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PHILOSOPHICAL TRANSACTIONS B

Comparison of Bayesian and numerical optimisation-based diet estimation on herbivorous zooplankton

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Summary

Consumer diet estimation with biotracer-based mixing models provides valuable information about trophic interactions and the dynamics of complex ecosystems. Here we assessed the performance of four Bayesian and three numerical optimisation-based diet estimation methods for estimating the diet composition of herbivorous zooplankton utilising consumer fatty acid profiles and resource library consisting of the results of homogenous diet feeding experiments. The method performance was evaluated in terms of absolute errors, central probability interval checks, the success in identifying the primary resource in the diet, and the ability to detect the absence of resources in the diet. Despite occasional large inconsistencies, all the methods were able to identify the primary resource most of the time. The numerical optimisation method QFASA utilising chi-squared (QFASA-CS) or Kullback-Leibler (QFASA-KL) distance measures had the smallest absolute errors, most frequently found the primary resource, and adequately detected the absence of resources. While the Bayesian methods usually performed well, some of the methods produced ambiguous results and some had much longer computing times than QFASA. Therefore, we recommend using QFASA-CS or QFASA-KL. Our systematic tests showed that FA models can be used to accurately estimate complex dietary mixtures in herbivorous zooplankton.

1 Introduction

Studying consumer-resource interactions in food webs advances understanding of the flow of energy through trophic levels and the carbon and nutrient cycles in ecosystems. Assimilable indicators, i.e., biotracers, are regularly used in diet research [1]. Stable isotopes (SI) have been used to estimate the importance of autochthonous and allochthonous carbon in aquatic food webs [2-4], but they provide only limited information

about consumer diets as often the number of resources exceeds the number of used isotopes [5]. Stable isotope mixing model outputs can also be strongly influenced by poorly constrained assumptions [6]. The number of fatty acids (FA), however, can easily exceed the number of resources in a diet due to the large number of different FAs present in living organisms [7,8]. FA profile-based diet estimation (hereafter "FA diet estimation") can be conducted using a numerical optimisation-based method Quantitative Fatty Acid Signature Analysis (QFASA) [7] or Bayesian methods initially developed for SI-based diet estimation and later adapted to FA diet estimation; Fatty Acid Source Tracing Algorithm for R (FASTAR) [9] and MixSIAR [10]. Compared to SI-based diet estimation, FA diet estimation can also provide information about the nutritional value of the assimilated resources [8] which enhances our understanding of consumers' nutritional ecology.

Diet estimation with FAs is relatively new, and both modelling approaches have been developed lately; QFASA was published in 2004 and FASTAR in 2014. The 2016 published MixSIAR is claimed to improve on the error structure of its predecessor FASTAR [11] but the differences are not compared in any FA diet estimation study we know of. Many different QFASA variants have also been introduced lately in 2014 [12] and 2017 [13] but their performance has not been simultaneously evaluated in any study. While simulation tests have been conducted separately on QFASA [14], FASTAR [9] and MixSIAR, although not with FA data [11], performance synthesis of the established FA diet estimation methods is still lacking. Numerical optimisation methods and Bayesian methods have been compared only in a recent concise study where QFASA utilising Aitchison's (QFASA-AIT) distance measure and FASTAR utilising the MixSIR model were compared using the FA profiles of two beluga whale individuals [15]. This comparison was, however, neither systematic nor extensive. Therefore, there is currently not enough evidence on how these different approaches perform comparatively when challenged in various ways.

When a resource is consumed by a consumer, dietary FAs are assimilated to the tissues of the consumer, and thus the FA profile of the consumer starts to resemble the FA profiles of its resources in varying degrees [16]. However, the FAs do not always directly assimilate but are often modified by the consumer metabolism to some extent [16,17]. Therefore, FA modification must be accounted for in FA diet estimation. Iverson et al. [7] introduced the concept of 'calibration coefficients', a coefficient calculated from empirical data for each FA to account for the FA modification and until recently it has been the main method of accounting for FA modification in FA diet estimation. The calibration coefficients attempt to capture the FA modification by constituting a linear mapping from resource to consumer. It is therefore possible to estimate the diet based only on the consumer and resource FA profiles. However, it has been shown that optimally with both QFASA and the Bayesian methods, homogenous diet feeding experiments with the consumers should be carried out when possible to better consider for the diet specific FA modification in the consumers [8,18]. In such experiments, the resource FA profiles are not directly observed, but rather the FA profiles of consumers that have consumed only single resource. Therefore, the FA modification already accounted for in the FA profiles and thus, a source

66 of uncertainty can be avoided altogether. Consequently, the resource library data used in this study are the

67 results of homogenous diet feeding experiments.

68 Whereas all the diet estimation methods utilise the exact same data, the problems they solve and hence

69 their estimates are essentially different. Each QFASA variant solves an optimisation problem by minimising a

70 given distance measure between the observed and predicted consumer FA profiles by finding a point estimate

71 for the resource proportion vector in the diet that minimises the specified distance measure. In contrast, the

72 Bayesian methods solve the joint posterior probability distribution, i.e., the conditional probability distribution

73 of the diet proportions given the observed consumer FA profiles. The posterior probability distribution can be

74 then used to calculate a desired point estimate to be used for comparison purposes.

75 All FA diet estimation methods require a resource library containing FA profiles of all the possible

76 resource types as an input [10,19]. Therefore, for a given consumer, the resource library will likely contain some

77 resources that are not part of that consumer's diet. The effects of these absent resources, or the number of

78 different resources on a diet, have not been well studied before.

79 To compare the performance of different FA models, it is beneficial to use a consumer with a fast and

80 conservative FA metabolism so that FA modification is minimal. Hence the differences in diet estimates are due

81 to the models and not the consumer data. Herbivorous zooplankton are important heterotrophic consumers

82 linking primary producers and planktivorous fish in aquatic food webs, and a crucial link in the transfer of

83 essential biomolecules through aquatic food webs [20,21]. *Daphnia* is an optimal consumer for testing different

84 FA models since they reproduce parthenogenetically, have rapid FA turnover [22,23] and have limited capacity

85 to synthesize essential FAs EPA or DHA from ALA [23,24]. Moreover, *Daphnia* feed primarily on different types

86 of phytoplankton and bacteria, but may also feed on terrestrial organic matter directly or in microbial-mediated

87 pathways [3,25,26] resulting in multiple potential resources (>10) in the diet. Furthermore, the role of

88 allochthonous carbon in the *Daphnia* diet has been debated [3,27,28], and the role of cyanobacteria as a resource

89 for zooplankton is of interest as lake occurrence and intensity of cyanobacteria blooming increases in a warming

90 climate [29-32].

91 Here, we assessed the performance of current FA-based diet estimation methods; two versions of

92 Bayesian FASTAR [9], two versions of Bayesian MixSIAR [10], and three versions of numerical optimisation-

93 based QFASA [7], which was performed with the QFASAR-package [33]. Systematic and extensive simulations

94 were conducted in R Statistical Software version 3.6.1 [34]. We evaluated the performance of the different

95 methods using a large testbench consisting of 100 replicas of 25 hypothetical *Daphnia* diets varying in the

96 number of resource items and their relative proportions. Each replica consisted of three simulated observations

97 of the consumer's FA profile. We measured the performance of the methods in terms of the absolute error

98 between the true diet and the point estimate, the frequency in which the true diet was within the 68% and 95%

99 central probability intervals, the methods' ability to detect the absence of resources in the diet, and the ability to

100 identify the primary resource in the diet. Computational efficiency was assessed alongside the method

101 performance evaluation. The effect of sample size was also explored by conducting simulations with one to five
102 observations on select hypothetical diets.

103

104

2 Material and methods

106

2.1 *Daphnia* fatty acid profile resource library

108 The *Daphnia* FA profile resource library used in this study was formed from the results of homogenous diet
109 feeding experiments conducted on *Daphnia* and presented as relative FA proportions. This means that the
110 resource FA profiles are not the FA profiles of the resources but instead they are the FA profiles of the consumer
111 that has eaten only a single resource. The library is based on the one presented by Galloway et al. [8] which
112 consisted of phytoplankton; cryptomonads (crypto), diatoms (diatom), dinoflagellate (dino), golden algae
113 (golden) and green algae (green), bacteria; actinobacteria (actino) and cyanobacteria (cyano), and terrestrial
114 particulate organic matter (t-POM). We expanded the library by adding previously unpublished data points for
115 existing resource items as well as introduced three completely new resource items, namely, euglenoids
116 (eugleno), methane oxidising bacteria (MOB), and microbes that had consumed t-POM (t-POMb).

117 The newly constructed library was further examined for consistency. We found that several resources
118 showed indications of multimodality consisting of two or more clusters in the FA tracer space, possibly resulting
119 from the feeding tests being carried out on different resource species. As multimodality in the resource FA
120 profiles implies a multimodal distribution of consumer FA profiles, which would complicate the comparison of
121 different methods by introducing untraceable errors in the estimates, we decided to modify the resource library
122 by keeping only the data points belonging to the primary mode or the cluster with the most data points to create
123 a more concise resource library of 49 FA profile observations for 11 resource items using 23 FA tracers (library
124 presented in supplementary: Table S1). Furthermore, a non-metric multidimensional scaling analysis (NMDS)
125 of Bray-Curtis similarity was conducted to assess the similarity of resource FA profiles as these similarities could
126 make differentiating between resources harder for the methods (Figure S1).

127 Some of the FA tracers are a sum of FA proportions specific to certain resources, i.e., diatoms, green
128 algae, and MOB all have a distinct FA group in the library (Table S1). The group BrFA includes all branched
129 FAs (*iso*-14:0, i*so*-15:0, *anteiso*-15:0, *iso*-17:0 and *anteiso*-17:0); group MOB MUFA includes 16:1ω8, 16:1ω6 and
130 18:1ω8; group Green PUFA includes 16:3ω3, 16:2ω6, 16:4ω3 and 16:4ω1; and group Diatom PUFA includes
131 16:3ω4, 16:2ω7 and 16:2ω4. This grouping saves computing time as the number of dimensions is reduced and it
132 should not affect the diet estimates.

133

2.2 Models

135   The examined seven methods use mixing models to estimate the diet of a consumer based on observations of

136   the consumer's FA profile and a library of observed resource FA profiles. The FA profiles are expressed as

137   relative proportions of the FAs. Common to these methods is that the models' predicted or the expectation of

138   the predicted consumer FA profile vector, $\boldsymbol{y} = (y_1, \ldots, y_M)$, is assumed to be a weighted average of the FA profiles

139   of the resources, $\boldsymbol{x}_i = (x_{i1}, \ldots, x_{iM})$, that the consumer has eaten, where the weights, π_i, are the relative

140   proportions of the different resources in the consumer's diet. If we collect the relative proportions to a *diet vector*

141    $\boldsymbol{\pi} = (\pi_1, \ldots, \pi_N)$, and denote the $N \times M$ matrix of M FA tracers in N resource items by X, the consumer FA profile

142   can be written as

$$y = \pi X.$$

144   Using the resource library and the observed consumer FA profiles, the mixing models estimate the unknown

145   diet vector, i.e., which resources items and how much of each has the consumer assimilated.

146   FASTAR implements two Bayesian models for diet estimation; MixSIR [35] and SIAR [36]. In FASTAR,

147   the observation model for the consumer FA tracer observations, y_k, is a normal distribution where the mean is

148   a weighted sum of the resource FA tracers, x_{ik}, which are inferred from the resource library, and the variance

149   is a weighted sum of the sample variances, ω_{ik}^2, of the resource FA observations in the resource library, where

150   the weights are the relative diet proportions squared,

$$y_k \sim N\left(\sum_i \pi_i x_{ik}, \sum_i \pi_i^2 \omega_{ik}^2\right).$$

152   In SIAR, an otherwise similar observation model is used except that an unknown tracer-specific residual

153   error term, σ_k^2, is added to the observation model variance term,

$$y_k \sim N\left(\sum_i \pi_i x_{ik}, \sum_i \pi_i^2 \omega_{ik}^2 + \sigma_k^2\right).$$

155   The initial MixSIAR article [11] discusses two new models which are compared to two previous SI

156   models; MixSIR and SIAR, also utilised by FASTAR. The new models 'Model 2: Consumers as perfect

157   integrators, but with residual error' and 'Model 4: Consumers between perfect specialists and perfect integrators

158   including multiplicative error' were developed to improve the error structure in these mixing models as the

159   previous models were seen to inadequately account for error by the authors. The authors describe the perfect

160   integrator as a consumer that randomly samples the resource distributions many times, effectively sampling the

161   resource mean. Perfect specialist is described as a consumer that samples exactly at one location from each

162   source distribution, thus all variability in consumer FA values result from individual specialisation and

163   sampling error. The residual error here means that the observed spread in consumer FA values is entirely due

164   to unexplained deviations from the mean. Multiplicative error here considers the consumption rate of the

165   resources. As these are descriptions for SI and not FA data, we decided to include both models in our

166   comparison to assess how well they are suited in FA diet estimation.

167   In Model 2 (hereafter MixSIAR 1), the observation model for the consumer FA tracer observations, y_k,

168   is a normal distribution where the mean is the weighted sum of the resource FA tracers, x_{ik}, which are inferred

169   from the resource library, and the variance, σ_k^2, is an unknown tracer-specific residual error term,

$$170 \qquad y_k \sim N(\textstyle\sum_i \pi_i x_{ik}, \sigma_k^2).$$

171   In Model 4 (hereafter MixSIAR 2), an otherwise similar observation model is used except that the

172   variance is a weighted sum of the sample variances, ω_{ik}^2, of the resource FA observations in the resource library,

173   where the weights are the relative diet proportions squared, multiplied by an unknown tracer-specific error

174   term, ε_k,

$$175 \qquad y_k \sim N(\textstyle\sum_i \pi_i x_{ik}, \sum_i \pi_i^2 \omega_{ik}^2 \times \varepsilon_k).$$

176   All of the Bayesian models use the symmetric and uninformative 'flat' Dirichlet prior distribution for

177   the diet vector, and the vector of the residual error terms, σ_k^2, in SIAR was given a Gamma prior whereas in

178   MixSIAR 1 a Wishart prior was used, while MixSIAR 2 uses a uniform distribution, $U(0,20)$, for the

179   multiplicative error term, ε_k.

180   The output of the Bayesian methods is the joint posterior probability distribution of the unknown diet

181   vector and the observation model parameters, which is obtained by updating the joint prior probability

182   distribution with the consumer FA profile observations using Bayes' rule. The resulting posterior distribution

183   then reflects the modeller's beliefs about the unknown variables naturally incorporating the degree of

184   uncertainty about the unknown variables, which is reflected in the spread of the distributions.

185   QFASA differs from the Bayesian methods as it does not aim to solve a probability distribution of the

186   unknown diet vector, but instead uses a numerical nonlinear optimisation algorithm to find a point-estimate

187   which minimises some objective function, Q, that is used to describe the similarity between the observed FA

188   profiles, $\boldsymbol{y}$, and the predicted FA profile $\hat{\boldsymbol{y}} = \boldsymbol{\pi}\bar{X}$, where $\bar{X}$ is a matrix consisting of the average FA profiles for

189   each resource in the library. We focus on three objective functions based on the Aitchison distance measure

190   (QFASA-AIT), the Chi-squared distance measure (QFASA-CS), and the Kullback-Leibler distance measure

191   (QFASA-KL).

192   The Aitchison distance measure [12] yields an objective function

$$193 \qquad Q_{AIT} = \sum_k \left(\sum_j \left(\log\left[\frac{y_{k,j}}{g(\boldsymbol{y}_k)}\right] - \log\left[\frac{\hat{y}_j}{g(\hat{y})}\right] \right)^2 \right)^2,$$

194   where $g(\cdot)$ denotes the geometrical mean.

195   The objective function for the Chi-squared distance measure (Stewart et al. 2014) is defined as

$$196 \qquad Q_{CS} = \sum_k \sum_j \frac{\left(y_{k,j} - \hat{y}_j\right)^2}{c_{k,j}},$$

197   where

$$198 \qquad c_{k,j} = \begin{cases} 1 & \text{if } y_{k,j} = \hat{y}_j = 0 \\ y_{k,j} + \hat{y}_j & \text{otherwise} \end{cases}.$$

199   Finally, the objective function based on the Kullback-Leibler distance measure is

$$200 \qquad Q_{KL} = \sum_k \left(\sum_j (y_{k,j} - \hat{y}_j) \log\left[\frac{y_{k,j}}{\hat{y}_j}\right] \right)^2.$$

To quantify the uncertainty about the point-estimate, QFASAR uses 100 sample bootstrapping to produce a variance estimate [37].

In the Bayesian models, the Dirichlet prior distribution forces each diet proportion to the open interval $(0,1)$, which does not include zero, and thus none of the resource proportions are estimated to the zero. However, the absence of resource items should be indicated by exceedingly small estimates of diet proportions. The QFASA variants QFASA-AIT and QFASA-KL have the limitation that none of the predicted FA tracers can be zero, and additionally in QFASA-AIT none of the observed consumer FA tracers can be zero, as these would result in division by zero in their respective objective functions.

2.3 Test bench

As consumer FA profiles for known diet vectors are difficult to obtain, we simulated "hypothetical" consumer FA profiles for a set of hypothetical diet vectors using a simulation model to test the seven diet estimation methods. Our simulation model utilises the resource library FA profile observations which are the observed consumer FA profiles resulting from homogenous diet feeding experiments conducted by feeding the consumer only one resource item. Thus, the resource library observation $\tilde{y}_k$ for the kth resource item corresponds to a known "mono-diet" vector, $\tilde{\pi}_k$, whose kth element is one and all the other elements are zero.

We built a Bayesian model for simulating the hypothetical consumer FA profiles utilising the resource library observations. We assumed that the resource library observations and the consumer FA profile observations are produced by the same process and inferred the statistical properties of the process using the resource library. The simulation model is constructed as follows. The expected value, $E(y|\pi, X)$, of an observed consumer FA profile y given a diet vector π and the matrix of resource FA tracers X is

$$E(y|\pi, X) = \pi X.$$

Since the observed consumer FA profile vector y consists of the relative proportions of each FA in the consumer tissue, and is thus a simplex vector, we used a Dirichlet observation model for the consumer FA profiles, i.e.,

$$y|\pi, \eta, X \sim \text{Dirichlet}(\eta \pi X),$$

where the natural parameter vector of the Dirichlet distribution is expressed as the product of its expected value and a concentration parameter, η, which attempts to capture the variability in the observation process.

The mono-diet observations can be written using this model as

$$\tilde{y}_k|\tilde{\pi}_k, \eta, X \sim \text{Dirichlet}(\eta \tilde{\pi}_k X),$$

and they are conditional on the same unknown concentration parameter η and the unknown matrix of resource FA tracers X. The pairs of library observations and known mono-diet vectors $(\tilde{y}_k, \tilde{\pi}_k)$ can be then used as calibration data to learn about the unknown model parameters (η, X) by updating the parameter prior distributions according to Bayes' rule. For each row, x_i, in the matrix X, we used a Dirichlet prior,

$$x_i \sim \text{Dirichlet}(1,1,\ldots,1),$$

whereas a uniform prior was assigned for the concentration parameter,

$$\eta \sim \text{Uniform}(0.0001, 2000).$$

7

After fitting the model to the resource library data, five hypothetical consumer observations were generated for each of the 25 hypothetical diet vectors by sampling from the posterior predictive distribution of the hypothetical consumer observation. This procedure was replicated 100 times resulting in a total of 2500 test cases. A graphical description of the simulation model is presented in Figure 1. All the hypothetical consumers include one to eight absent resources and in most of the hypothetical diets the number of absent resources is greater than the number of present resources. The hypothetical diet vectors are shown in Table S2.

The simulation model parameter inference was conducted using Hamiltonian Monte Carlo sampling with probabilistic programming language Stan version 2.9.0 [38,39]. We used a warmup period of 1000 samples after which the next 1000 samples were recorded. The Stan code for calibrating the simulation model is presented in the supplementary materials.

2.4 Evaluating method performance

The evaluations for all 25 hypothetical diets were conducted using three observations since often little FA consumer data is available due to time and cost constraints. The metrics were calculated for all cases, and additionally grouped by hypothetical diets to assess potential challenges created by different combination of resources and the effect of number of different resources in the diet, or by resources to assess *Daphnia* specific challenges to the methods. The effect of sample size was then separately assessed on four hypothetical diets selected based on the three-observation performance with the number of observations ranging from one to five. Notably, however, MixSIAR does not allow running MixSIAR 1 or 2 with only one observation and thus one-observation performance on MixSIAR could not be assessed.

We calculated absolute errors, i.e., the distances of the point estimates from the true diet proportions, for each case where in the case of the Bayesian methods (i.e., FASTAR and MixSIAR) the median of the marginal posterior distribution was used as the equivalent for the QFASA point estimate. The absolute errors were calculated separately for resource proportions resource proportions with only present resources and with only absent resources.

As the proper accounting for the uncertainty in the estimates is a desirable feature of the models, we calculated the 68% and 95% central probability intervals (CPI) for each present resource proportion and for each model fit to determine how often the true diet proportions were inside these intervals. For the Bayesian models the p-CPIs were calculated from the MCMC posterior samples such that the lower and upper endpoints of the intervals correspond with the $p/2$ and $(1 - p/2)$-quantiles of the MCMC samples, respectively. The CPIs for QFASA were calculated using the quantile function (i.e., the inverse of the cumulative distribution function) of a [0,1]-truncated normal distribution where the mean was the QFASA point estimate and the standard deviation was the square root of the reported variance in the covariance matrix produced by QFASAR as the bootstrapping samples of QFASAR cannot be accessed due to its implementation.

271   We examined the methods' ability to detect the absence of resources in the diet. As discussed earlier, in the
272   Bayesian models none of the diet proportions can be zero, and thus the absence of resources should be indicated
273   by exceedingly small estimates of diet proportions. Thus, we developed a heuristic rule for detecting absent
274   resources using the posterior marginal distributions of the Bayesian models and the constructed [0,1]-truncated
275   normal distribution for QFASA. We calculated the probability of the estimate being smaller than 0.02 and if this
276   probability was higher than 50%, we decided that the method had identified an absent resource, i.e., we checked
277   if $P(\pi_i < 0.02) > 0.50$. We then applied this heuristic rule for each absent resource in all the test cases. The zero
278   heuristic was also tested on resources that were present to assess whether the heuristic would falsely identify
279   present resources as absent.

280   Finding the primary resource in consumer diet is a desirable feature for a diet estimation method.
281   Therefore, we examined whether the methods could find the primary resource of the diet by checking if the
282   primary resource in the hypothetical diet was indeed the resource with the highest estimated proportion in the
283   diet. This examination was done only on hypothetical diets which had a distinct primary resource, therefore
284   excluding hypothetical diets H13-H16 and H19-H20 from this examination.

285
286

287   3 Results

288

289   QFASA-CS and QFASA-KL had the smallest mean absolute errors in present resource cases and absent resource
290   cases (Table 1). The frequency of errors larger than 0.1 was considerably higher on QFASA-AIT than other
291   methods (Figure 2). QFASA-CS and QFASA-KL were followed by FASTAR and MixSIAR methods, with
292   QFASA-AIT having the largest absolute error (Table 1). SIAR and MixSIAR 1 always had mean absolute errors
293   with absent resources above 0.005 (Figure 2). While most of the log-gradient for absolute error of absent
294   resources for MixSIR were near zero, the mean was not as mean is highly sensitive to outliers (Figure 2). Finding
295   the true diet proportion inside the 68% CPI and 95% CPI was most frequent in SIAR (Table 1). For 68% CPI
296   MixSIR, MixSIAR 1, QFASA-CS and QFASA-KL the frequencies were close to SIAR while MixSIAR 2 and
297   QFASA-AIT had smaller frequencies (Table 1). For 95% MixSIAR 1 was closest to SIAR while MixSIR, MixSIAR
298   2, QFASA-CS and QFASA-KL were less frequent, and QFASA-AIT was the least frequent (Table 1). MixSIR
299   detected most absent resources (86.3% of cases) but notably it also detected many present resources as absent
300   (40.5% of cases) (Table 1). QFASA-CS, QFASA-KL and MixSIAR 2 detected absent resources well, while SIAR,
301   MixSIAR 1 and QFASA-AIT had problems with the absent resource detection (Table 1, Figure 2). Only MixSIR
302   considerably falsely detected present resources as absent although QFASA-AIT had some problems as well,
303   while SIAR and MixSIAR 2 had the least false negatives (Table 1). Notably, however, MixSIR and QFASA-AIT
304   had cases where they detected the primary resource as absent.

305   Examination of the individual hypothetical diets revealed that while the absolute errors generally

306   stayed below 0.2, MixSIR and QFASA-AIT produced some high errors of over 0.5 on some hypothetical diets

307   and resources (Figures S2). Absolute errors for absent resources were generally below 0.1 but for some

308   hypothetical diets and resources the error was higher than 0.2 especially for MixSIR and QFASA-AIT (Figures

309   S3). Finding the true diet proportion inside the 68% CPI was especially problematic with hypothetical diets H17-

310   H18 and H21-H25 for all methods (Figure S4), and the same effect could be observed for 95% CPI as well (Figure

311   S5). H19 was the most troublesome hypothetical for detecting absent resources from the diet as only MixSIR

312   reached over 0.15 detection rate (Figure S6). Hypothetical diets H23 and H24 resulted in the highest number of

313   falsely detected absent resources (Figure S7).

314   Absolute errors for different resources were mostly below 0.1 for all methods except MixSIR and

315   QFASA-AIT (Figure S8). However, the errors on euglenoids were over 0.2 on all Bayesian methods and QFASA-

316   AIT (Figure S8). Notably some resources such as diatoms, green algae and MOB were estimated in QFASA

317   extremely close to zero when absent in the diet (Figure S9). Again, estimating euglenoids was the most

318   problematic for the methods as they were not frequently in the 68% CPI or even in the 95% CPI for all but

319   MixSIR, QFASA-CS and QFASA-KL (Figures S10 and S11). Compared to other methods, MixSIR had frequent

320   problems with actinobacteria, dinoflagellates and golden algae, whereas QFASA-AIT had problems with

321   diatoms, MOB and tPOM in both CPI ranges (Figures S10 and S11). Cyanobacteria and dinoflagellates were the

322   most problematic resources for the methods to detect as absent in general (Figure S12). The least amount of false

323   detections was with diatoms, euglenoids, green algae and MOB's in general, while cyanobacteria, tPOM and

324   tPOMb resulted in the highest number of false detections (Figure S13). Furthermore, the 70% similarities found

325   in the NMDS analysis between actinobacteria and euglenoids, and cryptomonads and golden algae (Figure S1),

326   did not result in observable problems for the methods.

327   Four hypothetical diets were selected to assess the effect of the number of observations on the diet

328   estimates. H2 was one of the easiest for the methods, whereas H17, H19 and H23 were problematic. The absolute

329   errors of diet estimates decreased as the number of observations increased in all cases except MixSIAR 1 in H19

330   (Figure 3). However, the decrease in the absolute error was notable only up to three observations after which

331   the error seemed to level off.

332

333

334   **4 Discussion**

335

336   We tested seven different FA diet estimation methods systematically and extensively and assessed their

337   performance by several criteria. Our results demonstrate that FA diet estimation is a powerful tool for

338   determining the diets of herbivorous zooplankton including 11 different resources. Moreover, it seems that

339   recent developments in diet estimation methods have improved their accuracy of diet estimation. In our

340 systematic testing, QFASAR utilising QFASA-CS or QFASA-KL estimated most accurately the contribution of

341 different phytoplankton, bacteria, and terrestrial organic matter in herbivorous zooplankton, showing that a

342 modern numerical optimisation method can unravel dietary components from a complex group of possible

343 resources. While QFASA-CS and QFASA-KL had mean absolute errors of ~0.056, the Bayesian methods SIAR,

344 MixSIAR 1 and MixSIAR 2 did not fall far behind (mean absolute error ~0.080). QFASAR utilising QFASA-AIT

345 and FASTAR utilising MixSIR, however, had problems in the estimates as seen in higher mean absolute errors

346 (>0.113). Furthermore, QFASA-AIT estimated the primary resource falsely in almost 60% of the cases, MixSIR

347 falsely estimated present resources as being absent in over 40% of the cases, and both of these methods had

348 several cases where the primary resource was estimated as being absent. In a prior study [40], QFASA-AIT

349 performed better than QFASA-KL, whereas Stewart [13] recommended the use of QFASA-CS but did not

350 compare it to either QFASA-KL or QFASA-AIT. The reason for the inferior performance of QFASA-AIT with

351 our dataset, however, could not be identified. Our tests with our *Daphnia* library suggest that FA diet estimates

352 are most accurate on QFASAR utilising QFASA-CS or QFASA-KL. Therefore, we recommend using these

353 methods in the estimation of different phytoplankton, bacteria, and terrestrial organic matter in *Daphnia* diet.

354   Hypothetical diets with the only three present resources (H17-H18 and H21-H25) were the most

355 problematic diets for the methods to estimate. In general, the smaller the number of present resources, the

356 greater the absolute errors were. However, the only hypothetical diet (H19) with one absent resource was

357 distinctly troublesome for the models to distinguish the absent resource as only MixSIR could adequately

358 identify the absence of that resource, although notably it also estimated 50% of the present diet items as absent.

359 Six of the hypothetical diets that did not have a primary resource (H13-H16 and H19-H20) represent generalists,

360 whereas the other hypothetical diets represent specialists. The absolute errors were smallest with evenly

361 distributed hypothetical generalist diets (H19-H20) which also had the least absent resources (one and three

362 respectively). This could support the conclusions of prior studies [18,41] that more evenly distributed diet

363 proportions of a generalist diet are easier for the methods to estimate. However, with the Bayesian methods this

364 might be explained by the prior distribution whose expected value is equal diet proportions. Furthermore, the

365 absolute errors for hypothetical diets with four 0.25 resource proportions (H13-H16) were among the highest.

366 Therefore, the concept of generalist diets being easier for the methods to estimate is questionable. Our tests

367 suggest that smaller absolute errors are mainly tied to smaller number of absent resources in the diet with all

368 estimation methods.

369   In the context of intriguing questions specific to zooplankton diets, QFASA (QFASA-CS and QFASA-

370 KL) and Bayesian methods (both of the MixSIAR methods, and SIAR) accurately estimated the proportions of

371 allochthonous resources in herbivorous zooplankton diet (H12) and were able to tell whether terrestrial

372 resources were consumed directly or via microbial loop pathway (H11). Therefore, our updated resource library

373 could be used to estimate the importance of allochthonous carbon for herbivorous *Daphnia* in different lakes

374 and seasons. Moreover, these methods accurately also estimated the utilisation of cyanobacteria (H9), and thus

FA diet estimation can accurately show if *Daphnia* have consumed (assimilated) cyanobacteria or not. Any of the tested FA diet estimation methods were not able to accurately estimate the contribution of Euglenoids in *Daphnia* diet, however, they were present in only one hypothetical diet (H6) as they are only common in eutrophic lakes and do not well support growth of *Daphnia* [20]. Having only one hypothetical diet include the resource is the most probable cause for the higher absolute errors and lower results in CPI checks. These results encourage the use of FA in diet estimation for *Daphnia* as all possible resources can be detected if they are a substantial part of the diet.

According to our simulations, the absolute errors decreased as the number of observations increased. However, the absolute error levelled off after three observations suggesting that no further improvements could be obtained with the inclusion of more observations. This is an interesting result as it confirmed our *a priori* belief that three observations would be reasonable for the tests, and it might also discourage researchers from conducting unnecessary expensive and time-consuming extra measurements. If empirical data can be assumed to resemble our simulated data, it is likely that adding more than three observations will result in diminishing returns also on empirical consumer data. Therefore, we recommend using at least three replicates for fatty acid estimations.

The implementation of FASTAR described in the initial article [9] treated each consumer FA observation separately and the resulting posterior MCMC samples were then concatenated across individuals. According to [9] the intention was to treat each consumer as a fixed effect, each having their own diet. Fitting the model separately for each consumer achieves that and provides correct posterior distribution for each consumer. When concatenating these posterior samples, the resulting aggregate represents the population level estimates (assuming the sampled consumers represent diets for unsampled individuals, and that the diets among unsampled individuals occur in proportion to those in the samples). Such a distribution does not represent any of the analysed consumers, but the population aggregate across individuals. These assumptions are a possible explanation for the poor results of MixSIR. Consequently, some MixSIR diet estimate distributions were bimodal or trimodal; in these cases, the calculated point estimates potentially represent none of the modes. While SIAR was the best performing method in both 68% and 95% CPI checks, it too, fits the model separately to each observation and sums up the resulting posterior densities instead of using all the observations at once. However, multimodality was not observed in SIAR diet estimates, most likely because there was more overlap in its single observation posterior densities due to larger uncertainty in its estimates. For analyses involving multiple individuals, we caution that if studies make assumptions different from those in FASTAR [9], they consider alternative methods.

The viability of a zero heuristic to detect absent resources of the model outputs is debateable. SIAR and MixSIR 1 produce wider posterior probability distributions than the other Bayesian methods, and thus their zero-heuristic results are inferior to other methods as their medians rarely reach less than 0.02. Generally, the less false identifications there are, the less true absent detections there are. Choosing a different value as the

410 limit would not therefore make much difference. Consequently, we do not believe a reliable heuristic to detect

411 absent resources could be created for current methods. Therefore, the uncertainty with low estimates is a

412 problem that scientists have to be aware of in the current methods.

413 The recent study by Choy et al. [15] compared QFASAR utilising QFASA-AIT to FASTAR utilising

414 MixSIR on beluga whale data and concluded that QFASAR were more accurate diet estimation method than

415 MixSIR. Our results show that all tested methods except QFASA-AIT and MixSIR can estimate well enough

416 herbivorous zooplankton diets, where QFASAR with QFASA-CS or QFASA-KL gave the most reliable diet

417 estimates. There are several possible reasons for the differing results as the tested species was a consumer with

418 a high trophic level, contrary to our first-degree heterotrophs, and only one observation was used instead of the

419 three observations we recommend. Furthermore, calibration coefficients – instead of homogenous diet feeding

420 experiments – were used to account for FA modification in QFASAR and incorporated into FASTAR library,

421 unlike in our work. We, however, recognise that homogenous diet feeding experiments are not feasible for all

422 consumers and thus calibration coefficients [7] or other methods of accounting for FA modification in the

423 consumer may be required. Regardless, the diet estimation methods should be tested prior the implementation

424 to field data.

425 Creating a well working resource library is difficult, since large variance and multimodality can

426 interfere with adequate differentiation between different resources. Large variance generally makes the

427 estimates less accurate, but this should not be a problem for modern FA diet estimation methods [42]. While

428 multimodality itself is not a problem, all current diet estimation methods assume unimodality, hence the

429 removal of multimodality from our FA library. The Bayesian methods utilise the mean and standard deviation

430 of each resource. Therefore, the possible multimodality or skewness can cause the expected value to be not

431 representative of the data. Moreover, QFASAR utilises only the mean of each resource, resulting in loss of

432 observed variation in the FA data. Therefore, we urge scientists to check for multimodality before conducting

433 studies. Furthermore, means for assessing the library variances and multimodality should be created, in future

434 modelling tools. A diet estimation method more in line with the simulation model presented in this study – here

435 utilised in creating the hypothetical consumer vectors – should be considered as the basis for the next generation

436 FA diet estimation methods, as the uncertainty would be fully incorporated into the model.

437 A feature of Bayesian modelling separating it from QFASA is the possibility to use prior information.

438 This information can be incorporated in the form of informative prior distributions for the model parameters,

439 which could have improved the performance of MixSIAR. Here we did not test informative priors as QFASA

440 cannot utilise such information and FASTAR does not have a readily available implementation for informative

441 priors. For herbivorous zooplankton the usefulness of adding prior information to the model has, however,

442 already been demonstrated [8]. If prior information is available, we recommend using MixSIAR methods for

443 diet estimation.

Lengthy computing times have been a challenge with Bayesian mixing models [41]. In our simulations the FASTAR and QFASAR methods computed a single test case with three observations on average in less than a minute, while MixSIAR 1 computation run lasted over an hour and MixSIAR 2 over four hours on a modern desktop computer. In this light, the recent advance in Bayesian modelling is questionable, tens or hundreds of times longer but with minimal improvement in estimate accuracy compared to both FASTAR and QFASA. However, Hamiltonian Monte Carlo, which is implemented in the probabilistic programming language Stan [38,39], and which we used in the generation of the simulation data for this article, should be explored in the future as a solution to the long computation time [43].

Based on our results, using at least three observations, and choosing either QFASA-CS or QFASA-KL produce the most accurate results. For the Bayesian methods recommending one method is not straightforward. While SIAR produced satisfactory estimates, the assumptions of FASTAR were not suitable for our case, and neither of the MixSIAR methods performed as well as QFASA-CS or QFASA-KL. The best Bayesian method for finding absent resources was MixSIAR 2 whereas otherwise SIAR was the most reliable. According to our experiments, MixSIR should not be used in any case when more than one observation is available as MixSIAR requires at least two observations. Adding more than three observations did not considerably improve the results with our data. Detecting absent resources is not reliable with current methods, and this is an uncertainty that must be accepted.

To summarise on the differences between the two approaches, QFASA is computationally fast and gives reliable estimates on our data, but there is no way to incorporate priors, uncertainty is not modelled, and the diet estimates are point estimates. Contrarily, the Bayesian framework offers the possibility to utilise prior information and to explicitly account for all possible sources of uncertainty, which are incorporated in the posterior probability distribution. However, especially MixSIAR is comparatively very slow to compute and adding complexity only increases computational requirements. At the time, the current Bayesian methods can be recommended only when prior information is available, which means that users should explore MixSIAR. Our systematic testing showed that the established FA diet estimation methods should be tested prior to use to understand the limitations and uncertainties of these methods. We conclude that QFASAR utilising QFASA-CS or QFASA-KL can accurately unravel complex dietary mixtures in herbivorous zooplankton and can be used to estimate the contribution of different phytoplankton, bacteria, and terrestrial organic matter in herbivorous cladoceran diet. There is, however, several means by which the modeling frameworks can be advanced.

Acknowledgments

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Figures and tables
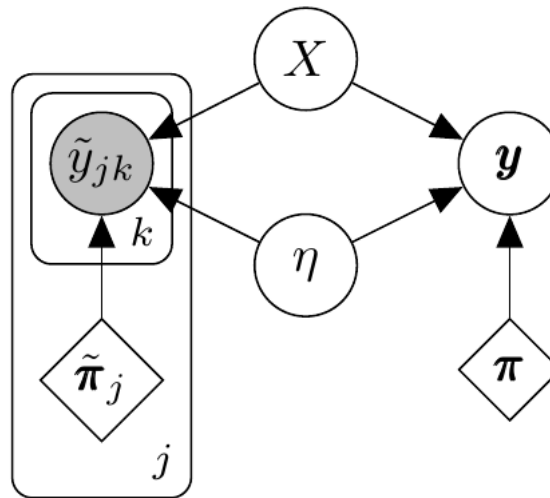
Figure 1

581
582 **Figure 1.** Graphical representation of the model used for creating the hypothetical consumer FA profiles. Here
583 X is the unknown matrix of resource FA tracers, $\tilde{y}_{jk}$ is the kth observation vector in the jth resource mono-diet
584 feeding test in the resource library, $\tilde{\boldsymbol{\pi}}_j$ is the jth mono-diet vector, $\boldsymbol{\pi}$ is the hypothetical diet vector input, $\boldsymbol{y}$ is
585 the hypothetical FA profile from which we generated our test data by drawing three random samples, and η is
586 the unknown parameter controlling the variability in the observation process. The grey circle represents the
587 observed nodes, the quadrangles represent known and input nodes, and the white circles represent unknown
588 parameters which are inferred using the resource library data.
589

590   Figure 2
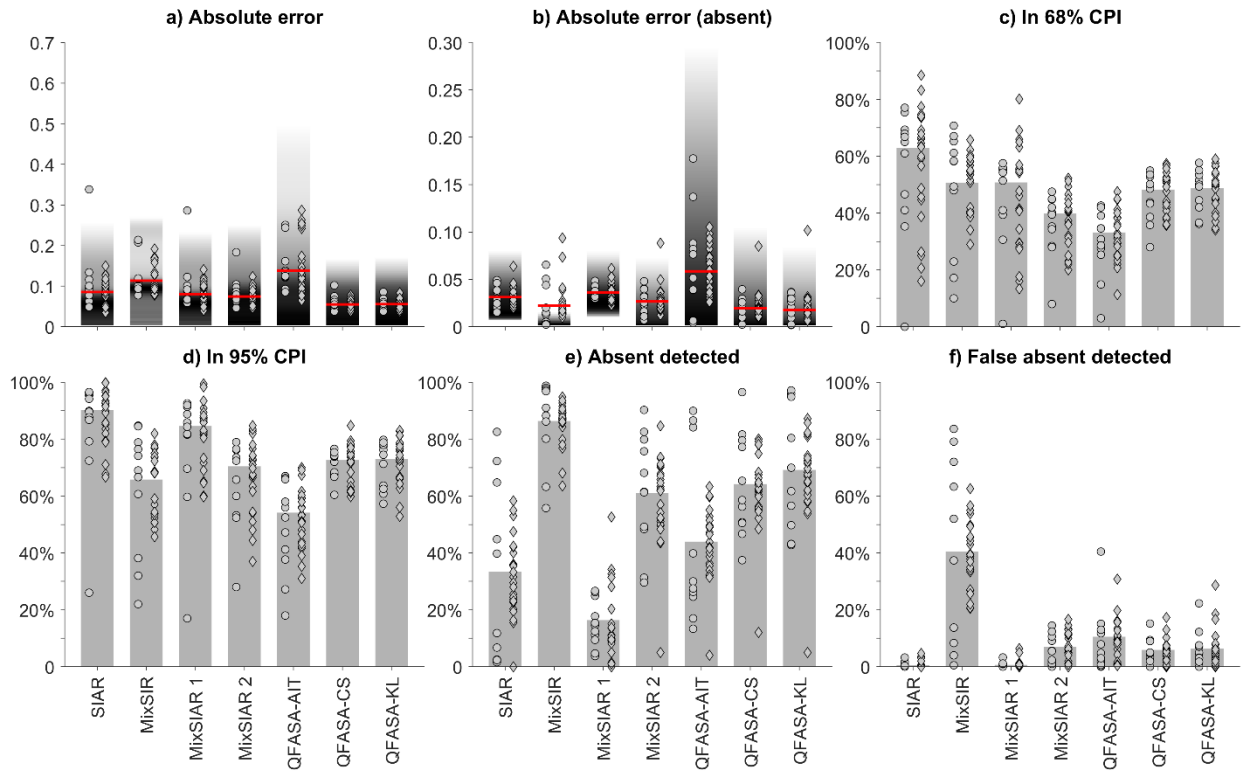
591

592   **Figure 2.** The summary of results for each method presented as a log density gradient for the absolute error

593   for present resource items (panel a) and absent resource items (panel b) with red vertical lines indicating the

594   mean. The log density gradients illustrate the distribution of the absolute errors where darker areas indicating

595   higher frequency of errors (N = 11500 and N = 16000, for present and absent resource cases, respectively). The

596   frequency of finding the true present resource proportion in the 68% and 95% CPI (panels c and d), the

597   frequency of identifying the absence of a resource (panel e), and the frequency of falsely identifying a resource

598   as absent when the resource was present (panel f), for all relevant resources are represented as bars. The

599   circles represent the averages for each 11 resources for all hypothetical diets and the rhombi represent the

600   averages for each 25 hypothetical consumers for all resources.

601

602 Table 1

603 **Table 1.** The averages and 0.95-quantiles (in parentheses) of absolute errors for present resource cases and

604 absent resource cases, frequencies of the true present diet proportion being inside 68% and 95% CPI, the

605 frequency of identifying the absence of resources over absent resource cases (true absent) and present resource

606 cases (false absent), and the frequency of detecting the primary resource for each method. The best performing

607 method for each criterion is bolded and underlined, while other well performing methods are only bolded.

		Absolute error		CPI		Zero heuristic		
Framework	Method	Present cases	Absent cases	68%	95%	Absent detected	False absent detected	Primary resource
FASTAR	SIAR	0.085 (0.282)	0.031 (0.069)	**<u>0.632</u>**	**<u>0.906</u>**	0.334	**<u>0.006</u>**	0.976
	MixSIR	0.113 (0.283)	0.022 (0.141)	0.507	0.661	**<u>0.863</u>**	0.405	0.809
MixSIAR	Model 1	0.080 (0.241)	0.036 (0.076)	0.509	**0.852**	0.164	**0.007**	**0.981**
	Model 2	0.075 (0.211)	0.027 (0.104)	0.400	0.715	0.611	0.069	0.972
QFASAR	QFASA-AIT	0.138 (0.504)	0.059 (0.236)	0.333	0.545	0.439	0.105	0.407
	QFASA-CS	**0.056 (0.141)**	**<u>0.018</u> (0.087)**	0.486	0.733	0.692	0.064	**0.996**
	QFASA-KL	**<u>0.055</u> <u>(0.139)</u>**	**0.020 <u>(0.084)</u>**	0.482	0.730	0.642	0.059	**<u>0.995</u>**

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610 Figure 3
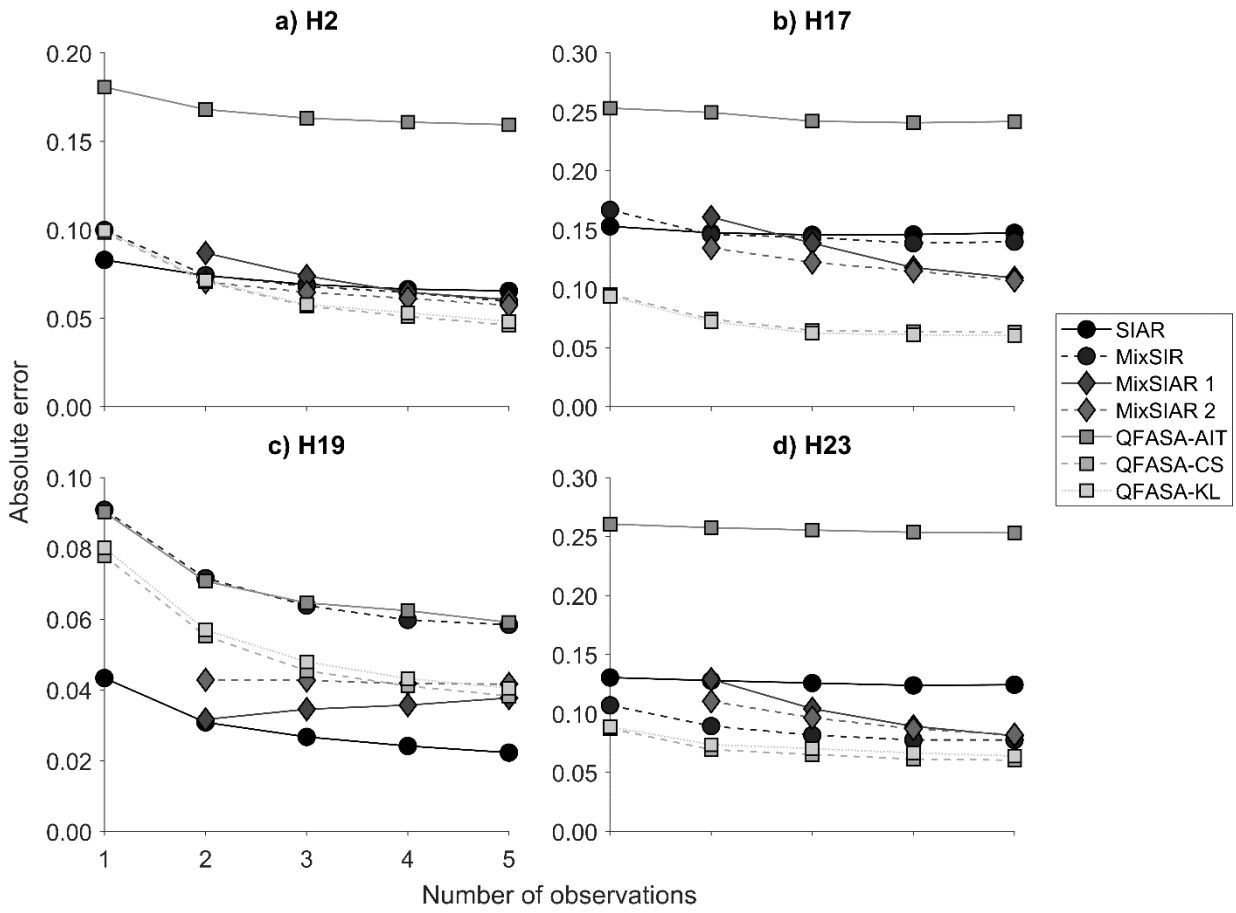
Figure 3. The effect of the number of observations on absolute error for present resources for hypothetical diets
H2, H17, H19 and H23. The tests were not conducted on MixSIAR with one observation since the software does
not allow it.