



Evaluation of different stable isotope methods to estimate trophic position of perch (*Perca fluviatilis*) in Swedish lakes

Utvärdering av stabila isotoper som metod för att uppskatta trofinivå i abborre (*Perca fluviatilis*) från Svenska sjöar

Caroline Ek¹, Marcus Sundbom², Sara Danielsson¹ and Suzanne Faxneld¹

¹Department of Environmental Research and Monitoring, Swedish Museum of Natural History, Stockholm

²Department of Environmental Science and Analytical Chemistry, Stockholm University, Stockholm

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Swedish Museum of Natural History
Department of Environmental Research and Monitoring
P.O. Box 50 007
104 05 Stockholm
Sweden



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Preparation of samples and biological parameters:

Swedish Museum of Natural History
Sara Bernhardt

Mercury analysis:

Department of Environmental Science and Analytical Chemistry, Stockholm University
Project leader: Marcus Sundbom

Stable isotope analysis:

Stable Isotope Facility, UC Davis
Stable Isotope Laboratory, Stockholm University

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Evaluation of different stable isotope methods to estimate trophic position of perch (*Perca fluviatilis*) in Swedish lakes

Report authors Caroline Ek ¹ , Marcus Sundbom ² , Sara Danielsson ¹ and Suzanne Faxneld ¹ ¹ Department of Environmental Research and Monitoring, Swedish Museum of Natural History, Stockholm ² Department of Environmental Science and Analytical Chemistry, Stockholm University, Stockholm	Responsible publisher Swedish Museum of Natural History Postal address Naturhistoriska riksmuseet Box 50007 104 05 Stockholm Telephone +46(0)8-519 540 00
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Summary The Water Framework Directive (WFD) requires member states to implement a strategy to meet the environmental quality standards (EQS) set by the WFD for a number of priority substances. Since the EQS listed in the WFD are derived to protect the most sensitive species in the ecosystem, often top predators or humans, it has been decided that for certain priority substances the quality standards should be compared to a monitoring species with a trophic position (TP) similar to the diet of the most sensitive species. To enable such adjustment require both knowledge about the monitoring species TP in the food web as well as the relationship between contaminant concentration and trophic position. In this study, we focus on finding a suitable method for TP estimates of perch in Swedish lakes by evaluating both traditional stable isotope analysis in bulk samples (BSIA) using different baseline matrices and the more recent development of compound-specific stable isotope analysis in amino acids (CSIA-AA). For this, three representative monitoring lakes were selected in which perch together with potential baseline matrices (bivalves, gastropods and sediment) were sampled. We applied triple-isotope analyses, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$, of bulk material of all sampled matrices, and in addition $\delta^{15}\text{N}$ in perch using CSIA-AA. Results showed that TP estimates derived from CSIA-AA were significantly ($p<0.001$) lower compared to all the BSIA-derived methods and further that the BSIA-derived TP estimates using gastropods as a baseline were significantly higher ($p<0.001$) than all other TP estimates. Since no statistical differences could be detected between TP estimates based on bivalves, sediment or a 'mixture' baseline these were assumed to produce similar results and therefore all valid baseline matrices for TP estimates of perch in these three lakes. In the present study we also attempted to adjust mercury contaminant data to a specific TP of 3.5 according to the WFD. The adjustment resulted in significantly different concentration for one of the two tested lakes but did not influence the chemical status classification as all lakes were well above the threshold for mercury in freshwater lakes.	

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1. Summary

Under the Water Framework Directive (WFD), Sweden is committed to protect and, when necessary, improve the water quality of water bodies found in Sweden. Specifically for contaminants, the WFD requires member states to implement a strategy to meet the environmental quality standards (EQS) set by the WFD for a number of priority substances. Since the EQSs listed in the WFD are derived to protect the most sensitive species in the ecosystem, often top predators or humans, it has been decided that for certain priority substances the quality standards should be compared to a monitoring species with a trophic position (TP) similar to the diet of the most sensitive species. To enable such adjustment require both knowledge about the monitoring species TP in the food web as well as the relationship between contaminant concentration and trophic position (Trophic Magnification Factor (TMF)). For many substances, estimates of the TMF can be found in scientific literature. However, a well-established and accurate method for TP estimation does not exist for Eurasian perch, the predominant monitoring species of the current Swedish National Monitoring Programme for Contaminants in Freshwater Biota.

In this study, we therefore focus on finding a suitable method for TP estimates of perch in Swedish lakes by evaluating both traditional stable isotope analysis in bulk samples (BSIA) using different baseline matrices, and the more recent development of compound-specific stable isotope analysis in amino acids (CSIA-AA). For this, three representative monitoring lakes were selected in which perch together with potential baseline matrices (bivalves, gastropods and sediment) were sampled. We applied triple-isotope analyses, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$, of bulk material of all sampled matrices, and in addition $\delta^{15}\text{N}$ in perch using CSIA-AA. TP estimates were derived using BSIA with different baseline matrices as well as a 'mixture' of these derived from the estimated source contribution of each produced using a Bayesian mixing model. In addition, TP estimates were also derived using $\delta^{15}\text{N}$ from CSIA-AA. Comparisons of TP estimates were done using a two-factor ANOVA with TP method and lake as factors and Tukeys HSD post-hoc test for group-wise comparisons. Results showed that TP estimates derived from CSIA-AA were significantly ($p < 0.001$) lower compared to estimates from all the BSIA-derived methods, and further, that the BSIA-derived TP estimates using gastropods as a baseline were significantly higher ($p < 0.001$) than all other TP estimates. Since no statistical differences could be detected between TP estimates based on bivalves, sediment or a 'mixture' baseline these were assumed to produce similar results and therefore all valid baseline matrices for TP estimates of perch in these three lakes. In the present study we also attempted to adjust mercury contaminant data to a specific TP of 3.5 according to the WFD. The adjustment resulted in significantly different concentration for one of the two tested lakes but did not influence the chemical status classification as all lakes were well above the threshold for mercury in freshwater lakes.

2. Background

Under the Water Framework Directive (WFD, 2000/60/EC), Sweden is committed to protect and, when necessary, improve the water quality of water bodies found in Sweden. Specifically for contaminants, the WFD requires member states to implement a strategy to meet the environmental quality standards (EQS) set by the WFD (The European Commission 2000) for a number of priority substances. EQS correspond to a threshold concentration where no adverse effects on the ecosystem are expected. An important part of this strategy is to undertake monitoring programmes designed to adequately classify and assess the chemical status of the water bodies with regard to environmental contaminants by checking compliance with the set EQSs. It is further also strongly encouraged that member states participate in large-scale geographical chemical status assessments of EUs combined water bodies.

What is noteworthy, however, is the lack of a clear description for how to establish contaminant monitoring programmes and therefore different member states have used different monitoring matrices with specific biological characteristics (e.g., fat content, dry weight content, trophic position in the food web etc.). Since some of these bio-characteristics are known to influence the concentrations of bioaccumulative substances, such differences between national monitoring programmes are likely to hamper the execution of harmonised EU-assessments of EQSs as well as prevent evaluations of the general geographical contaminant load found within the EU. As a result, the European Commission recommends, for contaminants where top predators or humans are considered as the most sensitive, that the concentrations measured in any monitoring matrix should be adjusted to a specific standardized “EU fish” with regard to bio-characteristics relevant for the specific substance or substance group in question (The European Commission 2014).

Since the EQSs listed in the WFD are derived to protect the most sensitive species in the ecosystem, often top predators or humans, it has been decided that for certain priority substances the quality standards should be compared to a monitoring species with a trophic position (TP) similar to the diet of the most sensitive species. As monitoring species rarely have a TP similar to the average diet of e.g. human populations, the WFD currently suggests that the contaminant concentrations should be normalized to this TP (The European Commission 2014). To enable such adjustment require both knowledge about the monitoring species TP in the food web as well as the relationship between contaminant concentration and trophic position. Such relationships can often be simplistically described by the trophic magnification factor (TMF) that is defined as the ratio between the concentrations in consumer and its diet organisms at steady state. For many substances, estimates of the TMF can be found in scientific literature. However, a well-established and accurate method for TP estimation does not exist for Eurasian perch, the predominant monitoring species of the current Swedish National Monitoring Programme for Contaminants in Freshwater Biota.

Stable isotope analysis (SIA) is a common method to estimate the TP of a species in a food web and traditionally stable nitrogen isotopes (^{15}N : ^{14}N , $\delta^{15}\text{N}$ expressed relative to an international standard) have been analysed in a bulk sample (BSIA) of a tissue or whole animal (Fry 2007). This method assumes an average increase in $\delta^{15}\text{N}$ of approximately 2-4 ‰ per trophic level, i.e. between consumer and diet (Minagawa 1984, Post 2002, Vanderklift and Ponsard 2003), and the $\delta^{15}\text{N}$ value can hence be considered a relative measure of a species TP in relation to other individuals or species within the same food web. Alone, the value can be used in e.g., the evaluation of time series (Hebert and Weseloh 2006), but to translate the $\delta^{15}\text{N}$ value to a trophic position, the baseline $\delta^{15}\text{N}$, i.e. the isotopic composition at the base of the food web, is also required. Since the isotope composition of primary producers at the base of the food web is known to vary over time and space in coastal areas (Rolff 2000) and lakes (Vander Zanden and Rasmussen 1999, Leggett et al. 2000, Xu et al. 2005), primary producers would require repeated sampling over a longer time to get a representative baseline. An

alternative, and more common, approach is to conduct a simultaneous sampling of the baseline and target monitoring organisms and then chose a baseline organism, typically a primary consumer, which is expected to integrate the primary producer $\delta^{15}\text{N}$ variability over time scales that are representative for monitoring species nitrogen turnover.

A more recent development in SIA is to measure $\delta^{15}\text{N}$ in specific compounds (e.g., fatty acids and amino acids) instead of bulk material – a method known as compound-specific isotope analysis (CSIA). The use of CSIA of N in amino acids (AA, CSIA-AA) has proven advantageous as there exists two types of AAs; essential AA that undergo little or no ^{15}N fractionation between diet and consumer and remain stable in the food web (*source* AAs), and AA for which $\delta^{15}\text{N}$ changes between diet and consumer (*trophic* AAs) (Popp et al. 2007). By analysing $\delta^{15}\text{N}$ in both source and trophic AAs from one sample, it is possible to retrieve information on both the $\delta^{15}\text{N}$ value at the base of the food web and the organism's trophic position in the food web (Chikaraishi et al. 2009). To estimate a species' TP based on this method has proven superior to the more traditional bulk method in several studies (Steffan et al. 2013, Bowes and Thorp 2015). Still, however, are trophic position estimates largely done using $\delta^{15}\text{N}$ in bulk samples as this method is well-established and the analytical costs relatively low. This despite the method being associated with uncertainties pertaining to both the assumption about the trophic shift being 3.4 ‰ while it in reality can vary between -3.2 ‰ and +9.7 ‰ (Cabana and Rasmussen 1996, Vander Zanden and Rasmussen 1999, McCutchan et al. 2003), and to the choice and sampling of a relevant baseline matrix.

In the ongoing Swedish National Monitoring Programme for Contaminants in Freshwater Biota (SNMPCFB), $\delta^{15}\text{N}$ in bulk samples are determined annually. However, as there is no simultaneous sampling of a baseline matrix, estimation of TP and subsequent adjustment of concentrations for priority substances to the specific TP set by WFD are not feasible. The objective of this study is therefore to evaluate different methods for TP estimation of the main monitoring species in Swedish lakes.

In the majority of the sampled lakes within the SNMPCFB the monitoring matrix is perch (*Perca fluviatilis*), a common fish species found in most lakes and coastal waters in Sweden. Perch is an opportunistic predator that typically undergoes an ontogenetic diet shift as it grows. The shift is characterized by a gradual transfer from feeding mainly on smaller invertebrates to feeding on fish (Estlander et al. 2010) which may be accompanied by a switch from a littoral to more pelagic food sources. Unfortunately, the quite narrow size interval of individual perch selected for SNMPCFB is likely to coincide with the size range for the ontogenetic diet shift. This is problematic as then there is not clear which baseline matrix that would be the most relevant, and it may also be more appropriate to consider using a mixture of both a pelagic baseline matrix (e.g., bivalves feeding primarily on phytoplankton and small zooplankton) and a benthic baseline matrix (e.g., gastropods feeding on epiphytes and dead plant and animal material). Sediment can be a diverse matrix with possible contributions from both the pelagic and benthic system (Pati et al. 1999) and therefore a suitable baseline matrix for perch in this size range.

The stable isotope ratio of carbon ($^{13}\text{C}:^{12}\text{C}$, $\delta^{13}\text{C}$ expressed relative to an international standard) remains fairly stable in the food web compared to $\delta^{15}\text{N}$ (Fry 2007) and can therefore be used to separate different potential baselines as $\delta^{13}\text{C}$ can differ between pelagic and benthic/littoral algae constituting the food sources for bivalves and gastropods, respectively (France 1995). For some lakes, with deep waters, which lack benthic primary production, it is uncertain whether $\delta^{13}\text{C}$ is sufficient to separate food webs. In these lakes, the sublittoral and profundal zones are dominated by sediment dwelling detritus feeders like Chironomidae, an important food source for perch (Estlander et al. 2010). Studies have shown that stable sulphur isotopes ($^{34}\text{S}:^{32}\text{S}$, $\delta^{34}\text{S}$ expressed relative to an international standard) differ between organisms inhabiting the pelagic zone compared to those

associated with the sediment and similar to $\delta^{13}\text{C}$ remain fairly stable in the food web (Croisetiere et al. 2009). For these reasons, $\delta^{34}\text{S}$ can be used complementary to $\delta^{13}\text{C}$ to separate food webs based on organic material originating from sediment from pelagic food webs. In the former, organic reduced sulphur is the primary S source whereas in the latter dissolved oxidized sulphur as sulphate constitute the primary S source. The method utilize that reduced and oxidized S typically have different isotopic composition.

Since the isotopic composition of perch likely is a mixture of several food webs, it is possible to evaluate a mixture of baselines from different food webs using a tracer mixing model (Phillips 2001, Phillips and Gregg 2001, Stock et al. 2018), with multiple tracers wherefrom the contribution of the individual baselines can be assessed and used to calculate a new $\delta^{15}\text{N}$ -baseline relevant for these specific individuals.

With the main objective of evaluating different methods for TP estimation of the prevailing species in Swedish monitoring of lakes and to evaluate the TP adjustment of concentrations of selected priority substances, we have sampled multiple baseline matrices (bivalves, gastropods and sediment) in addition to perch in three representative lakes within the SNMPCFB. We apply triple-isotope analyses, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$, of bulk material of all sampled matrices, and in addition $\delta^{15}\text{N}$ in perch using CSIA-AA.

3. Material and Methods

3.1 Sampling of material and sample preparation

In this study, perch (*Perca fluviatilis*), mussels (*Anodonta anatine* and *Unio tumidus*), snails (*Viviparus viviparus*), and sediment were collected from three lakes in Sweden; Lake Mälaren (Södra Björkfjärden; X: 6577141, Y: 1592387), Lake Tärnan (X: 6606880, Y: 1644780), and Lake Älgsjön (X: 6552750, Y: 1532340). The collection of specimen took place between August and October 2018. At each lake, 12 samples per matrix were taken. The mussel *Unio tumidus* was only sampled from Lake Mälaren whereas specimens of *Anodonta anatine* were sampled from all lakes.

Snails and mussels were collected by hand during snorkelling, and they were mostly collected close to the shore. The specimens were placed in plastic containers with seawater and additional air. They were transported back to the laboratory at the Department of Environmental Research and Monitoring at the Swedish Museum of Natural History where they were allowed to stay overnight. Thereafter they were taken out of the water and the shells were wiped with paper before frozen until preparation. For mussels, approximately 1 g from each individual was used for the analyses. Prior preparation, mussels were thawed and then opened with a scalpel. Excess water was allowed to pour out before the weight was taken. Each mussel was then scraped out and the fresh tissue was put in a jar – one jar for each mussel. The fresh weight was taken. Thereafter each mussel was homogenized in their respective jar and 1 g from each homogenate was put in another jar (one jar for each mussel). The prepared samples were then put in the freezer prior analyses.

For snails, whole individuals were used and hence the sample amount varied from around 0.5 g to 2.5 g. The snails were thawed and then the soft tissue was taken out using a hooked knife spatula. The recovered soft tissue was weighted, put in pre-weighed plastic capsules and frozen.

Perch were collected with gill nets. Only healthy looking specimens with undamaged skin were selected and placed individually in polyethene plastic bags. Thereafter they were frozen as soon as possible and transported to the sample preparation laboratory at the Swedish Museum of Natural History and stored frozen until preparation. Before sample preparation of the fish, total body weight,

total length, body length, sex, age, gonad weight, liver weight and body condition of each individual were registered as well as the sample weight. The age of perch was determined by counting the otoliths growth zones.

Fish muscle samples were prepared for stable isotope analyses. The epidermis and subcutaneous fatty tissue were carefully removed prior to the sampling of the muscle.

For each fish, approximately 2 g was taken for the analyses and the muscle pieces were put in pre-weighed plastic capsules that were immediately frozen.

Table 3.1. Summary of biological variables for perch and water content of sediment sampled in the three different lakes. Values are presented as mean (\pm SEM); n=12.

		Age (yr)	Total weight (g)	Total length (cm)	Sediment water cont. (%)
Lake	Älgsjön	5.6 (\pm 0.3)	33.9 (\pm 1.2)	15 (\pm 0.2)	78.7 (\pm 1.8)
	Mälaren	3.3 (\pm 0.1)	48.9 (\pm 2.2)	16.4 (\pm 0.2)	92.1 (\pm 0.3)
	Tärnan	6.5 (\pm 0.5)	38.9 (\pm 1.8)	16.7 (\pm 0.2)	93.4 (\pm 0.3)

Sediment was taken from 12 shallow locations in each lake using a sediment core sampler (Hydro, Bioas; Kiel, Germany). From each core, approximately the top 2 cm was transferred to plastic jars and kept frozen until further preparation. Three water samples were taken dispersed in the water column (surface, mid and bottom water) and about 6 litre from each lake were stored cold and dark.

All biological, sediment and water samples were delivered to the Department of Environmental Science and Analytical Chemistry, Stockholm University, for further sample preparation and stable isotope analysis. Biota samples were freeze-dried for one week and individual tissue % dry weight was estimated. The whole sediment samples (110-150 g) were first freeze dried. However, some clay-rich samples appeared not to be completely dry even after one week of freeze drying. To ensure that no water remained – especially important for S isotope analysis – homogeneous sub-samples were further dried for 20 hours at 55°C. Dissolved sulphate in the water samples were collected on an ion exchange column, eluted with 0.5 M NaCl and precipitated with 0.5 M BaCl₂. The precipitate was collected on filter and kept dry until ³⁴S-SO₄ analysis.

Dried samples were homogenized with scissors or a mortar before carefully being weighed into tin capsules (BSIA) or borosilicate glass vials (CSIA) for stable isotope analysis. Sample weights followed closely the guidelines of the Stable Isotope Facility, UC Davis, that performed analyses of BSIA of C and N in biota and sediment, S isotopes in biota and CSIA-AA in fish, or the Stable Isotope Laboratory at Stockholm University that performed the S isotope analysis of sediments and dissolved sulphate (the latter still pending). Large plant detritus was avoided when packing sediment samples.

3.2 Calculation of trophic position (TP) estimates using different methods and baseline matrices

To evaluate the performance of different methods for the estimation of trophic position of perch sampled in different Swedish lakes, both BSIA and CSIA-AA were used.

For BSIA, we applied the equation proposed by Post (2002) with a diet-consumer discrimination ($\Delta^{15}\text{N}_{\text{Bulk}}$) factor of 3.4 ‰ (Equation 1). Several TP estimates were done for each individual by using different baseline $\delta^{15}\text{N}$ values ($\delta^{15}\text{N}_{\text{base}}$) depending on the selected baseline matrix (*bivalve*, *gastropod* and *sediment*) or a weighted combination (*mixture*, derived from a tracer mixing model). For each baseline matrix, the average $\delta^{15}\text{N}$ value was used and as a TP_{base} the primary consumers bivalves and gastropods were assigned a TP of 2. The TP of sediment was assigned to 1.5 as sediment is likely to

contain organic material originating from higher trophic positions such as resting eggs from zooplankton, heterotrophic bacteria and excrements from other organisms in addition to phytoplankton (Pati et al. 1999, Karlson and Viitasalo-Frösen 2009). Based on the assumption that most of the sediment $\delta^{15}\text{N}$ signal originate from the pelagic food web we also compared our assigned TP of 1.5 with another calculated estimate derived from the difference between $\delta^{15}\text{N}$ in sediment and $\delta^{15}\text{N}$ in bivalves across the three lakes (Lake et al. 2018). The mean difference between the two matrices (1.94 ‰) were subtracted from 6.8 ‰ (equal to a TP of 2 for the mussel), which resulted in a value of 4.9 ‰ or an alternate TP of 1.4 for sediment. This value was close to our assigned value of 1.5, which can therefore be assumed reasonable.

$$TP_{Bulk} = (\delta^{15}N_{Perch} - \delta^{15}N_{base})/\Delta^{15}N_{Bulk} + TP_{base} \quad \text{Equation 1}$$

For CSIA-AA, we applied an equation for TP estimate based on data from Chikaraishi et al. (2009) for the two AAs phenylalanine (Phe, source AA) and glutamic acid (Glu, trophic AA) (Equation 2). The difference between Glu and Phe at the base of the food web was therefore assumed to be 3.4 ‰ and the diet-consumer discrimination factor ($\Delta^{15}\text{N}_{Glu-Phe}$) to 7.6 ‰.

$$TP_{AA} = (\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta_{Glu/Phe})/\Delta^{15}N_{Glu-Phe} + 1 \quad \text{Equation 2}$$

3.3 Source contribution using Bayesian statistics

To evaluate the relative contribution of baseline matrices (i.e. different food webs) to the perch diet and isotope composition, the Bayesian tracer mixing model MixSIAR was used (Stock et al. 2018) in the statistical software R (R Core Team 2018). The settings for the model was a 3 tracer ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$) source model where the diet-consumer discrimination factor ($\Delta^{15}\text{N}$) was assumed to be zero for both sulphur and carbon as these isotope ratios are known to be fairly stable in the environment (Peterson and Fry 1987). For nitrogen, this value was set to 3.4 ‰ ($\pm 1\%$ SD) in accordance with literature data (Minagawa 1984, Post 2002). However, since the difference between our sampled baseline matrices and perch is not only one trophic level we assigned a hypothetical TP of the perch to 3.2 based on the proportion of fish in the diet reported at this size interval (Jacobson et al. 2019) and multiplied the differences in TP between perch and the baseline matrix with the enrichment factor. Residual and processes error were set to TRUE and source data were loaded as mean \pm SD. All biological $\delta^{13}\text{C}$ values were lipid corrected according to Post et al. (2007) as lipids are known to be ^{13}C -depleted and therefore risk to confound the model output.

Model output was evaluated using the Gelman-Rubin and Geweke convergence diagnostics. In the Gelman-Rubin diagnostics, the MCMC convergence is analysed by comparing differences between multiple Markov chains. The assessment is based on the comparison of estimated between-chains and within-chain variances for each model parameter and where a large difference indicate nonconvergence (Gelman and Rubin 1992, Brooks and Gelman 1998). The Geweke diagnostics on the other hand analyse convergence using a time series approach, comparing the mean and variance of segments from the beginning (initial 10% i.e. the burn-in period) and the end (the final 50%) of a single chain. The diagnostic is a standard z-score calculated for the difference between the initial and final segment in each chain and analysed using a standard normal assumption that the majority (95%) of the values should fall within approximately 2 standard deviations of zero (Geweke 1991).

Because of the assumptions related to the $\Delta^{15}\text{N}$ value, a sensitivity analysis was also conducted to evaluate how sensitive the model outcome is to the $\Delta^{15}\text{N}$ parameter. A range of $\Delta^{15}\text{N}$ values (0-10 ‰ and with a SD of 0) were modelled for each lake and the proportions of the respective baseline matrices over the range were plotted.

3.4 Case study: Trophic position adjusted mercury (Hg) concentrations

As a case study, we attempted to adjust total mercury (Hg) concentrations analysed within the SNMCFB in perch from Lake Älgsjön (n=10) and Lake Tärnan (n=10) using estimated TPs (based on sediment as a baseline matrix) from this study. Hg concentrations and $\delta^{15}\text{N}$ were measured in subsamples from the same individuals. The threshold for mercury is an Environmental Quality Standard set to protect against secondary poisoning ($\text{EQS}_{\text{biota}}$) with a value of $20 \mu\text{g kg}^{-1}$ wet weight. Therefore, in accordance with guidelines for adjusting contaminant concentrations in freshwater environments, a critical concentration was assumed to be attained at a TP of 3.5 (The European Commission 2014). To adjust measured Hg concentrations ($\text{Conc}_{\text{meas}}$), a literature Trophic Magnification Factor (TMF) generic for freshwater systems ($\text{TMF}=4.3$) was applied (Lavoie et al. 2013) together with the critical TP of 3.5 (Equation 3). The adjusted concentrations ($\text{Conc}_{\text{TP-adj}}$) were compared with unadjusted Hg concentrations within and between lakes.

$$\text{Conc}_{\text{TP-adj}} = \text{Conc}_{\text{meas}} \times \text{TMF}^{3.5-\text{TP}(x)} \quad \text{Equation 3}$$

3.5 Statistical analysis

To evaluate the different strategies for TP estimation of perch we analysed the five different alternatives (CSIA-AA, bivalve, gastropod, sediment and “mixture”) using a 2-factor ANOVA with TP estimation method and Lake as factors. This approach allowed us to assess both if there were differences in TP estimates between methods but also if there were differences in TP estimates for perch sampled from different lakes as well as if patterns of TP estimates using different methods were consistent in all lakes. The model was evaluated by examination of standardized residuals using a QQ plot and Shapiro-Wilks test for normality as well as Levene’s test for homogeneity of variance. Tukey HSD test was used for multiple comparisons of means. Since the initial model produced residuals that did not fulfil Shapiro-Wilks test for normality, a secondary model was also performed excluding the interaction term (TP method x Lake) before using Tukey HSD test to validate our findings from the initial model.

Comparisons between adjusted and unadjusted Hg concentrations within and between lakes were done by using paired (within lakes) and unpaired t-tests (between lakes). Due to several pairwise comparisons (n=4) the p-value was adjusted using the Bonferroni correction to a significance level of $p \leq 0.0125$.

All statistical analyses were performed using the statistical software R, version 3.5.2 (R Core Team 2018). Data are presented as arithmetic mean \pm standard error of mean (SEM) and the significance level set to $\alpha \leq 0.05$ if not specified otherwise.

4. Results

4.1 Source contribution

The isospace of the different food web components in different lakes is a good indication of how well tracer mixing models are expected to perform. Lake Älgsjön showed a clear overlap between the two baseline matrices, bivalves and gastropods, whereas the sediment was somewhat separated when looking at biplots combining the three tracers $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ (see Figure 4.1.A). For Lake Mälaren and Lake Tärnan overlaps were less or non-existing between bivalves and gastropods and sediment signals were instead associated with bivalves and gastropods for Lake Tärnan and Lake

Mälaren, respectively (Figure 4.1.B-C). As expected, perch had higher $\delta^{15}\text{N}$ values compared to all baseline matrices.

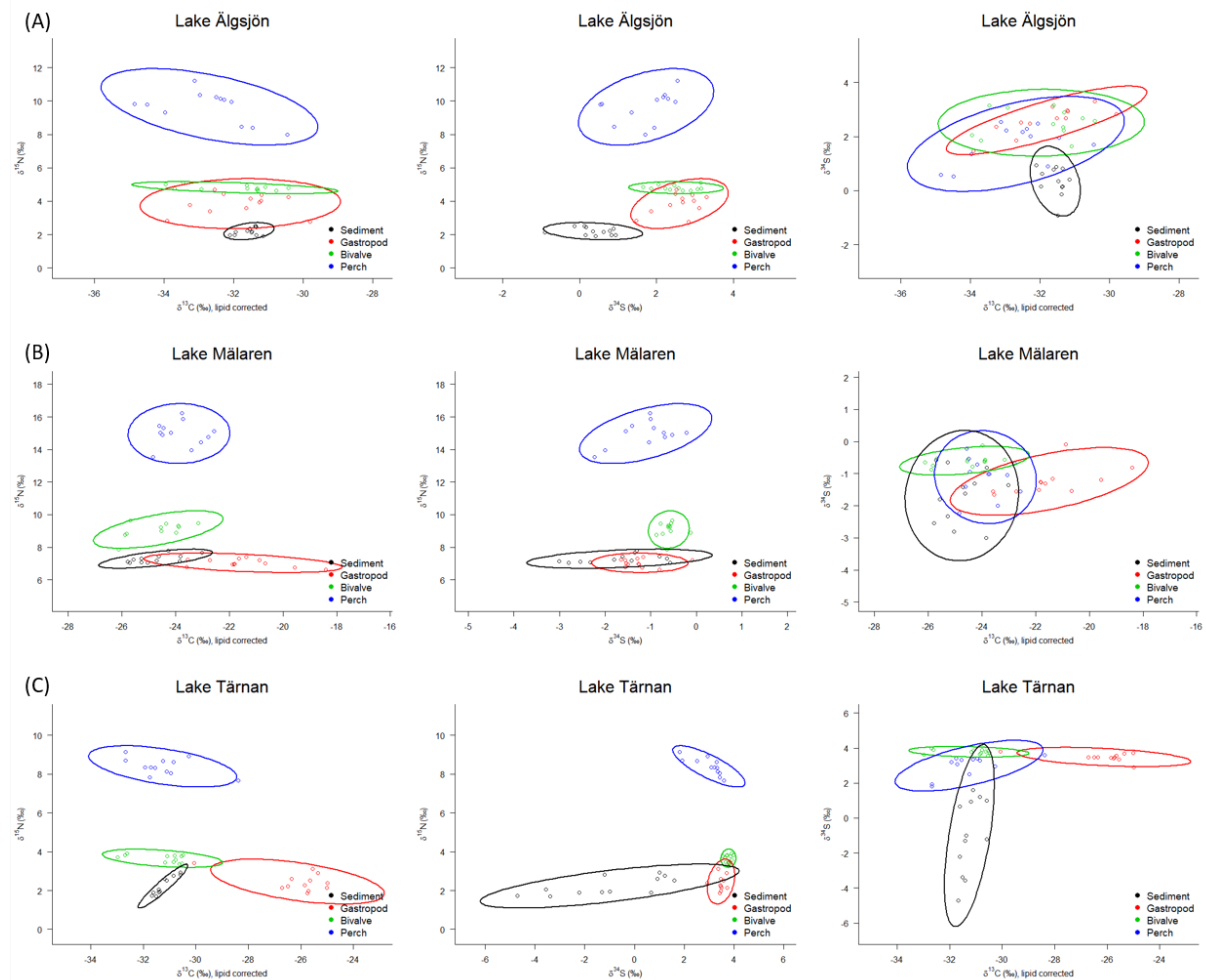


Figure 4.1. Isospace plots for the three different study lakes (A) Lake Älgsjön, (B) Lake Mälaren and (C) Lake Tärnan by combination of the three tracers ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$) used to estimate source contribution of the different baseline matrices (sediment, gastropods and bivalves). Ellipses represent 95 % of the observations.

The Bayesian tracer mixing model generated similar results for all three lakes with the largest contribution of the three sources originating from bivalve, between 49-79%, although the 95% confidence interval was occasionally overlapping between sources. The second largest contributor was sediment with an estimated source contribution between 16-32% and gastropods only contributed between 5-19% (Table 4.1). Based on the results from the tracer mixing model a “mixture” baseline $\delta^{15}\text{N}$ value was calculated using the relative proportion of each baseline matrix.

The sensitivity analysis showed that model output was most sensitive when the diet-consumer discrimination factor was underestimated i.e. when the true value was higher than 3.4 ‰ (see Figure 4.2). Noteworthy is the apparent mismatch between proportions assumed by the sensitivity analysis, which showed the highest proportion of gastropods, compared to the applied model output for Lake

Älgsjön. This can to some degree be explained by the considerably large 95%-CI for the estimated proportions but highlights the overall uncertainty coupled to tracer mixing models in general.

Table 4.1. The estimated contribution of different baseline matrices to the perch diet in different lakes (Lake Älgsjön, Lake Mälaren and Lake Tärnan).

	Proportion of:	Bivalve (95%-CI)	Gastropod (95%-CI)	Sediment (95%-CI)
Lake	Älgsjön	0.49 (0.14-0.8)	0.19 (0.01-0.51)	0.32 (0.09-0.52)
	Mälaren	0.52 (0.18-0.79)	0.18 (0.02-0.35)	0.3 (0.05-0.64)
	Tärnan	0.79 (0.65-0.89)	0.05 (0-0.15)	0.16 (0.08-0.28)

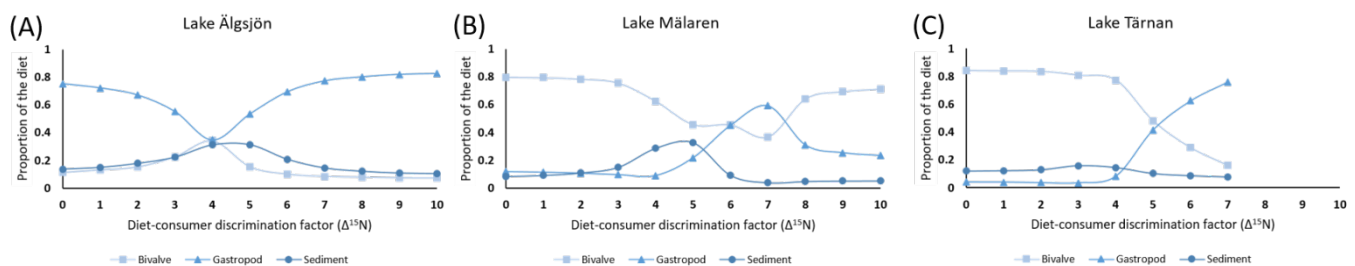


Figure 4.2. Sensitivity analysis of the effect of the diet-consumer discrimination factor ($\Delta^{15}N$) on the model outcome for the three lakes; (A) Lake Älgsjön, (B) Lake Mälaren, (C) Lake Tärnan.

4.2 Evaluation of TP estimates using different methods and baseline matrices

In general, trophic position estimates based on CSIA-AA had the lowest values whereas the highest estimates were produced using the gastropod (and sediment for Lake Älgsjön) as a baseline (see Table 4.2).

Table 4.2. Trophic position (TP) estimates using different methods (CSIA-AA and BSIA) and baseline matrices (BSIA; bivalve, gastropod, sediment and mixture). Values are shown as arithmetic mean (\pm SEM). $n=12$

	TP:	CSIA-AA	Bivalve	Gastropod	Sediment	Mixture
Lake	Älgsjön	2.9 (± 0.1)	3.4 (± 0.1)	3.7 (± 0.1)	3.7 (± 0.1)	3.6 (± 0.1)
	Mälaren	3.0 (± 0.03)	3.7 (± 0.1)	4.3 (± 0.1)	3.8 (± 0.1)	3.9 (± 0.1)
	Tärnan	3.0 (± 0.04)	3.4 (± 0.04)	3.8 (± 0)	3.3 (± 0.04)	3.4 (± 0.04)

The two-factor ANOVA showed that there were significant differences in TP estimates between methods and between lakes. Moreover, there were also a significant interaction effect between TP method and lake indicating that the pattern of different methods varied between lakes (see Table 4.3 and Figure 4.3). Tukeys multiple comparisons of means revealed that using CSIA-AA with the equation provided by Chikaraishi et al. (2009) for glutamic acid and phenylalanine provided significantly lower estimates compared to all other methods (see Table 4.4). In comparison, did the BSIA method with gastropods as baseline matrix generate significantly higher TP estimates compared to all other methods (CSIA-AA and the other baseline matrices). Whereas for the combinations between bivalves, sediment, and mixture no significant differences could be detected. For lake as a

factor, significant differences were found between Lake Mälaren and Lake Älgsjön and between Lake Mälaren and Lake Tärnan, whereas no significant difference could be detected between Lake Älgsjön and Lake Tärnan.

Table 4.3. Two-factor ANOVA evaluating the effect of the two factors Trophic position (TP) method and Lake on trophic position estimates.

	df	SS	MS	F	p-value
TP method	4	18.26	4.57	108.88	<0.001
Lake	2	4.31	2.15	51.38	<0.001
TP method x Lake	8	2.18	0.27	6.50	<0.001
Residuals	165	6.92	0.042		

Table 4.4. Tukey HSD test for multiple comparisons of means. Difference between group mean values with 95% confidence interval. P-values in bold are significant.

	Group-wise comparison	Difference (95%-CI)	P value
TP method	CSIA-AA-Bivalve	-0.57 (-0.7--0.44)	<0.001
	Gastropod-Bivalve	0.41 (0.28-0.54)	<0.001
	Mixture-Bivalve	0.04 (-0.09-0.18)	0.892
	Sediment-Bivalve	0.07 (-0.06-0.2)	0.579
	Gastropod-CSIA-AA	0.98 (0.85-1.11)	<0.001
	Mixture-CSIA-AA	0.61 (0.48-0.75)	<0.001
	Sediment-CSIA-AA	0.64 (0.51-0.77)	<0.001
	Mixture-Gastropod	-0.37 (-0.5--0.23)	<0.001
	Sediment-Gastropod	-0.34 (-0.47--0.21)	<0.001
	Sediment-Mixture	0.03 (-0.11-0.16)	0.980
Lake	Mälaren-Älgsjön	0.28 (0.19-0.36)	<0.001
	Tärnan-Älgsjön	-0.08 (-0.16-0.01)	0.106
	Tärnan-Mälaren	-0.35 (-0.44--0.26)	<0.001

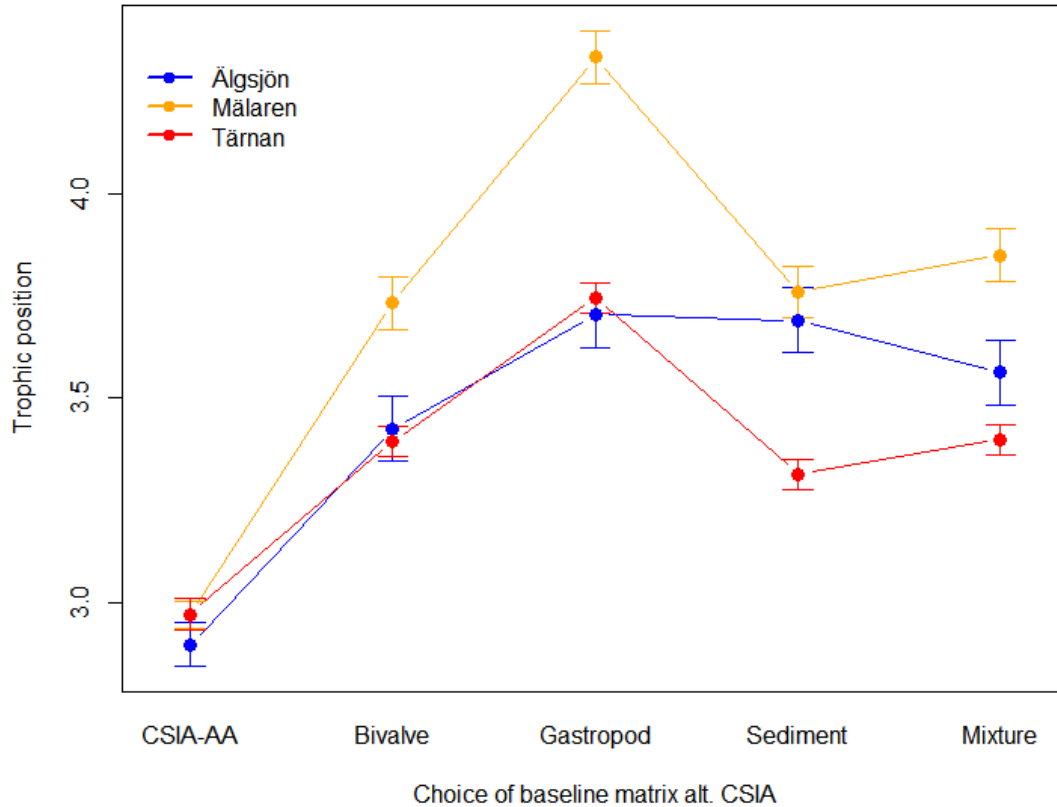


Figure 4.3. A two-factor interaction plot showing trophic position (TP) estimates using different methods (CSIA-AA and BSIA) and baseline matrices (BSIA; Bivalve, Gastropod, Sediment and Mixture) for three different lakes (Lake Älgsjön, Lake Mälaren and Lake Tärnan). Error bars are standard error of mean.

4.3 Trophic position adjusted mercury concentrations

The adjustment of Hg concentrations to a TP of 3.5 did not significantly change the average concentration for Lake Älgsjön ($p=0.8$) whereas for Lake Tärnan the adjustment resulted in significantly ($p<0.01$) higher concentrations compared to unadjusted concentrations (see Figure 4.4). Moreover, the adjustment also increased the coefficient of variation (CV) for mercury concentrations in both lakes. A comparison between lakes also showed that significant differences exist between lakes with generally higher concentrations in Lake Tärnan compared to Lake Älgsjön ($p<0.003$ and $p<0.002$ for unadjusted and adjusted Hg concentrations, respectively).

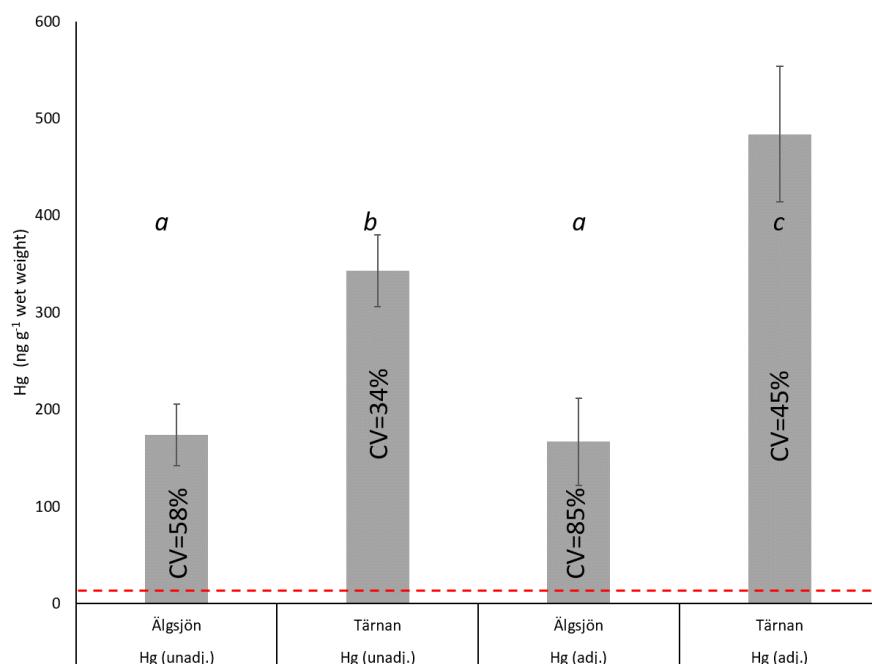


Figure 4.4. Barplot showing mercury (Hg) concentration before (Hg unadj.) and after (Hg adj.) trophic position adjustments in Lake Älgsjön and Lake Tärnan. CV: Coefficient of Variation. Error bars represent standard error of mean. Red dashed line indicates the threshold for mercury (EQS_{biota} 20 ng g⁻¹ wet weight). Different letters indicate significant differences between groups ($p \leq 0.125$). $n=10$.

5. Discussion

The aim of this study was to find a suitable method to estimate TP for perch in Swedish lakes included in the Swedish National Monitoring Programme for Contaminants in Freshwater Biota. To address this, we have compared several possible strategies for estimating TP based on stable isotope data using both traditional bulk SIA (BSIA) and CSIA-AA.

The use of CSIA-AA for TP estimates resulted in significantly lower estimates compared to BSIA, regardless of the selected baseline matrix. The average TP for perch using CSIA-AA was approximately a TP of 3 for all lakes, thus representing a scenario where perch would feed exclusively on primary consumers like zooplankton, grazers etc. This is in direct contradiction to the observed proportions of higher trophic level feed (fish) in the perch diet from the Baltic Sea (Jacobson et al. 2019) as well as data provided by the Swedish University of Agricultural Sciences on stomach content in perch from the same size range for the specific sampled lakes (Nationellt Register över Sjöprovfisken – NORS 2019). The apparent underestimation of TPs in this study is also in line with findings from other studies (Vokhshoori and McCarthy 2014, Ek et al. 2018) and is likely the result of “mixed” systems with several different plant sources supporting the perch diet. In Chikaraishi et al. (2010), data on the offset between Glu and Phe ($\beta_{Glu/Phe}$) in autotrophs show that the value differs between cyanobacteria and algae (+3.4 ‰), terrestrial C3 plants (-8.4 ‰) and terrestrial C4 plants (+0.4 ‰). Since the traditional equation for estimating TP from Glu and Phe is based on the assumption of a purely pelagic food web supported by only cyanobacteria and algae (Chikaraishi et al. 2009), any other system where other plant sources are dominating or a mixture occurs will render inaccurate TP estimates. In here, the lakes can be assumed to also have input from terrestrial material

at different degrees, which would influence the offset value between Glu and Phe and generate erroneous TP estimates. To circumvent this problem, Choi et al. (2017) proposed that by having knowledge of the contribution of different basal resources to the diet/system, a system-specific “mixture” offset values for Glu and Phe could be calculated. In the national monitoring programme, such assessment for every lake is hardly feasible and hence is the applicability of CSIA-AA in the monitoring programme not an option today.

Traditional bulk stable isotope analysis is associated with uncertainties related to both the diet-consumer discrimination factor but maybe more so to the selected baseline matrix which should have a $\delta^{15}\text{N}$ value representing source $\delta^{15}\text{N}$ with the same temporal resolution as the target organism. In here, focus has been to evaluate a suitable baseline matrix given the ongoing ontogenetic diet shift in perch at this size range. Between the three different baseline matrices (bivalves, gastropods and sediment) sampled and a “mixture” signal, significant differences in TP estimates were only found between gastropods and all other matrices. The gastropod represents a food web in the littoral zone where they feed by scraping off microalgae from surfaces as well as living of detritus material (i.e. dead animals and plants). The results from the tracer mixing model showed that this “food web” was not the best predictor for perch as the bivalve food web, representing filtration of microalgae and occasionally other particulate organic matter, generated the highest contribution in all lakes, between 49-79%. The gastropod food web only contributed less than 20% to the perch diet in Lake Mälaren, Lake Tärnan (18% and 5%, respectively). In Lake Älgsjön was the contribution similar to Lake Mälaren (19% compared to 18%), although using gastropods as a baseline matrix did not generate different TP estimates compared to the sediment matrix in that lake. The overall highest TP estimate for perch was retrieved by using gastropods as a baseline matrix in Lake Mälaren (TP of 4.3) – a large eutrophic system where perch can be expected to have more opportunities for feeding on pelagic fish than in the smaller forest lakes, especially in humic Älgsjön. This is supported by the considerably faster growth rates (cf. Table 3.1) in Lake Mälaren, as the shift to piscivorous diet is known to enhance growth of perch (e.g. Hargeby et al. (2005)). It is however unlikely that perch in that particular size range would feed exclusively on fish and more so, on fish from higher trophic levels. If perch generally feed on small fish in this lake, it would suggest gastropods not to be a suitable baseline matrix here and that bivalves, representing a pelagic food web, would generate more accurate TP estimates.

For the baseline matrices bivalves, sediment and “mixture”, no significant differences could be detected except for Lake Älgsjön where a significant difference was found between TP estimates based on bivalves and sediment. Lake Älgsjön is a lake surrounded for the most part of mixed coniferous and deciduous forest, containing a lot of humic substances potentially driving a high content of terrestrial material in the sediment. Such contribution could influence both the selected value for TP_{base} (closer to a TP of 1 than 1.5) as well as the $\delta^{15}\text{N}_{\text{base}}$ value, both of which could result in an apparent higher TP for the perch. It is reasonable to think that a high contribution of terrestrial plant material can obscure the pelagic signal in the sediment and thus driving the $\delta^{15}\text{N}$ value to lower values as algae generally have higher $\delta^{15}\text{N}$ values than terrestrial vegetation and supra-littoral vegetation (Romanuk and Levings 2005, Chikaraishi et al. 2009, Chikaraishi et al. 2010). In all, the data suggest that the sediment-based TPs from Lake Älgsjön could be overestimated and that the three alternatives bivalve-sediment-mixture produce similar results. With the exception of Lake Älgsjön, the TP estimates retrieved from these baselines are also in the same range as expected values (approximately a TP of 3.2) based on literature data on perch diet (Jacobson et al. 2019). Noteworthy are the overall higher TP estimates in Lake Mälaren compared to the other two lakes. This difference is statistically significant and highlights the importance of TP-adjusted contaminant concentrations to allow for accurate between-lake comparisons of contaminant status also within the Swedish monitoring programme. In summary, these findings suggest that primarily bivalves or secondary sediment would be top choices for a baseline matrix for TP estimation, at least for the three study lakes. However, because the occurrence of bivalves in the monitoring lakes is limited and in addition time consuming

to sample, bivalves as a baseline matrix is not considered the best option from a practical perspective. Instead, sediment is proposed as a suitable baseline matrix provided that the contribution of terrestrial carbon is not too high. Sediment is already sampled from 60% of all monitoring lakes annually and long time series of sediment samples are stored in the Environment Specimen Bank at the Swedish Museum of Natural History. We therefore conclude that continuous, and possibly retrospective, studies focusing on sediment as a baseline matrix is a natural progression from this study. Further evaluation of sediment as a baseline matrix is needed, and should include comparisons between different types of lakes (e.g., high/low input of terrestrial carbon) as well as retrospective studies of stored sediment samples. Sample preparation of sediment should be carefully considered and consistently performed because lake sediment is a heterogeneous matrix and subsamples prepared for BSIA are very small (milligram). For example, improved methods for isolating fine organic material and homogenization should be investigated in future studies.

As a case study, we adjusted mercury concentrations analysed in the same perch to a TP of 3.5 as specified by the WFD. Adjusted concentrations were significantly higher for Lake Tärnan, and for Lake Älgsjön the adjustment did not significantly change the average Hg concentration. The chemical status classification was not affected for neither of the lakes. Our data also indicate that the observed difference in average perch Hg concentrations between the two lakes still exists after TP-adjustment. This suggests, as expected, that other factors such as Hg load and bioavailability are setting the general fish Hg-levels, rather than differences in TP among lakes. In contrast, the observed increase in the CV caused by TP-adjustment of Hg concentrations may appear counter-intuitive. However, the variance propagation pattern that is inherent of the adjustment approach applied here (Eq. 3) is dependent on the relationship between Hg and TP. A positive relationship would decrease the CV, while a negative relationship, as observed for all three lakes in this study would increase the CV ($r \approx -0.5$). For perch, a positive relationship between Hg and TP is expected, at least over a wider size range. The negative relationships observed for Lake Tärnan and Lake Älgsjön may of course just be a coincidence for these quite small samples. However, revisiting the SNMCFB time series reveals that these two lakes show negative Hg- $\delta^{15}\text{N}$ relationships in most years since 2005. Although among SNMCFB lakes, it's still more common with a positive relationship. Out of the 28 perch lakes, the Hg- $\delta^{15}\text{N}$ relationship is significantly positive for 14 lakes, non-significant for 10 lakes and significantly negative for 4 lakes.

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