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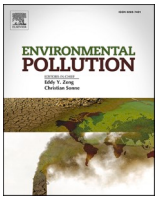

Compound-specific stable nitrogen isotope analysis of amino acids shows that bulk methods provide higher estimates of mercury biomagnification in the Gulf of St. Lawrence☆

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ABSTRACT

Compound-specific stable isotope analysis of amino acids (CSIA-AA) provides a method to estimate baseline $\delta^{15}N$ values of food chains, allowing less biased estimates of trophic positions for organisms. Greater accuracy in trophic positions can improve estimates of contaminant biomagnification. We calculated trophic positions with various CSIA-AA equations for four species of fish and northern gannets (*Morus bassanus*) from the Gulf of St. Lawrence. We examined the effect of CSIA-AA-derived trophic positions on mercury biomagnification metrics (trophic magnification factors (TMF) and biomagnification factors) and compared these with trophic position estimates and metrics obtained from traditional bulk stable isotope analysis. The TMFs for the CSIA-AA equations ranged from 10 to 19, and bulk stable isotope analysis produced TMFs of 43, one of the highest TMFs recorded yet in the literature. Biomagnification factors between prey and northern gannets ranged from 20 to 42 using dietary observations and stable isotope mixing models. Our study demonstrates that discrepancies in biomagnification assessed using different approaches may go undetected when using a single approach.

1. Introduction

Mercury is a naturally occurring metal found in air, water, and soil, which cycles in the environment (Driscoll et al., 2013). Humans have greatly disrupted the natural mercury cycle, drastically increasing emissions through activities such as mining, metal processing, fossil fuel combustion, industry, and small-scale artisanal gold mining (Driscoll et al., 2013; Esdaile and Chalker, 2018). Mercury is a contaminant of concern in ecosystems and food webs because of its potential to cause harm to living organisms. The organic form of mercury, methylmercury, is ecologically relevant because it biomagnifies through food webs, leading to high concentrations in top predators (Atwell et al., 1998; Anderson et al., 2009). Methylmercury can impede reproductive success, cause malformations, change behaviour in maladaptive ways, and even cause death (Friedmann et al., 1996; Scheuhammer et al., 2007; Ackerman et al., 2016). Thus, understanding the dynamics of mercury, both in its inorganic and organic forms (total mercury; THg) through food webs and into top predators is vital to preserving wildlife health.

Bulk stable isotope analysis (bulk SIA) is widely used to study the biotic transfer of contaminants through the environment. They can provide insight into diet, foraging habitats, and the transfer efficiency of contaminants through food webs (Hobson et al., 2002). $\delta^{15}N$ values are commonly linked to trophic position, as $\delta^{15}N$ values increase predictably with each trophic step (Peterson and Fry, 1987). As such, $\delta^{15}N$ can be used to calculate biomagnification metrics, linking trophic position to

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THg concentrations. $\delta^{13}C$ values are used to infer foraging habitat, where signatures are differentiated by the main type of vegetation due to differences in photosynthetic pathways (DeNiro and Epstein, 1978). Higher $\delta^{13}C$ values are associated with benthic/inshore algae and lower $\delta^{13}C$ values are associated with pelagic phytoplankton (France, 1995). $\delta^{34}S$ values help differentiate between benthic, inshore, and offshore habitats and further refine habitat estimates provided by $\delta^{13}C$ values (Connolly et al., 2004). $\delta^{34}S$ values are lower in benthic environments due to sulfate reduction, and are higher in the oceanic water column (Connolly et al., 2004; Elliott and Elliott, 2016). When combined, these three SIA ratios can provide powerful insight into food web dynamics and the movement of THg from the base to the top of food webs (Cabana and Rasmussen, 1994).

Bulk SIA is particularly useful for assessing a consumer's trophic position when calculating biomagnification (Won et al., 2018). Biomagnification metrics rely on the trophic position and contamination level of each organism in the food web to assess the rate of increase of contaminants. However, bulk SIA may provide a skewed estimate of trophic positions, due to baseline differences (e.g. primary producer) $\delta^{15}N$ signatures (McClelland and Montoya, 2002; McMahon and McCarthy, 2016). These baseline differences can be due to biotic (e.g. nitrogen fixation, denitrification) or abiotic (e.g. runoff, atmospheric deposition) factors, and are most evident when studying organisms that forage in or originate from multiple locations (Montoya, 2007). The analysis of stable isotope values of amino acids (compound-specific stable isotope analysis; CSIA-AA) is meant to remove this bias stemming from baseline differences (McClelland and Montoya, 2002). The $\delta^{15}N$ values of certain amino acids (AAs) increase in a stepwise manner with trophic position, as is expected with bulk $\delta^{15}N$ values (i.e. "trophic AAs", most commonly glutamic acid; McClelland and Montoya, 2002). The $\delta^{15}N$ values of other AAs do not increase significantly between trophic levels (i.e. "source AAs", most commonly phenylalanine; Chikaraishi et al., 2009). When using trophic and source AAs in combination, $\delta^{15}N$ signatures can be corrected for potential baseline differences to estimate an organism's trophic position. Comparisons of trophic positions for organisms from different habitats may therefore be more robust (McClelland and Montoya, 2002; Chikaraishi et al., 2009), and improve contaminant biomagnification relationships. Previous work on THg and methylmercury biomagnification has shown CSIA-AA produces higher biomagnification metrics than bulk SIA approaches in some studies (Elliott et al., 2021; Zhang et al., 2021), but with other contaminants, the opposite relationship is observed (Kobayashi et al., 2019). The mechanisms behind these relationships have not yet been identified.

Seabirds have been used extensively as health indicators of marine ecosystems (Furness and Camphuysen, 1997; Montevecchi, 2007; Elliott and Elliott, 2013; Braune et al., 2019; Bianchini et al., 2022). Particularly, their foraging ecology has helped predict important ecosystem shifts, such as fish stock collapse and shifting ranges for forage fish (Montevecchi, 2007; Guillemette et al., 2018). Their use as indicators of marine food web contamination is also highly relevant, as most seabirds are piscivorous predators, and are likely to accumulate contaminants at relatively high concentrations (Le Croizier et al., 2022). Northern gannets (*Morus bassanus*; henceforth "gannets") are large, piscivorous seabirds that travel great distances during the breeding season to feed their nestling (Garthe et al., 2007; Montevecchi et al., 2012; Guillemette et al., 2018). In the Gulf of St. Lawrence (Atlantic Canada), they feed on commercially relevant fish such as Atlantic mackerel (*Scomber scombrus*), Atlantic herring (*Clupea harengus*), as well as on capelin (*Mallotus villosus*), and sandlance (*Ammodytes* spp.) (Guillemette et al., 2018). During the breeding season, gannets regurgitate spontaneously when stressed at their nest site (Guillemette et al., 2018). Thus, northern gannets provide researchers with the non-lethal opportunity to study THg biomagnification to a top predator from their regurgitated prey samples.

Our objectives were to 1) estimate trophic positions of fish and gannets from the Gulf of St. Lawrence using CSIA-AA, and 2) contrast CSIA-AA with bulk SIA when assessing the biomagnification of THg in this food chain. We hypothesized that THg biomagnification would be greater when using CSIA-AA compared to bulk SIA, as has been reported in previous Hg studies (Elliott et al., 2021; Zhang et al., 2021).

2. Methods

2.1. Sample collection

Fieldwork was conducted at the gannet colony on Bonaventure Island in Percé, Québec, Canada during the 2017 and 2018 breeding seasons. Gannets and their nests were continuously monitored during the breeding season (June to August) and were caught routinely. During captures, blood (less than 5 mL) was drawn from the gannets' medial metatarsal vein and centrifuged in the field as described in Pelletier et al. (2023). The whole blood was immediately separated into the red blood cell and plasma fractions in cryotubes and placed in liquid nitrogen and soon after stored in −80 °C freezers. Only the red blood cell fraction was used for our study (henceforth referred to as "blood"). Regurgitated fish samples were collected opportunistically at the gannet colony. During captures, gannets may regurgitate due to stress, allowing for easy sampling of their stomach contents (Guillemette et al., 2018). Contents of the regurgitations were sorted and quantified by species and quantity (i.e. number of complete fish, heads, and tails). Regurgitations were frozen in a standard freezer by the end of the same day of collection and later stored long-term in −20 °C freezers until sample processing. Seabird red blood cells have a longer half-life, representing diet over the weeks preceding the sampling date, while fish muscle has a longer turnover rate, representing diet over the previous months (Vander Zanden et al., 2015; Shoji et al., 2021). Together, the fish muscle and gannet red blood cells provide insight into gannet THg exposure during the breeding season, when gannets are present and foraging in the Gulf of St. Lawrence.

2.2. Sample analysis

Regurgitations were thawed and dissected to retrieve approximately one cubic cm of undigested dorsal muscle tissue from each species present in the sample. We collected muscle from four species of fish; Atlantic mackerel ($n = 21$), Atlantic herring ($n = 13$), capelin ($n = 22$), and sandlance ($n = 17$). The fish muscle tissue and gannet blood ($n = 40$) were freeze-dried using an FTS Flexi-Dry compact freeze-dryer (Triad Scientific) for 48 h, powdered, and homogenized.

2.2.1. Mercury analysis

We analysed all samples (fish muscle and freeze-dried gannet blood) for THg following US EPA method 7473. Briefly, 25–40 mg of sample was combusted at 650 °C, Hg was accumulated on a gold-coated sand amalgamator followed by heat desorption and quantification by CV-AAS (cold vapor atomic absorbance spectrometer; Direct Mercury Analyser, DMA-80 evo, Milestone). Certified reference materials TORT-3 (lobster hepatopancreas) and DORM-4 (fish protein), certified by the National Research Council of Canada were analysed and had mean (±sd) recoveries of 100.0 ± 2.0% (n = 22) and 99.3 ± 2.8%, (n = 6), respectively. The DMA-80 detection limit was 0.02 ng/g, while the quantification limit was 0.06 ng/g. We assumed that around 100% of THg was methylmercury in seabird red blood cells and in fish muscle (as reported in Lavoie et al., 2010 (seabird red blood cells) Carbonell et al., 2009 (fish muscle)).

2.2.2. Bulk stable isotope analysis

Fish muscle and gannet blood were analysed for bulk SIA of nitrogen ($\delta^{15}N$ values), carbon ($\delta^{13}C$ values), and sulfur ($\delta^{34}S$ values) at the Ján Veizer Stable Isotope Laboratory (Ottawa, Ontario, Canada). Stable isotopes of carbon, nitrogen and sulfur are reported in Delta notation $\delta = ((R_x - R_{std}))/R_{std})*1000$ where R is the ratio of the abundance of the

heavy to the light isotope, x denotes sample and std is an abbreviation for standard. $\delta^{15}N$ is the ratio of $^{15}N/^{14}N$, $\delta^{13}C$ the ratio of $^{13}C/^{12}C$, and $\delta^{34}S$ the ratio of $^{34}S/^{32}S$. The samples were combusted in a Vario EL Cube (Elementar, Germany) EA-IRMS interfaced via Conflo IV to Delta Advantage isotope ratio mass spectrometer (Thermo, Germany - ($\delta^{15}N$ and $\delta^{13}C$) or the Delta Plus XP IRMS (ThermoFinnigan, Germany). The raw isotope data were referenced to the VPDB (carbon), AIR (nitrogen), and scales using six calibration standards (IAEA-N1, IAEA-N2, USGS-40, USGS-41, NBS-22, and IAEA-CH-6). Four internal check standards were included in the analytical runs: C-51 Nicotiamide ($\delta^{13}C$: −23.0‰; $\delta^{15}N$: +0.1‰), C-52 mix of ammonium and sucrose ($\delta^{13}C$: −11.9‰; $\delta^{15}N$: +16.6‰), C-54 caffeine ($\delta^{13}C$: −16.6‰; $\delta^{15}N$: −34.5‰), and AG-2 argentite ($\delta^{34}S$: −0.6‰). The analytical error was monitored using a blind standard (C-55, glutamic acid, $\delta^{13}C$: −4.0‰; $\delta^{15}N$: −28.5‰) and was better than ±0.1‰ for carbon and nitrogen. Ten percent of samples were randomly duplicated. Standard deviations for duplicates of gannet blood averaged 0.1‰ for $\delta^{13}C$, 0.06‰ for $\delta^{15}N$ and 0.3‰ for $\delta^{34}S$ and standard deviations for duplicates of fish muscle averaged 0.1‰ for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$. $\delta^{13}C$ values were mathematically normalized for lipid content according to the methodology detailed by Post et al. (2007). For the mean values of bulk SIA and THg for all species, refer to Table 1.

2.2.3. Compound-specific stable isotope analysis

Compound-specific stable isotope analysis of amino acids was carried out for a subset of fish muscle and gannet blood (n = 39; see Table S1 in Supplemental Materials) at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks (Alaska, USA). These methods are detailed elsewhere, by Barst et al. (2020, 2021). Briefly, samples were digested in 6 M HCl for 20 h at 100 °C. A 6:5 mixture of hexane and dichloromethane was added to each sample and vortexed. The acidic fraction of each sample was spiked with an internal standard of nor-Leucine (nLeu) and evaporated under nitrogen gas. Amino acids were then methylated with an acidified methanol solution and heated at 75 °C for 1 h. The samples were nitrogen evaporated again, until near dryness, and an acetylation mixture of triethylamine, acetone, and acetic anhydride was added. Samples were heated again at 60 °C for 10 min and returned to the nitrogen evaporator until near dryness. A mixture of potassium phosphate buffer and chloroform was then added to the samples, and they were centrifuged to isolate the organic phase and purify the derivatized AAs. The samples were returned to the nitrogen evaporator to eliminate the chloroform, and ethyl acetate was added to the vials once dry. Samples were finally spiked with internal standards of caffeine before being capped and analysed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to obtain $\delta^{15}N$ values of individual AAs. The instrumentation and parameters are described in detail in Barst et al., 2020; Barst et al., 2021. The analysis yielded data for seven trophic AAs (alanine, isoleucine (Ile), aspartic acid, proline (Pro), glutamic acid (Glu), valine, leucine) and a single source AA (phenylalanine (Phe)) which were consistently reliable (low standard deviation between triplicates). All samples were run in triplicate and the mean $\delta^{15}N$ value for each AA from a sample was used in data analysis.

The average nLeu (internal standard) $\delta^{15}N$ value in all samples was 18.7 ± 0.5 ‰ (mean ± sd) which corresponds to the known value of 19.3 ‰, and the average measured for caffeine was −3.1 ± 0.2 ‰ which corresponds to the known value of −3.3 ‰. Two mixed standards of AAs with different known $\delta^{15}N$ values were derivatized and analysed with each sample batch (n = 3). The measured values (n = 12 per AA) for both standards did not significantly differ from the expected values (Pearson's correlation test: standard 1: $t = 52.6$, $p < 0.001$; standard 2: $t = 52.3$, $p < 0.001$). We also digested and derivatized subsamples of the same sample (mackerel: 17–30) with each batch which we used to verify there was no systematic bias among batches: the internal standards nLeu (18.8 ± 0.5 ‰) and caffeine (−3.0 ± 0.2 ‰) corresponded to the known values in all batches.

2.3. Statistical analysis

To compare THg within a species between 2017 and 2018 and between gannet sexes, we verified there were no significant differences in THg data by running Shapiro-Wilks tests to verify normality and conducted student t-tests (or Wilcox tests for non-normally distributed THg data: gannets and sandlance). We used linear mixed-effects models (package lme4; Bates et al., 2009) to determine which stable isotopes best predicted log-transformed THg and set species as a random effect. We then compared all models using Akaike's information criterion for small sample sizes (AICc) to identify the best-fitted model (Burnham and Anderson, 2004).

To compare the bulk SIA and CSIA-AA derived TPs (see Trophic position calculations section below), we used reduced major axis (RMA) regression because both variables were dependent and contained uncertainty (error) in their measurements (Harper, 2016).

2.4. Trophic position calculations

2.4.1. Bulk stable isotopes

We calculated the trophic position (TP) for each sampled organism based on bulk $\delta^{15}N$ values, using capelin as a benchmark for which trophic position had been reported by previous work in the Gulf of St. Lawrence (Lavoie et al., 2010). Trophic positions for all fish were calculated using a modified trophic level equation from Lavoie et al. (2010) and capelin $\delta^{15}N$ value:

$$TP_{bulk\ fish} = \frac{\delta^{15}N_{fish} - \delta^{15}N_{capelin}}{3.4} + 3.9 \quad (1)$$

where $\delta^{15}N_{consumer}$ is the $\delta^{15}N$ value of the fish and $\delta^{15}N_{capelin}$ is the mean $\delta^{15}N$ value for capelin in this study (12.7 ± 0.4 ‰; mean ± sd; n = 22), 3.4 ‰ is the typical isotopic trophic discrimination factor (TDF) used for most organisms (Post, 2002) and 3.9 is the estimated TP for our reference organism (Lavoie et al., 2010). For gannets, we used a trophic discrimination factor of 3.0 ‰, determined for double-crested cormorants (*Phalacrocorax auritus*) in captive studies (Craig et al., 2015):

$$TP_{bulk\ gannet} = \frac{\delta^{15}N_{gannet} - \delta^{15}N_{capelin} - 3.0}{3.4} + 4.9 \quad (2)$$

All components of the equation are the same as in Equation (1), but the estimated TP for our reference organism (capelin: 3.9) is adjusted for trophic fractionation for gannets (4.9).

Table 1
Sample size and mean (±sd) values by species for $\delta^{15}N$, $\delta^{13}C$, $\delta^{34}S$, and total mercury concentration ([THg] dry weight) for samples of fish muscle and northern gannet red blood cells collected in 2017 and 2018 combined.

Taxa	n	$\delta^{15}N$ (‰)			$\delta^{13}C$ (‰)			$\delta^{34}S$ (‰)			[THg] (μg/g)		
Northern gannet (*Morus bassanus*)	40	15.0	±	0.1	−18.6	±	0.2	19.8	±	0.5	2.389	±	0.547
Atlantic mackerel (*Scomber scombrus*)	21	13.3	±	0.7	−19.3	±	0.7	18.7	±	0.6	0.207	±	0.080
Atlantic herring (*Clupea harengus*)	13	13.1	±	0.3	−19.1	±	0.2	19.0	±	0.2	0.208	±	0.081
Capelin (*Mallotus villosus*)	22	12.5	±	0.6	−19.5	±	0.3	18.8	±	0.5	0.037	±	0.019
Sandlance (*Ammodytes* spp.)	17	11.2	±	0.5	−20.2	±	0.7	19.3	±	0.6	0.041	±	0.024

2.4.2. Compound-specific stable nitrogen isotopes of amino acids

To test the CSIA-AA equations used to calculate TPs for our study organisms, and compare them to TP_{bulk}, we tested multiple equations from the literature on our fish and gannet samples. The first CSIA-AA equation we tested was from Chikaraishi et al. (2009) and has been widely used in the literature:

$$TP_{Glu-Phe} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta}{TDF} + Y \quad (3)$$

where $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ are the measured $\delta^{15}N$ values for the glutamic acid and phenylalanine AAs respectively, β is the difference between trophic and source AAs in primary producers, Y is the base TP of the primary producer, and TDF is the trophic discrimination factor, or how much the trophic AA's isotopic signature will change between a prey and its predator. Most of the TP equations we tested are modifications of Equation (3), and all β, Y, and TDF values we used can be found in Table 2. The second category of TP equation we tested used the average of multiple trophic AAs which are each normalized relative to Glu. This equation was suggested by Nielsen et al. (2015), who conducted a meta-analysis of CSIA-AA nitrogen data from an array of taxa, and proposed that this method would remove bias associated with the use of a single AA. The equation was modified to reflect the use of a single source AA (Phe):

$$TP_{Mult.\ AAs} = \left(\frac{\sum \left(\delta^{15}N_x i + \delta^{15}N_{diff}\, i \right) \Big/ X - \delta^{15}N_{Phe} - \beta_{Glu-Phe}}{TDF_{Glu-Phe}} \right) \quad (4)$$

where $\delta^{15}N_x i$ and $\delta^{15}N_{diff} i$ are the trophic AA $\delta^{15}N$ value, and the correction term which normalizes a given trophic AA relative to Glu, respectively. All the correction terms used for this equation were from Nielsen et al. (2015) and are listed in Table 2. The term *X* is the number of trophic AAs used to calculate an organism's trophic position, and $\beta_{Glu\text{-}Phe}$ and $TDF_{Glu\text{-}Phe}$ are the specific terms used for Glu and Phe, because all trophic AAs were normalized relative to Glu. Equation (5) stems from Equation (4) and only uses Glu as the trophic AA. We also tested the use of Pro and Ile as the trophic AAs for gannets and fish, respectively (Equations (6) and (7)), due to their good performance at separating these organisms into the appropriate expected TPs when we plotted the trophic isoclines for the trophic AAs (as in Chikaraishi et al., 2014; see Table 1 and Fig. S1 in the Supplemental Materials). We also identified three TP equations (Equations (8)–(10)) that used seabird-derived β and TDF values and have been used previously for seabirds (see Table 2).

We tested combinations of fish-specific and seabird-specific TP equations, based on the literature, by plotting the experimental TPs assessed using Equations (3)–(10) with bulk TP (Equations (1) and (2)); using reduced major axis regression) and with log(THg) (linear regression), as both these relationships have been established in the literature (see, for example, Lavoie et al., 2013; Nielsen et al., 2015; Ohkouchi et al., 2017). The three best-performing equation combinations in terms of R^2 with log(THg) and bulk TP were selected for the biomagnification assessment. We then calculated the mean and standard deviation of the absolute difference between TP calculated using bulk SIA and using CSIA for the three equations.

2.5. Biomagnification assessment

To assess biomagnification within the fish-to-gannet food chain, we plotted the three best-performing TP equations against log-transformed THg values for the four species of fish and gannets.

$$Log_{10}[THg] = b(TP) + a \quad (11)$$

where TP was the organism's trophic position calculated using one of the three CSIA-AA or the bulk SIA TP equations, *b* was the slope and *a* was the intercept of the relationship between log(THg) and TP. The trophic magnification factor (TMF), also referred to as the food web magnification factor, is used to indicate the average change in contaminant concentrations at each step of the food chain (Riget et al., 2007). We calculated the TMF for each of the four TP equations used (three best-performing CSIA-AA equations and the bulk SIA equation) using Equation (12) from (Lavoie et al., 2013):

$$TMF = 10^b \quad (12)$$

where *b* is the slope from Equation (11). We also calculated the biomagnification factor (BMF) for gannets in both 2017 and 2018, while accounting for the composition of gannet diet in each year, as in Lavoie et al. (2010). The BMF is the ratio of a contaminant concentration in a consumer relative to the concentration in its diet (Riget et al., 2007).

$$BMF = \frac{[THg_{gannet}]}{\sum_{i=1}^{n} \left([THg_{fish\ i}] * f_{fish\ i} \right)} \quad (13)$$

Where THg_{gannet} and THg_{fish} are the raw THg concentrations for the gannets and fish, respectively, and f_{fish} is the proportion of gannet diet that each fish species made up for the targeted year. We used f_{fish} values from two sources. First, we used regurgitation observations recorded in the field, that described the proportions of each of the four species of fish in gannet diet. Secondly, we used f_{fish} values obtained from stable isotope mixing models.

We ran stable isotope mixing models to determine gannet diet composition, because regurgitation observations may be biased towards the prey items gannets are providing their chicks, and diets differing between adults and their chicks have been documented in seabirds (Brown and Ewins, 1996; Barrett et al., 2007). We used the MixSIAR package (Stock et al., 2018), which resolves stable isotope mixing equations using Bayesian framework using JAGS (Just Another Gibbs Sampler). We included the mean and standard deviation of bulk $\delta^{15}N$,

Table 2
Compound-specific trophic position equations tested in this study for fish species and northern gannets. The listed variables include the trophic and source amino acids (AA), the trophic discrimination factor (TDF), β value, and base trophic position of primary producers (Y). An additional column includes the correction terms required when using Equation (4), to normalize individual AAs to Glutamic Acid (Glu). "Target Organisms" indicates the taxa these equations have been tested with in the literature.

Equations #	Trophic AA	Source AA	TDF	β	Y	Target Organisms	Source Article
Eq. (3)	Glu	Phe	7.6	−3.4	1	Fish, Invertebrates	Chikaraishi et al., (2009)
Eq. (4)	Glu, Ala, Ile, Val, Asp, Pro	Phe	6.6	−2.9	1	All taxa	Nielsen et al., (2015) [1]
Eq. (5)	Glu	Phe	6.6	−2.9	1	All taxa	Nielsen et al., (2015)[1]
Eq. (6)	Pro	Phe	5.7	−3.1	1	This study: Seabirds	Chikaraishi et al., (2009)
Eq. (7)	Ile	Phe	4.4	−2.9	1	This study: fish	Chikaraishi et al., (2009)
Eq. (8)	Glu	Phe	3.5	−3.4–7.6	2	Seabirds	Wu et al., (2018)
Eq. (9)	Glu	Phe	5.39	−3.4	1	Seabirds	Hebert et al., 2016
Eq. (10)	Glu	Phe	6.2	−3.4–4	2	Seabirds	Quillfeldt and Massello 2020

[1] Correction terms($\delta^{15}N_{diff}i$) to standardize all trophic AAs relative to Glu: Ala = 0.59, Ile = 2.63, Val = −3.35, Asp = −1.78, Pro = −1.39. The correction terms were omitted in Equation (5) because Glu was the only trophic AA used in that iteration of the equation.

$\delta^{13}C$, $\delta^{34}S$, and nitrogen, carbon, and sulfur composition (%) for all the fish and gannet samples for 2017 and 2018 in our mixing models. Including a third isotope ($\delta^{34}S$) in our mixing models allowed higher resolution to differentiate between prey sources in gannet diet (Connolly et al., 2004). We used TDFs calculated from double-crested cormorants that were fed catfish (*Ictalurus punctatus*) in controlled experiments (Craig et al., 2015; $\delta^{15}N = 3.0$, $\delta^{13}C = -0.8$, $\delta^{34}S = 0.1$) for our herring and mackerel, and TDFs calculated for Peruvian boobies (*Sula variegata*) feeding on Peruvian anchovy (*Engraulis ringens*) for our capelin and sandlance samples (Le Croizier et al., 2022; $\delta^{15}N = 2.3$, $\delta^{13}C = 1.3$, $\delta^{34}S = 0.1$). Species-specific TDFs were not available in the literature for gannets, so we used factors for genetically close species (Peruvian boobies) and for fish species constituting gannet prey (catfish substituting for herring and mackerel, and anchovy as a stand-in for smaller capelin and sandlance). We also included our regurgitation observations of diet for both studied years as a prior to inform our model. We ran the jags model with a chain length of 100 000, burn = 50 000, thin = 50, and chains = 3. Once we calculated the dietary proportion of each of our prey species (mackerel, herring, capelin, and sandlance) in gannet diet from our isotope mixing models, we used these to calculate the BMF and compare them to the BMFs calculated based on dietary observations.

All statistical analyses were run in R 4.1.2 (R Core Team, 2021

3. Results

3.1. Trophic position calculations

While testing combinations of TP equations, Equation (4) from Nielsen et al. (2015), had the highest coefficient of determination with both bulk TP and log(THg) between the fish-specific TP equations (see Table S2). For this reason, when we tested the bird-specific equations on the gannets, we used Equation (4) to calculate the TPs for all fish, to find the best TP equation combination for both taxa. Fig. 1 shows the relationship between TP for all organisms calculated using bulk SIA and CSIA-AA equations. In the case of the gannet samples, bulk SIA provided much more consistent TP estimates (y-axes Fig. 1), while CSIA-AA TP estimates were much more variable (x-axes Fig. 1). This was not observed for the fish samples in our study. Additionally, using the same CSIA-AA equation for seabirds and fish was not appropriate (Fig. 1). For example, using Equations (4) and (5) for both taxa yielded steep slopes due to the placement of gannets at very low trophic positions. The best-performing TP equation combinations based on the R^2 for reduced major axis regression were Equations (8) & (4) (henceforth "Eq. (8)"), Equations 9 & 4 ("Eq. (9)"), and Equations (10) & (4) ("Eq. (10)"). The average difference in TP estimates calculated using bulk SIA and CSIA-AA was close to half a trophic level for fish (means ± sd: Eq. (4) = 0.65 ± 0.17) and more variable for gannets (Eq. (8) = 0.06 ± 0.40, Eq. (9) = 0.56 ± 0.26, Eq. (10) = 0.60 ± 0.23). Average TPs for the three CSIA-AA and bulk equations can be found in Supplemental Table S3. These three equations also best correlated with log(THg), as is expected

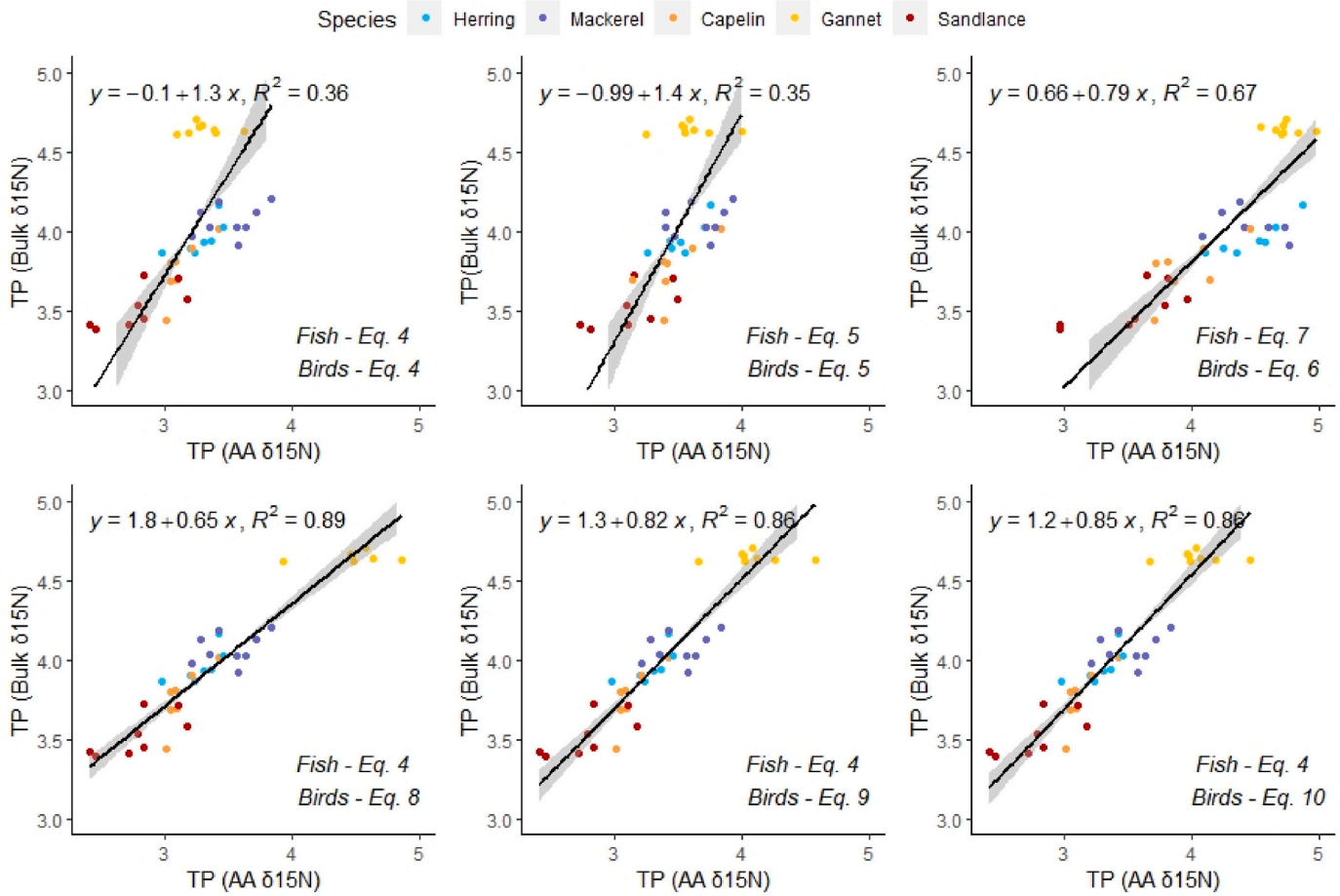

Fig. 1. Relationship between trophic positions (TP) calculated using bulk stable nitrogen isotopes ($\delta^{15}N$ values) and TPs calculated from experimental equations using compound-specific stable isotope analysis of amino acids (AA) in northern gannets and their prey. The shaded region represents the 95% confidence interval of the slope of each relationship.

with biomagnifying contaminants such as Hg (Fig. 2; Lavoie et al., 2013). Eqs. (8)–(10) were used to assess the biomagnification potential of THg in the fish-to-gannet food chain.

3.2. THg concentrations in gannets

In order to compare our THg concentrations in gannet red blood cells to other studies and toxicity thresholds, we converted our dry-weight red blood cell concentrations to a wet-weight, whole blood equivalent following the methodology detailed in Ackerman et al. (2016). In our study, all sampled gannets had concentrations of THg which placed them in the low-risk category, after conversion from dry to wet weight concentrations (mean ± sd of THg in 2017 = 0.40 ± 0.05 μg/g ww, n = 20 and 2018 = 0.28 ± 0.04 μg/g ww, n = 20; see Supplemental Fig. S2). THg concentrations were significantly different between years (t = 9.0, df = 35.8, p = <0.0001) but not between sexes (t = −1.1, df = 24.0, p = 0.3).

3.3. Biomagnification of mercury

Our linear mixed-effects models suggest that when taking the effect of species on THg concentrations into account, $\delta^{15}N$ value is the most important bulk SIA ratio to consider (Table 3). The values of $\delta^{15}N$ (11.1–15.2 ‰), $\delta^{13}C$ (−21.2 to −17.9 ‰), and $\delta^{34}S$ (17.7–20.9 ‰) did not show a large amount of variation among our samples. Our values of $\delta^{15}N$ in AAs were more varied (Glu: 17.7–27.2 ‰, Ala: 13.5–25.2 ‰, Val: 16.8–27.9 ‰, Ile: 15.1–25.3 ‰, Asp: 13.7–28.4 ‰, Pro: 18.8 to 5.6 ‰) in terms of trophic AAs. Baseline values varied on a similar scale to the bulk stable isotopes (Phe: 1.3–5.6 ‰).

The trophic magnification slopes (*b*) and the TMF values for each of the four TP equations ranged from 10.0 to 42.7 (Table 4) and were all

Table 3
Linear mixed-effects models to determine the influence of stable isotopes of $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ on log-transformed total mercury (log_{10}(THg)) concentrations in four species of fish (Atlantic mackerel, Atlantic herring, capelin, and sandlance) and one seabird, northern gannets. Akaike's information criterion for small sample sizes (AICc) and the difference between the most supported and other models (ΔAICc) are reported.

model	df	ΔAICc	Akaike Weight
$log_{10}(THg) \sim \delta^{15}N + (1 \mid Species)$	4	0.00	0.36
$log_{10}(THg) \sim \delta^{15}N + \delta^{34}S + (1 + \delta 15\ N \mid Species)$	7	1.61	0.16
$log_{10}(THg) \sim \delta^{15}N + \delta^{13}C + (1 + \delta^{15}N \mid Species)$	7	1.72	0.15
$log_{10}(THg) \sim \delta^{15}N + \delta^{34}S + (1 \mid Species)$	5	2.20	0.12
$log_{10}(THg) \sim \delta^{15}N + \delta^{13}C + (1 \mid Species)$	5	2.20	0.12
$log_{10}(THg) \sim \delta^{15}N + \delta^{13}C + \delta^{34}S + (1 + \delta^{15}N \mid Species)$	8	3.64	0.06
$log_{10}(THg) \sim \delta^{15}N + \delta^{13}C + \delta^{34}S + (1 \mid Species)$	6	4.44	0.04
$log_{10}(THg) \sim \delta^{13}C + \delta^{34}S + (1 \mid Species)$	5	51.15	0.00
$log_{10}(THg) \sim \delta^{15}N$	3	109.98	0.00

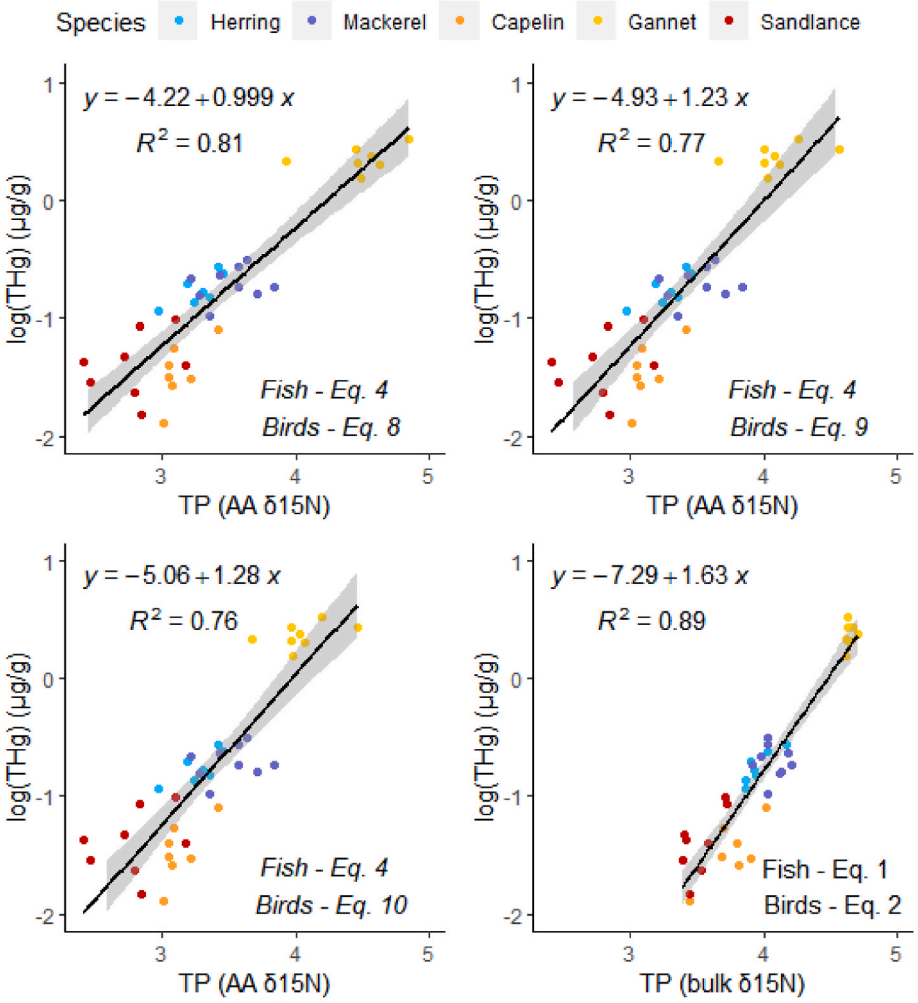

Fig. 2. Relationship between log-transformed total mercury (log(THg)) and trophic position (TP) calculated from experimental equations using compound-specific stable isotope analysis of amino acids (AA) in northern gannets and their prey. The shaded region represents the 95% confidence interval of the slope of each relationship.

Table 4
Trophic magnification slope (TMS) and factor (TMF) for each of the tested trophic position equation combinations. Equations (4) and (8)–(10) are equations using the $\delta^{15}N$ values from compound-specific stable isotope analysis in amino acids (CSIA-AA) and Equations (1) and (2) use the traditional bulk stable isotope (bulk SIA, indicated by the asterisk) approach. Groups denoted by the same superscript letter are not significantly different from each other.

Equation	Slope	95% Confidence Interval	Trophic Magnification Factor
Eq. (8) & Eq. (4)	1.00 [a]	0.78–1.08	10.0
Eq. (9) & Eq. (4)	1.23 [ab]	1.01–1.46	17.1
Eq. (10) & Eq. (4)	1.28 [ab]	1.04–1.51	18.8
Eq. (1) & Eq. (2) *	1.63 [b]	1.44–1.82	42.7

close to (Eq. (8)) or greater than one (Eqs. (9), (10), and bulk SIA), confirming that Hg biomagnification is occurring in our specific food chain. The intercepts of these four relationships are all very low. The back-transformed intercept values for Eqs. (8)–(10), and bulk SIA were all <0.00006 μg/g, indicating the THg values at the base of this food chain approach 0, regardless of the equation used. BMFs calculated using diet observations (proportions detailed in Supplementary Table S4) and stable isotope mixing models were 22.8 and 37.7 respectively for 2017, and 20.2 and 35.8, respectively for 2018.

4. Discussion

Understanding the biomagnification of Hg, a highly toxic contaminant to wildlife, is important when assessing ecosystem health. Our objective was to assess the use of compound-specific stable isotope analysis of amino acids (CSIA-AA) in biomagnification studies. To do this we compared CSIA-AA methods to calculate an organism's trophic position (TP) against the well-established bulk stable isotope analysis (bulk SIA). Then, we assessed the biomagnification level in our fish-to-gannet food chain in two ways: 1) by calculating trophic magnification factors (TMFs), which indicated the extent to which total mercury (THg) is amplified over the entire food chain and 2) by calculating biomagnification factors (BMFs) which informed on the extent of THg biomagnification between gannets and their prey. We found that differences between bulk SIA and CSIA-AA TMFs were stark: the true TMF value for our food chain is likely somewhere in between the two extreme values and may be closer to the middle values estimated by two of the three CSIA-AA equations. Thus, bulk SIA seems to overestimate the extent of biomagnification in the gannet food chain in the Gulf of St. Lawrence, while some CSIA equations may underestimate it.

4.1. Trophic position calculations

Three CSIA-AA equations (Eqs. (8)–(10)) produced TPs that matched our studied species' expected TPs and corresponded well with TPs calculated using bulk SIA (Fig. 1). These three equations also had positive relationships with THg (Fig. 2). The similarity between R^2 values between the three CSIA-AA equations might be due to the fish samples, for which trophic positions were calculated the same way (using Eq. (4)), leaving only the gannet samples to vary and change the slope and coefficients of determination. The gannet TPs estimated by bulk SIA were much more consistent and repeatable than those estimated by CSIA-AA, which showed more variability (see x-axes of Fig. 1). It is difficult to determine whether the spread of the gannet TPs is due to the incorporation of more noise in the CSIA-AA data or of more nuances that bulk SIA failed to account for. It was important to thoroughly test the different trophic position equations using CSIA-AA available in the literature for our study organisms, as many different equations are available in the literature, and these may yield different results depending on the study objective. Using data collected from captive fish and bird populations would be a valuable next step to validating CSIA-AA equations.

One limitation of our trophic calculation comparison was the difficulty of validating CSIA-AA approaches independently from TPs assessed using other approaches. As discussed previously, bulk SIA has been shown to be biased due to baseline differences in some studies (McClelland and Montoya, 2002). Diet observations may be biased, especially for nesting seabirds, to represent the diet of the young rather than the adult (Barrett et al., 2007). Other methods to assess TP, such as analysing stomach contents, can be biased towards organisms with harder structures, such as bones and otoliths, which are digested slowly (Buckland et al., 2017). Thus, when trying to validate TPs derived from CSIA-AA by using other, biased approaches as a benchmark, we run the risk of choosing a CSIA-AA equation that also produces biased TP estimates. However, TPs calculated using bulk SIA remains the best available tool in the literature and the most widespread practice to compare TPs calculated using CSIA-AA (see for example Wu et al., 2018; An et al., 2020; Zhang et al., 2021). However, the CSIA-AA approach is important to develop because it corrects for baseline differences across space and time. For studies sampling from different ecosystems or food webs within an ecosystem, bulk SIA cannot account for these discrepancies, unless baseline organisms are used. Collecting baseline organisms is not always possible and CSIA-AA is the only alternative way to correct for these potential biases in those instances.

The CSIA-AA equations mainly produced lower TP estimates, especially for fish (using Eq. (4)) than when using bulk SIA. This is because when baseline $\delta^{15}N$ values are accounted for, by subtracting source AA $\delta^{15}N$ values from trophic AA values, the overall $\delta^{15}N$ signature decreases, resulting in lower TP estimates (An et al., 2020; Zhang et al., 2021). For many gannets that were estimated to be at the same TP using bulk SIA, the CSIA-AA equations differentiated them more due to differences in Phe values, which represent baseline $\delta^{15}N$ signatures. There is also consistent evidence for individual specialization in seabirds, including gannets (Wakefield et al., 2015), which may help in differentiating individuals when using CSIA-AA. The differences in TP estimates calculated using bulk SIA and CSIA-AA were variable (fish: 0.65, gannets: 0.06–0.60), and were mostly higher than other studies that compared TPs calculated from bulk SIA or diet to TPs calculated from CSIA-AA (0.23 ± 0.06: Wu et al., 2018, 0.14 ± 0.08: Thébault et al., 2021), or were comparable to a study in coastal fish (0.6 ± 0.32 in fish; An et al., 2020). Interestingly, Eq. (8), derived from Wu et al. (2018) produced TP estimates that were very similar to our bulk TPs in the case of northern gannets (see Supplemental Table S4), yet in their study produced CSIA-AA TPs that were lower than bulk TPs. Considering that gannets forage over extensive areas and in different regions of the Gulf of St. Lawrence (Guillemette et al., 2018), differences in baseline $\delta^{15}N$ values are expected.

4.2. THg contamination in northern gannets

Toxicity thresholds for THg in many bird taxa have been studied in the literature. We used thresholds suggested by Ackerman et al. (2016): background concentrations of THg in blood consist of concentrations <0.2 μg/g ww, low-, moderate-, and high-risk concentrations are 0.2–1.0 μg/g ww, 1.0–3.0 μg/g ww, and 3.0–4.0 μg/g ww, respectively, and lethal-risk concentrations are >4.0 μg/g ww. In our study, all sampled gannets had concentrations of THg which placed them in the low-risk category, after conversion from dry-to wet-weight concentrations (see Supplemental Fig. S2). Negative biological effects have been observed in other seabirds with similar THg concentrations. In double-crested cormorants, gene expression was shown to have been altered, which may lead to oxidative stress of genes related to cellular stress (Gibson et al., 2014), and in black-legged kittiwakes (*Rissa tridactyla*), males were unable to successfully raise two chicks compared to males with lower THg concentrations (Tartu et al., 2016). Thus, there

may be small, undetected fitness impacts in our gannet population caused by THg concentrations higher than background levels.

4.3. Biomagnification of THg

Our linear mixed-effects models using three isotopes revealed that when accounting for species as a random effect, $\delta^{15}N$ values alone are best suited as an indicator of log(THg) concentrations in this food chain. This result is expected, as the relationship between trophic level and Hg burden has been well-established (Cabana and Rasmussen, 1994; Atwell et al., 1998; Hobson et al., 2002). However, our findings also contrast with other biomagnification studies, such as Góngora et al. (2018) and Elliott et al. (2021), who found that combined $\delta^{15}N$ and $\delta^{34}S$ values or simply $\delta^{34}S$ values were the best indicators of THg in Arctic marine ecosystems. Elliott and Elliott (2016) also recommended pairing $\delta^{15}N$ values with $\delta^{34}S$ values when studying sources of Hg. In our samples, $\delta^{34}S$ signatures did not show much variation (range 17.7–20.9‰). It is likely because our samples were from similar mid-water habitats and, consequently, $\delta^{34}S$ values did not exert much weight in our models.

An important consideration when selecting a TP equation is the impact of the equation on the relationships that are being studied. Between the three CSIA-AA and the bulk SIA equations, the steepness of the relationship with THg (the slope, *b*) is quite different. The slope of the relationship is commonly used as a factor to calculate measures of biomagnification, such as TMF (Riget et al., 2007; Lavoie et al., 2010). Therefore, when conducting a biomagnification study, the choice of TP equation will affect the results and interpretations stemming from it.

4.4. Biomagnification factors

When we looked at the biomagnification of THg in this food chain, the choice of CSIA-AA or bulk SIA to calculate TP yielded very different results. We chose to compare our three best CSIA-AA TP equations to the bulk SIA approach to demonstrate the range of TMFs that were estimated from the same samples. Eq. (8) produced a milder TMF estimate of 10.0, signifying that THg increased by ten times on average between each trophic level in our studied food chain. Eqs. (9) and (10) produced similar TMFs (17.1 and 18.8) that were steeper, but not significantly different from Eq. (8), and bulk SIA produced a higher TMF, of 42.7, than Eq. (8). This contrasts previous findings by Elliott et al. (2021) who reported that TMF for THg in an Arctic ecosystem was lower when assessed using bulk SIA and over three times greater when assessed using CSIA-AA. Further, in a lacustrine ecosystem, Zhang et al. (2021) described a higher TMF for methylmercury when assessed using CSIA-AA than with bulk SIA (9.5 compared to 5.7, respectively). Using bulk SIA to assess TP in our study produced TMFs greater than any other previously reported in the Gulf of St. Lawrence (range 3.8–6.5; Lavoie et al., 2010) and elsewhere (range 0.2–4.3; Riget et al., 2007; Elliott et al., 2021; Vainio et al., 2022). This is a further indicator that the choice of TP equation in a biomagnification study is a critical step, and bulk SIA may be overestimating the level of biomagnification in ecosystems if the study organisms are highly mobile, as northern gannets are. Because gannets travel long distances to forage, they likely feed in many different habitats, and thus, from different food chains. The bulk approach likely could not account for variation in the baseline nitrogen signatures, therefore producing an exceedingly high TMF. It is also important to note, however, that other studies which calculated TMFs included organisms from lower trophic positions, such as invertebrates. The inclusion of these organisms might have decreased the slope of the TP to log(THg) relationship in our study, which in turn would cause TMFs (both bulk and CSIA-AA) to be lower.

We obtained two BMF values for each year; one was calculated from regurgitation observations, and the other was calculated using diet composition proportions from bulk stable isotope mixing models. The BMFs calculated from regurgitation observations are lower than the mixing model BMFs, potentially due 1) to fluctuations in gannet diet during the breeding season at the Bonaventure Island colony (Guillemette et al., 2018; Pelletier and Guillemette, 2022), 2) diet observations may be skewed to represent nestling diet (Barrett et al., 2007), or 3) the presence of individual specialists in our sub-sampled populations (Wakefield et al., 2015) which may influence the mixing model output. Our BMFs from mixing models and our TMF derived from bulk (42.7) were very similar, indicating consistency between these two approaches using bulk SIA. The true value of the BMF for each year is likely somewhere in between the BMF from diet observations and the BMF from mixing models. One recent study investigated the use of CSIA-AA, specifically $\delta^{15}N$ signatures of Phe, to calculate a BMF providing more insight into dietary sources (Kim et al., 2023). The development of this method is likely to offer better accuracy of BMF metrics, and more studies are required to refine the method. The BMFs for our study are comparable to previously reported BMFs for THg in the Gulf of St. Lawrence: Lavoie et al. (2010) tested piscivorous seabirds and calculated BMFs ranging between 11.8 and 42.5. In the Barents Sea, BMFs for seabirds feeding on polar cod (*Boreogadus saida*) and herring yielded values between 25.4 and 107.3 (Jæger et al., 2009). Our results correspond well with these BMF estimates, although the range of reported values is wide. Our results were also slightly higher than BMFs calculated by Le Croizier et al. (2022) for Peruvian boobies, a close congener to northern gannets, in the Humboldt current ecosystem (range: 3–15). This may be due to differences in THg burden between the species' ecology, such as diet, and ecosystem. Overall, both of our BMF estimates produced are well within the magnitude of Hg biomagnification reported by other seabird studies.

5. Conclusion

Overall, the extent of biomagnification of THg in the Gulf of St. Lawrence when using BMFs is comparable to other seabirds and ecosystems but is very high when assessed with TMFs calculated using bulk SIA data. Our study was the first of its kind, to our knowledge to rigorously test TP equations using CSIA-AA and bulk SIA $\delta^{15}N$ data to examine Hg biomagnification in the Gulf of St. Lawrence. When assessing biomagnification, different approaches yielded largely different results for TMFs. Our results suggest a potential overestimate of Hg biomagnification extent when using bulk SIA compared to the compound-specific approach due to spatial differences in baseline $\delta^{15}N$. Thus, when assessing biomagnification, the methodology may greatly impact the outcome, and investigators should choose the appropriate method for their study organism. To ensure discrepancies between the two approaches are accounted for, we suggest that a subsample of the data be analysed for both bulk and CSIA-AA $\delta^{15}N$, so the bulk SIA data may be corrected for baseline values. A subsample of $n = 3$ per group should be sufficient to capture the baseline variability within the sample environment (Elliott et al., 2021). Our results are relevant to understanding how contaminants of concern, such as Hg, biomagnify through marine food chains and impact wildlife health.

CRediT authorship contribution statement

Rose M. Lacombe: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. **Benjamin D. Barst:** Formal analysis, Resources, Writing – review & editing. **David Pelletier:** Investigation, Resources, Writing – review & editing, Funding acquisition. **Magella Guillemette:** Resources, Writing – review & editing, Funding acquisition. **Marc Amyot:** Resources, Writing – review & editing. **Raphaël A. Lavoie:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Kyle H. Elliott:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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