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# 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

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

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

53 When a resource is consumed by a consumer, dietary FAs are assimilated to the tissues of the consumer,  
54 and thus the FA profile of the consumer starts to resemble the FA profiles of its resources in varying degrees  
55 [16]. However, the FAs do not always directly assimilate but are often modified by the consumer metabolism to  
56 some extent [16,17]. Therefore, FA modification must be accounted for in FA diet estimation. Iverson et al. [7]  
57 introduced the concept of ‘calibration coefficients’, a coefficient calculated from empirical data for each FA to  
58 account for the FA modification and until recently it has been the main method of accounting for FA  
59 modification in FA diet estimation. The calibration coefficients attempt to capture the FA modification by  
60 constituting a linear mapping from resource to consumer. It is therefore possible to estimate the diet based only  
61 on the consumer and resource FA profiles. However, it has been shown that optimally with both QFASA and  
62 the Bayesian methods, homogenous diet feeding experiments with the consumers should be carried out when  
63 possible to better consider for the diet specific FA modification in the consumers [8,18]. In such experiments,  
64 the resource FA profiles are not directly observed, but rather the FA profiles of consumers that have consumed  
65 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

## 105 2 Material and methods

106

### 107 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, *iso*-15:0, *anteiso*-15:0, *iso*-17:0 and *anteiso*-17:0); group MOB MUFA includes 16:1 $\omega$ 8, 16:1 $\omega$ 6 and  
130 18:1 $\omega$ 8; group Green PUFA includes 16:3 $\omega$ 3, 16:2 $\omega$ 6, 16:4 $\omega$ 3 and 16:4 $\omega$ 1; and group Diatom PUFA includes  
131 16:3 $\omega$ 4, 16:2 $\omega$ 7 and 16:2 $\omega$ 4. This grouping saves computing time as the number of dimensions is reduced and it  
132 should not affect the diet estimates.

133

### 134 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,  $\mathbf{y} = (y_1, \dots, y_M)$ , is assumed to be a weighted average of the FA profiles  
 139 of the resources,  $\mathbf{x}_i = (x_{i1}, \dots, x_{iM})$ , that the consumer has eaten, where the weights,  $\pi_i$ , 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  $\boldsymbol{\pi} = (\pi_1, \dots, \pi_N)$ , 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

$$143 \quad \mathbf{y} = \boldsymbol{\pi}X.$$

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,  $\omega_{ik}^2$ , of the resource FA observations in the resource library, where  
 150 the weights are the relative diet proportions squared,

$$151 \quad y_k \sim N(\sum_i \pi_i x_{ik}, \sum_i \pi_i^2 \omega_{ik}^2).$$

152 In SIAR, an otherwise similar observation model is used except that an unknown tracer-specific residual  
 153 error term,  $\sigma_k^2$ , is added to the observation model variance term,

$$154 \quad y_k \sim N(\sum_i \pi_i x_{ik}, \sum_i \pi_i^2 \omega_{ik}^2 + \sigma_k^2).$$

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,  $\sigma_k^2$ , is an unknown tracer-specific residual error term,

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

In Model 4 (hereafter MixSIAR 2), an otherwise similar observation model is used except that the variance is a weighted sum of the sample variances,  $\omega_{ik}^2$ , of the resource FA observations in the resource library, where the weights are the relative diet proportions squared, multiplied by an unknown tracer-specific error term,  $\varepsilon_k$ ,

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

All of the Bayesian models use the symmetric and uninformative ‘flat’ Dirichlet prior distribution for the diet vector, and the vector of the residual error terms,  $\sigma_k^2$ , in SIAR was given a Gamma prior whereas in MixSIAR 1 a Wishart prior was used, while MixSIAR 2 uses a uniform distribution,  $U(0,20)$ , for the multiplicative error term,  $\varepsilon_k$ .

The output of the Bayesian methods is the joint posterior probability distribution of the unknown diet vector and the observation model parameters, which is obtained by updating the joint prior probability distribution with the consumer FA profile observations using Bayes’ rule. The resulting posterior distribution then reflects the modeller’s beliefs about the unknown variables naturally incorporating the degree of uncertainty about the unknown variables, which is reflected in the spread of the distributions.

QFASA differs from the Bayesian methods as it does not aim to solve a probability distribution of the unknown diet vector, but instead uses a numerical nonlinear optimisation algorithm to find a point-estimate which minimises some objective function,  $Q$ , that is used to describe the similarity between the observed FA profiles,  $\mathbf{y}$ , and the predicted FA profile  $\hat{\mathbf{y}} = \boldsymbol{\pi}\bar{\mathbf{X}}$ , where  $\bar{\mathbf{X}}$  is a matrix consisting of the average FA profiles for each resource in the library. We focus on three objective functions based on the Aitchison distance measure (QFASA-AIT), the Chi-squared distance measure (QFASA-CS), and the Kullback-Leibler distance measure (QFASA-KL).

The Aitchison distance measure [12] yields an objective function

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

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

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

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

where

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

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

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

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

203 In the Bayesian models, the Dirichlet prior distribution forces each diet proportion to the open interval  
 204 (0,1), which does not include zero, and thus none of the resource proportions are estimated to the zero. However,  
 205 the absence of resource items should be indicated by exceedingly small estimates of diet proportions. The  
 206 QFASA variants QFASA-AIT and QFASA-KL have the limitation that none of the predicted FA tracers can be  
 207 zero, and additionally in QFASA-AIT none of the observed consumer FA tracers can be zero, as these would  
 208 result in division by zero in their respective objective functions.

209

### 210 2.3 Test bench

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

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

$$222 E(\mathbf{y}|\boldsymbol{\pi}, X) = \boldsymbol{\pi}X.$$

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

$$225 \mathbf{y}|\boldsymbol{\pi}, \eta, X \sim \text{Dirichlet}(\eta\boldsymbol{\pi}X),$$

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

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

$$229 \tilde{\mathbf{y}}_k|\tilde{\boldsymbol{\pi}}_k, \eta, X \sim \text{Dirichlet}(\eta\tilde{\boldsymbol{\pi}}_kX),$$

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

$$234 \mathbf{x}_i \sim \text{Dirichlet}(1,1, \dots, 1),$$

235 whereas a uniform prior was assigned for the concentration parameter,

$$236 \eta \sim \text{Uniform}(0.0001, 2000).$$

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

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

247

#### 248 **2.4 Evaluating method performance**

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

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

262 As the proper accounting for the uncertainty in the estimates is a desirable feature of the models, we  
263 calculated the 68% and 95% central probability intervals (CPI) for each present resource proportion and for each  
264 model fit to determine how often the true diet proportions were inside these intervals. For the Bayesian models  
265 the  $p$ -CPIs were calculated from the MCMC posterior samples such that the lower and upper endpoints of the  
266 intervals correspond with the  $p/2$  and  $(1 - p/2)$ -quantiles of the MCMC samples, respectively. The CPIs for  
267 QFASA were calculated using the quantile function (i.e., the inverse of the cumulative distribution function) of  
268 a [0,1]-truncated normal distribution where the mean was the QFASA point estimate and the standard deviation  
269 was the square root of the reported variance in the covariance matrix produced by QFASAR as the  
270 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  $\sim 0.056$ , the Bayesian methods SIAR,  
344 MixSIAR 1 and MixSIAR 2 did not fall far behind (mean absolute error  $\sim 0.080$ ). 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

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

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

390 The implementation of FASTAR described in the initial article [9] treated each consumer FA observation  
391 separately and the resulting posterior MCMC samples were then concatenated across individuals. According to  
392 [9] the intention was to treat each consumer as a fixed effect, each having their own diet. Fitting the model  
393 separately for each consumer achieves that and provides correct posterior distribution for each consumer. When  
394 concatenating these posterior samples, the resulting aggregate represents the population level estimates  
395 (assuming the sampled consumers represent diets for unsampled individuals, and that the diets among  
396 unsampled individuals occur in proportion to those in the samples). Such a distribution does not represent any  
397 of the analysed consumers, but the population aggregate across individuals. These assumptions are a possible  
398 explanation for the poor results of MixSIR. Consequently, some MixSIR diet estimate distributions were bimodal  
399 or trimodal; in these cases, the calculated point estimates potentially represent none of the modes. While SIAR  
400 was the best performing method in both 68% and 95% CPI checks, it too, fits the model separately to each  
401 observation and sums up the resulting posterior densities instead of using all the observations at once. However,  
402 multimodality was not observed in SIAR diet estimates, most likely because there was more overlap in its single  
403 observation posterior densities due to larger uncertainty in its estimates. For analyses involving multiple  
404 individuals, we caution that if studies make assumptions different from those in FASTAR [9], they consider  
405 alternative methods.

406 The viability of a zero heuristic to detect absent resources of the model outputs is debateable. SIAR and  
407 MixSIAR 1 produce wider posterior probability distributions than the other Bayesian methods, and thus their  
408 zero-heuristic results are inferior to other methods as their medians rarely reach less than 0.02. Generally, the  
409 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.

444 Lengthy computing times have been a challenge with Bayesian mixing models [41]. In our simulations  
445 the FASTAR and QFASAR methods computed a single test case with three observations on average in less than  
446 a minute, while MixSIAR 1 computation run lasted over an hour and MixSIAR 2 over four hours on a modern  
447 desktop computer. In this light, the recent advance in Bayesian modelling is questionable, tens or hundreds of  
448 times longer but with minimal improvement in estimate accuracy compared to both FASTAR and QFASA.  
449 However, Hamiltonian Monte Carlo, which is implemented in the probabilistic programming language Stan  
450 [38,39], and which we used in the generation of the simulation data for this article, should be explored in the  
451 future as a solution to the long computation time [43].

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

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

473

474

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476

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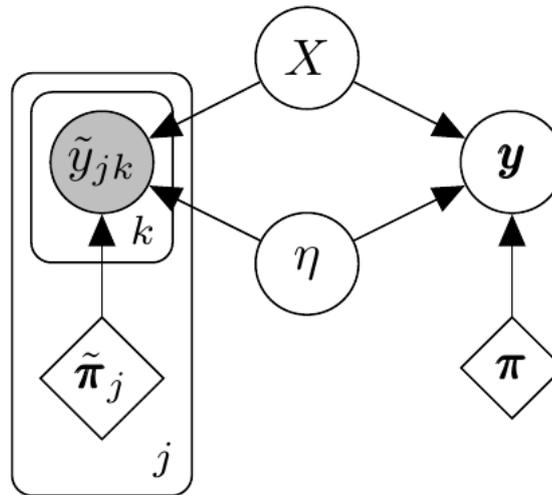
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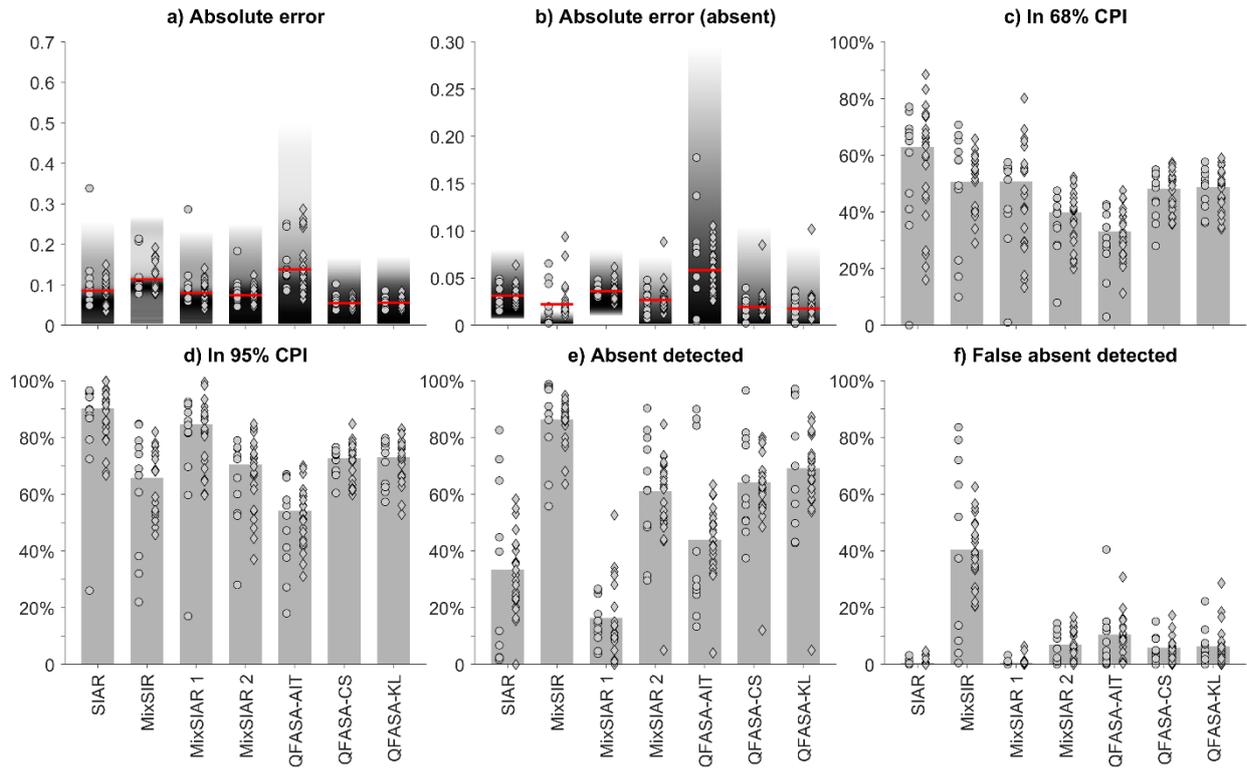


581

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  $k$ th observation vector in the  $j$ th resource mono-diet  
 584 feeding test in the resource library,  $\tilde{\pi}_j$  is the  $j$ th mono-diet vector,  $\pi$  is the hypothetical diet vector input,  $y$  is  
 585 the hypothetical FA profile from which we generated our test data by drawing three random samples, and  $\eta$  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

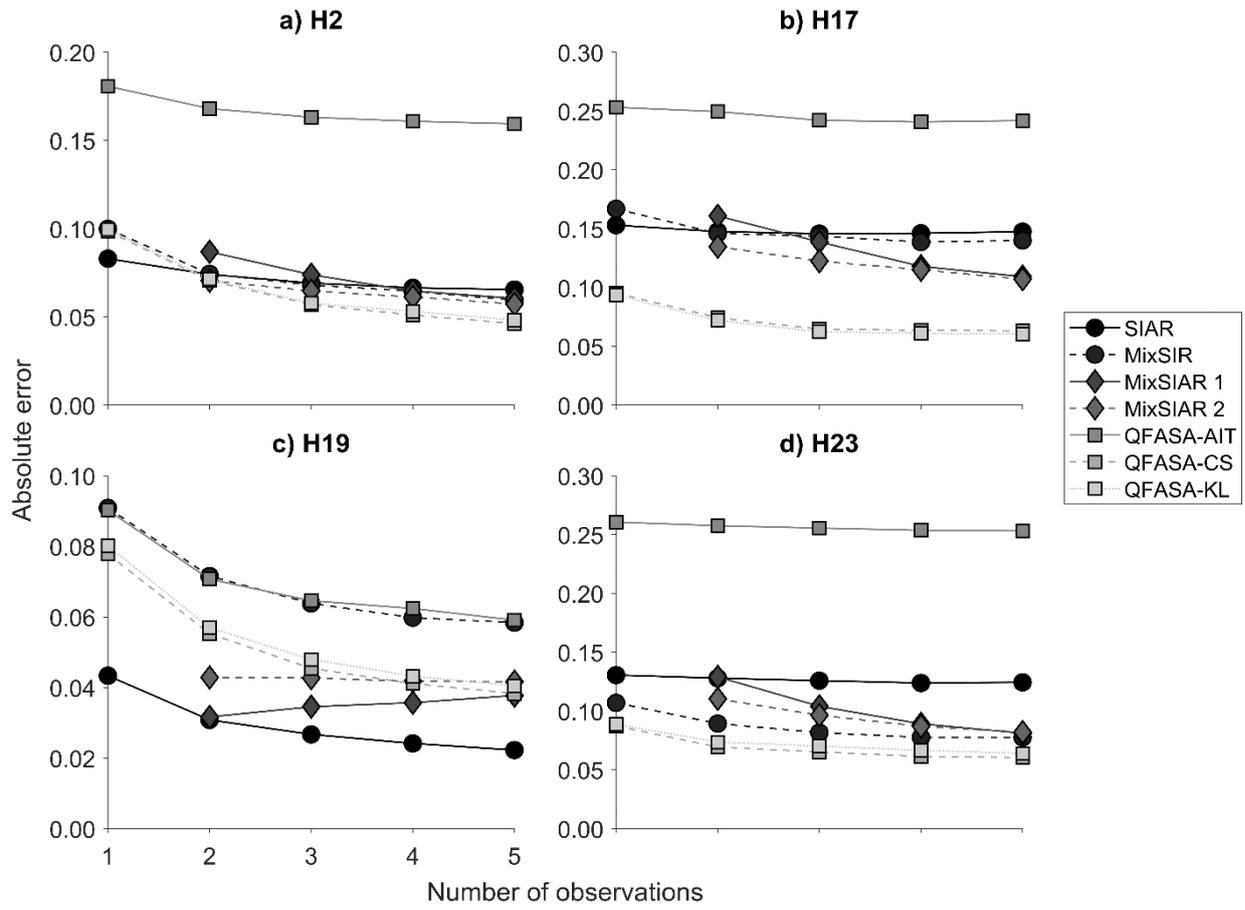
602 **Table 1**

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.

Framework	Method	Absolute error		CPI		Zero heuristic		Primary resource
		Present cases	Absent cases	68%	95%	Absent detected	False absent detected	
FASTAR	SIAR	0.085 (0.282)	0.031 (0.069)	<b><u>0.632</u></b>	<b><u>0.906</u></b>	0.334	<b><u>0.006</u></b>	0.976
	MixSIR	0.113 (0.283)	0.022 (0.141)	0.507	0.661	<b><u>0.863</u></b>	0.405	0.809
MixSIAR	Model 1	0.080 (0.241)	0.036 (0.076)	0.509	<b>0.852</b>	0.164	<b>0.007</b>	<b>0.981</b>
	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	<b>0.056</b> <b>(0.141)</b>	<b><u>0.018</u></b> <b>(0.087)</b>	0.486	0.733	0.692	0.064	<b>0.996</b>
	QFASA-KL	<b><u>0.055</u></b> <b>(0.139)</b>	<b>0.020</b> <b>(0.084)</b>	0.482	0.730	0.642	0.059	<b><u>0.995</u></b>

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609

610 Figure 3



611

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