ORCID

Raphaël Lagarde https://orcid.org/0000-0001-9809-1673

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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