Triple stable isotope analysis to estimate the diet of the Velvet Scoter (*Melanitta fusca*) in the Baltic Sea

Rasa Morkūnė[1], Jūratė Lesutienė[1], Julius Morkūnas[1], Rūta Barisevičiūtė[2]

[1] Marine Research Institute, Klaipeda University, Klaipėda, Lithuania

[2] Center for Physical Sciences and Technology, Vilnius, Lithuania

Corresponding Author:

Rasa Morkūnė

Marine Research Institute, Klaipeda University, H. Manto str. 84, LT-92294, Klaipėda, Lithuania

Email address: rasa.morkune@apc.ku.lt

Abstract

This study quantifies contributions of different food sources in the winter diet of the Velvet Scoter (*Melanitta fusca*) in coastal waters of the Lithuanian Baltic Sea using non-lethal avian sampling. We highlight the application of stable sulphur isotope ratios as complementary to stable carbon and nitrogen isotope analysis in order to discriminate sandy bottom macrozoobenthos organisms as potential food sources for the Velvet Scoter. Selection of the most relevant trophic enrichment factors and Monte Carlo simulations in order to choose the best fitted model were provided. A stable isotope mixing model revealed the main contributions of a group of bivalves, *Mya arenaria* and *Cerastoderma glaucum*, to be 46-54%, and while the crustacean, *Saduria entomon,* comprised one third of its diet, other food sources were responsible for the remaining contributions.

Introduction

Many studies have revealed relationships between the distribution of wintering marine ducks and macrozoobenthos communities (Kube, 1996; Loring et al., 2013; Žydelis et al., 2009). Anthropogenic activities such as commercial harvesting of benthic organisms, trawling, development of wind parks, introduction of new species, eutrophication, and climate change might have negative consequences on the composition and productivity of benthic communities.

27 Alterations in the availability of feeding resources or the extent of feeding habitat degradation are

28 mentioned as important issues contributing substantially to the decline in the number of wintering

29 ducks in the Baltic Sea (Skov et al., 2011). However, they have not been directly reported for the

30 Velvet Scoter (*Melanitta fusca*), although regular observations of the winter diet composition and

31 foraging grounds of this species might be important for an analysis of declines and conservation

32 management.

33   The Velvet Scoter is considered a vulnerable species over its entire distribution (BirdLife

34 International, 2016). In the Baltic Sea, the total number of its wintering population was reported

35 as having decreased by 60% over the last two decades (Skov et al., 2011). Mid-winter surveys in

36 the Lithuanian coastal zone of the south-eastern Baltic Sea showed an 80% decline (from 40,000

37 to 8,000) in wintering scoters (Švažas, 2001; Šniaukšta, 2012, 2014, 2015, 2016). These midwinter

38 estimates of scoters did not include concentrations known to be offshore at depths <35 m (Daunys

39 et al., 2015). Nevertheless, inadequate study of trophic ecology research, limits understanding of

40 factors behind any changes in the distribution and number of wintering Velvet Scoters.

41   Outside its breeding period, Velvet Scoters mainly feed upon marine bivalves that live on the

42 surface or within the upper sandy substrates <20 m deep. Crustaceans, including isopods and

43 amphipods, annelids, echinoderms and fish had been also found in the oesophagus contents

44 (Žydelis, 2002; Fox, 2003). Since a single species can often dominate the scoter's diet, this food

45 must be of sufficient local abundance to fulfil the nutritional needs of ducks. It is assumed that

46 most scoters feed in shallow areas, where the highest density of suitable prey biomass occurs.

47 Moreover, flights by scoter flocks among different coastal areas likely help to find the best feeding

48 habitats. Research on habitat use and foraging ecology through direct observations is difficult to

49 conduct in a marine environment, so that most studies of seaduck foraging ecology and diet have

50 been based mostly on analysis of gut contents from bycaught specimens (Duffy and Jackson, 1986;

51 Fox, 2003; Barrett et al., 2007).

52   Declining wintering populations of scoters have led to fewer bycaught birds available for

53 dietary studies. Moreover, insufficient fishery regulations and a protection status targeted towards

54 a zero bycatch mortality led to an unwillingness of fisherman to deliver specimens they have

55 caught for scientific studies. This has resulted in a search for alternative non-lethal methods to

56 investigate the feeding habits of marine birds. Stable isotope analysis (SIA) of blood samples from

57   living birds provides opportunities for non-lethal dietary studies, which is important for the

58   protection of threatened species and ethical reasons (e.g. Jardine et al., 2003; Cherel et al., 2008;

59   Morkūnė et al., 2016). The stable isotope (SI) approach has been widely applied to estimate energy

60   flows and food web interactions. However, this method has been particularly powerful when

61   isotopic patterns ('isoscapes') in a study ecosystem are known and the appropriate food sources

62   differ isotopically among each other (Phillips et al., 2005). In the Baltic Sea, riverine discharge

63   and nitrogen-fixing cyanobacteria blooms complicates isotopic differentiation between carbon

64   ($^{13}C/^{12}C$, $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$, $\delta^{15}N$) isotopes because of highly variable SI values in the

65   primary organic matter sources (Rolff and Elmgren, 2000; Antonio et al., 2012; Lesutienė et al.,

66   2014). However, our previous study on the inclusion of sulphur ($^{34}S/^{32}S$, $\delta^{34}S$) isotopes in analysis

67   of Baltic Sea food webs (Morkūnė et al., 2016) revealed the possibility to distinguish food sources

68   that were either derived from benthic production influenced by sulphur reduction, or from pelagic

69   well-oxygenated water layers (Connolly et al., 2004; Croisetiere et al., 2009, Fry and Chumchal,

70   2011).

71   This study aims to quantify the contributions of different food sources in the winter diet of the

72   Velvet Scoter based on triple SIA in blood samples in the Baltic Sea. It highlights the application

73   of $\delta^{34}S$ as complementary to $\delta^{13}C$ and $\delta^{15}N$ ratios to discriminate sandy bottom macrozoobenthos

74   organisms as potential food sources for the Velvet Scoter. Gut content analyses from bycaught

75   Velvet Scoters was used to verify and complement SI mixing model results from this study.

76   **Methods**

77   **Study site**

78   The study site is located in the Lithuanian coastal zone of the south-eastern Baltic Sea. It is an

79   open coastal area with dominant sandy benthic habitats which serve as important wintering

80   grounds for Western Palearctic concentrations of the Velvet Scoter. Due to permanent sand

81   transfer, wave and current actions, as well as the absence of macrophytes and boulders, benthic

82   species biomass in the shallow mobile sand habitat <6 m depth is low and dominated by burrowing

83   infaunal (polychaetes, bivalves *Macoma balthica*) and actively swimming nectobenthic common

84   shrimps (*Crangon crangon*). The deeper (up to 30 m depth) benthic community is mostly

85 represented by *M. balthica*, *Mya arenaria*, *Cerastoderma glaucum,* polychaetes*,* and nectobenthic
86 isopods *(Saduria entomon)* (Olenin and Daunys, 2004).

Collection of ducks from fishery bycatch and gut content analysis

88 Diet composition was estimated for 71 Velvet Scoters. These birds drowned in gillnets during
89 regular fishery activities throughout March and November of 2012 and from November 2015 to
90 April 2016 at depths ranging from 2 to 22 meters above the sandy benthic habitat (Fig. 1).
91 Carcasses were supplies voluntarily by coastal commercial fishermen. In a laboratory, esophagi
92 and gizzards contents were sorted by animal prey item. Most collected birds contained some
93 pebbles, which were excluded from further calculations. The diet composition was assessed
94 according to the total wet weight (g) of prey and the proportion of the total wet weight (%),
95 including mollusc shells. The ash-free dry weight (AFDW) of the prey in grams and % represented
96 a measurement of the weight of organic material and was calculated according to Rumohr et al.
97 (1987) and Timberg et al. (2001). The frequency of the occurrence of various prey items found in
98 gut contents was expressed as a % of the total number of ducks used for the diet analysis.

Sample collection for stable isotope analysis and measurements

100 Wintering velvet scoters were captured using the night lighting technique (Whitworth et al.,
101 1997) from November 2012 to February 2013 over waters 5-15 m in depth in the Lithuanian coastal
102 zone (Fig. 1). Permits to capture, use and release birds were obtained from the Environmental
103 Protection Agency of Lithuania (No 7, 2012, and No 1, 2013). Blood (0.5–1 ml) was obtained
104 from the medial metatarsal vein of live birds (Arora, 2010). The blood samples were stored frozen
105 at -20°C in cryogenic vials. Whole blood samples were freeze-dried for 48 hours, weighed, and
106 placed in tin capsules (0.5–0.7 mg for carbon and nitrogen, 1.7–2.0 mg for sulphur) for SIA.

107 Macrozoobenthic organisms were collected for SIA in two foraging areas important for velvet
108 scoters in December 2012 (Fig. 1). A Van Veen sampler was used to collect macrozoobenthos
109 (bivalves, polychaetes) in the coastal sandy bottom area at a depth range from 10 to 15 m.
110 However, as crustaceans *S. entomon* were not found in the samplers, they were collected from a
111 scientific bottom trawl on the sandy Klaipeda-Ventspils Plateau at a depth of 35 m in the northern
112 part of the study site. Information about the distribution and biomass of *S. entomon* is not extensive
113 for the Lithuanian coastal zone because the species prefers deeper habitats in the Baltic Sea.

114   However, it is known that after disruption of the thermocline during the second part of winter, *S.*

115   *entomon* migrate to near-shore coastal areas (Bacevičius, 2013) and become available for coastal

116   predators such as benthivorous ducks and fish (based on preliminary stomach analysis; Žydelis,

117   2002; Šiaulys et al., 2012), including the area where birds were caught for this study. Moreover,

118   the area where *S. entomon* were sampled, has been designated as an important marine area for

119   marine birds, particularly due to their stable numerous concentrations during winter time (Daunys

120   et al, 2015). As we assume that *S. entomon* must be available prey on the coastal zone at least at

121   the second part of winter, and that Velvet Scoters could move between main coastal areas and

122   deeper sandy Klaipeda-Ventspils Plateau, the *S. entomon* sampling site were representative for this

123   study.

124   Entire polychaetes, muscle tissue of crustaceans, and soft tissues of bivalves were taken for

125   SIA. The sampled material was dried at 60°C for 48 hours and then was stored frozen until

126   analysis. Unfrozen samples were ground into a fine powder in an agate mortar, weighed and placed

127   into tin capsules (0.5-0.7 mg for $\delta^{13}C$ and $\delta^{15}N$ analysis and 1.1-2.3 mg for $\delta^{34}S$ analysis).

128   Isotope-ratio analysis involved precise measurement by mass spectrometry of the less

129   abundant heavy isotope relative to the more abundant light isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$)

130   of the carbon dioxide (CO_2), nitrogen gas (N_2), or sulphur dioxide gas (SO_2) generated from the

131   combustion of the sample material. The $\delta^{13}C$ and $\delta^{15}N$ ratios in the samples were determined using

132   a Thermo Scientific Delta V Advantage mass spectrometer coupled to a Flash EA 1112 elemental

133   analyser at the State Research Institute Center for Physical Sciences and Technology, Lithuania.

134   The $\delta^{34}S$ values were determined using a SerCon elemental analyser and custom cryofocusing

135   system interfaced to a SerCon 20-22 IRMS (Sercon Ltd., Cheshire, UK) at the Stable Isotope

136   Facility, University of California, USA.

137   The results of the isotopic ratios were compared to conventional standards, i.e., Vienna Peedee

138   Belemnite (VPDB), for carbon, atmospheric N_2 for nitrogen, and Vienna Canyon Diablo troilite

139   (VCDT) for sulphur, defined as δ values: $\delta X = [(R_{sample}/R_{standard}) - 1)] \times 10^3$ (‰), where $X = {}^{13}C$, ^{15}N

140   or ^{34}S, and $R = {}^{13}C/^{12}C$, $^{15}N/^{14}N$ or $^{34}S/^{32}S$. For calibration of the CO_2 and N_2 reference gases, the

141   international standards from the International Atomic Energy Agency (Vienna) were used: IAEA-

142   600 (Caffeine, $\delta^{13}C = -27.771 \pm 0.043‰_{VPDB}$) and NBS-22 (Oil $\delta^{13}C = -30.031 \pm 0.043‰_{VPDB}$)

143   were used for ^{13}C and IAEA-600 (Caffeine, $\delta^{15}N = 1 \pm 0.2‰_{air\,N2}$) for ^{15}N. Repeated analyses of the

144   homogeneous material yielded standard deviations of less than 0.08‰ for carbon and 0.2‰ for

145   nitrogen. For calibration of the SO_2 reference gases, three laboratory standards were calibrated

146   directly against IAEA-S-1 (Silver Sulphide, $\delta^{34}S = -0.30‰_{VCDT}$), IAEA-S-2 (Silver Sulphide,

147     $\delta^{34}S = 22.7 \pm 0.2‰_{VCDT}$), and IAEA-S-3 (Silver Sulphide, $\delta^{34}S = -32.3 \pm 0.2‰_{VCDT}$) were used.

148   Repeated analysis of the three laboratory standards yielded standard deviations of less than 0.3‰.

149   The long-term reproducibility of $\delta^{34}S$ measurements is $\pm 0.4‰$.

150   Lipid removal in the benthic samples was not performed in order to keep the $\delta^{15}N$ values

151   unaffected by treatment (Post et al., 2007). The C:N ratios the in majority of the benthos samples

152   were higher than the recommended limit for aquatic organisms (C:N>3.5), at which a lipid

153   correction should be performed (Table 1). Therefore, we corrected their $\delta^{13}C$ values using an

154   arithmetic lipid normalization equation proposed by Post et al. (2007): $\delta^{13}C = \delta^{13}C_{untreated} - 3.32 +$

155     $0.99 \times C:N$. Lipid correction for bird blood was not applied (Cherel et al., 2005).

156   **Analysis of stable isotope ratios**

157   The SPSS statistical software (SPSS/7.0) and R software (R Core Team, 2013) were used for

158   the calculations and presentations of the results.

159   The food sources were defined when a significantly different isotopic composition of at least

160   one isotope existed. The differences of SI ratios among species were compared using a multivariate

161   analysis of variance (MANOVA). Tukey's Honestly Significant Difference (HSD) test was used

162   to detect significantly different means. Levene's test was used to test the homogeneity of variances.

163   **Selection of trophic enrichment factors**

164   Different sets of trophic enrichment factors (TEFs) for carbon and nitrogen were used in a

165   number of SI models (Table 2). For Model0, carbon TEF was calculated for each food source

166   individually by applying a function of $-0.199 \times \delta^{13}C_{source} - 3.986$ as suggested by Caut et al. (2009);

167   the values ranged from -0.2 to 0.4‰ for individual species and/or combined sources. The standard

168   error for the carbon TEF of the combined sources was determined by first-order error propagation

169   of uncertainties (Annex 1). Nitrogen TEF for bird blood was set at $2.25 \pm 0.20‰$ following Caut

170   et al., (2009) who suggested the method to adjust isotope discrimination values for different

171   consumer groups and their tissues according to the isotope composition of diet sources. As this

172   method was criticized by Perga and Grey (2010) due to an inapplicable use of a variable TEF

173   without specific knowledge of the predator-prey fractionation dynamics, we applied more sets of

174   TEFs (Table 2) to assess sensitivity of inferences to variation in TEFs. In ModelA and ModelB,

175   TEFs of carbon and nitrogen were used in order to prove the selection of the TEF values for

176   Model0. In ModelC, we applied the TEF values obtained from Caut et al. (2009), but used averaged

177   single values (Table 2).

178   The mean reported trophic shift for sulphur ($0.5 \pm 0.56‰$) is not significantly different from

179   zero (Peterson and Fry, 1987; McCutchan et al., 2003). Thus, we did not apply any TEF for sulphur

180   in any of the SI models of this study.

181   **A Monte Carlo simulation of mixing polygons**

182   A Monte Carlo simulation of mixing polygons (Smith et al., 2013) was used to apply the point-

183   in-polygon assumption to the models. Convex hulls (*mixing polygons*) were iterated using

184   distributions of dietary sources (Fig. 2) and different sets of TEFs (Table 2), and probabilities for

185   consumers being in the mixing polygons were calculated. This provided a quantitative basis for

186   consumer exclusion (those outside the 95% mixing region) or model rejection/validation.

187   In Model0, one individual Velvet Scoter was excluded from further analysis (Fig. 3A-F).

188   Regarding the $\delta^{34}S$ values, that individual had higher $\delta^{34}S$ values which were outside the 95%

189   mixing region of the food sources. Consequently, Bayesian mixing models were calculated only

190   for the seven Velvet Scoters that were determined to be within the 95% mixing region of the

191   sources by three isotopes considered. As the TEF for carbon in ModelC differed only slightly from

192   the one in Model0, the fit of both models to the mixing polygons were very similar. Thus, further

193   Bayesian mixing modelling for ModelC were used for the seven Velvet Scoters (see mixing

194   polygons, Annex 4).

195   In ModelA and ModelB, relatively high TEFs affected the extents of mixing polygons which

196   did not validate these models. Most consumers were characterized with very low probabilities to

197   occur within the mixing polygons (Annex 2 and 3). Thus, we rejected these models as unsuitable

198   for diet estimation for the Velvet Scoters with the current food sources known to be available

199   within the Lithuanian coastal zone.

200   **Stable isotope mixing models**

201   Models, which were validated by Monte Carlo simulations of mixing polygons (i.e. Model0

202   and ModelC), were used for mixing modelling in the package SIAR (Stable Isotope Analysis in

203   R; Parnell et al., 2010). The triple $\delta^{34}S \& \delta^{15}N \& \delta^{13}C$ values were applied to estimate multiple food

204   source contributions to composite diets. Additionally, we used three different information sets for

205   mixing models: A) no prior data, B) prey proportions based on ash-free dry weight and C) those

206   based on wet weight as prior data from gut content analysis. The mean percentage with standard

207   deviation (SD) and the 95% credibility interval (CI_{95}) were outputs from isotopic mixing models.

208   **Results**

209   **Diet composition by gut content analysis**

210   Ninety-four % of bycaught individuals contained at least some food remains in their esophagi

211   and gizzards. Five species of soft bottom molluscs, two species of crustaceans, and benthic fish

212   species were identified in the guts (Table 3). Soft bottom molluscs dominated in the diet, according

213   to wet weights. *C. glaucum* bivalves dominated among the identified molluscs by wet weight,

214   while the estimation of AFDW revealed that all three bivalve species were equally important in

215   the diet. *S. entomon* were identified as important prey objects by estimations of both wet weight

216   and AFDW. Fish only accounted for a trace portion of the prey items found in the gut content

217   (Table 3). *C. glaucum* was the most frequent item, while half of the ducks also had other bivalves

218   in their guts. *S. entomon* was consumed by the one third of ducks analysed.

219   **Stable isotope ratios of Velvet Scoters and their food sources**

220   The SI ratios found within the blood samples of the eight Velvet Scoter individuals ranged by

221   0.4, 1.7 and 3.9‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, accordingly (Table 4). There were six main taxa of

222   sandy bottom macrozoobenthos that significantly differed in isotopic composition (MANOVA,

223    $F_{15, 86}=107.6$, $p<0.05$; Fig. 2; Table 1). Because of similar values of three SI ratios bivalves, *C.*

224   *glaucum,* and *M. arenaria* were pooled into one homogeneous group (HSD, p>0.05). The

225   polychaetes and *M. balthica* had similar $\delta^{34}S$ values (HSD, *p*>0.05), but might still be separated

226   by $\delta^{15}N$ values (HSD, *p*<0.001). The *C. crangon* and *S. entomon* crustaceans differed significantly

227   in their $\delta^{13}C$ values (HSD, p<0.05).

228   According to the defined SI values for the homogeneous groups, five benthic food sources

229   could be distinguished: 1) *S. entomon*, 2) *C. crangon,* 3) *M. balthica,* 4) *M. arenaria* and *C.*

230 *glaucum*, 5) polychaetes. These groups could be included as separate end-points into the mixing
231 model.

Mixing model results

233   The mixing models were run for Model0 (further description in the text and Table 5) and
234 ModelC (Annex 5; not described due to similarities to Table 5). They revealed that the main food
235 sources for Velvet Scoters derived from the *M. arenaria* and *C. glaucum* group of bivalves, which
236 contributed to 46 to 52% of the diet (Table 5; Fig. 4; Fig. 5). The proportions of other food sources
237 varied due to the different application of prior information into the mixing models. The prior
238 information enhanced the importance of the *S. entomon* and *M. balthica*, and decreased the
239 proportions of the *C. crangon* and polychaetes in diet estimations. Moreover, according to standard
240 deviations and CI$_{95}$, prior information resulted in slightly more accurate diet estimates.

241   By comparing models results based on different prior information (Table 5), it was clear that
242 inferences based on AFDW reduced the importance of the *S. entomon* to the diet of Velvet Scoters.

Discussion

Approaches of triple stable isotope measurements and gut content analysis for winter
diet estimation for the Velvet Scoter

246   In this study, triple SI measurements and gut content analysis provided relevant estimates of
247 the Velvet Scoter's diet in the wintering grounds of the Lithuanian Baltic Sea coastal zone.
248 However, as the applied methods have specific limitations and require some assumptions, diet
249 estimations might differ. Velvet Scoters, as other marine ducks, are mobile consumers and even in
250 winter, when their forage is largely restricted to marine environment, they can move large distances
251 as hydrological conditions change (Cherel et al., 2008). As the isotopic signature of tissues in
252 newly arrived individuals might acquire in previous feeding habitats (Phillips and Gregg, 2001),
253 the SIA results should be interpreted with the assumption that the tissues analysed have reached
254 an isotopic equilibrium before sampling at any particular wintering site. The isotopic half-life of
255 the bird blood was estimated as being approximately two weeks, while complete equilibrium could
256 take longer (Vander Zanden et al., 2015). Therefore, in this study, we checked the isotopic
257 equilibrium in the blood of Velvet Scoters, according to SI ratios in food sources and different sets
258 of TEFs (Fig. 3; according to Smith et al., 2013).

259   The selection of the most suitable TEFs for this particular study was a very important

260   conjecture. It is known that TEFs may vary depending on a consumer's type, its nutritional status,

261   diet quality, size, age, dietary ontogeny, tissue, elemental composition, and the isotopic value of

262   their diet objects (e.g. McCutchan et al., 2003). We used a method by Caut et al. (2009) to calculate

263   TEFs for carbon and nitrogen from the SI ratios of food sources, depending on the consumer

264   classes and types of tissue. As this method was found to be contradictory (Perga and Grey, 2010),

265   we also showed the effects of different sets of TEFs to final estimations about the winter diet for

266   Velvet Scoters. Model0 assumed TEFs from suggested by Caut et al. (2009) (i.e. varied TEFs for

267   carbon according SI values of the selected food sources; Table 2). Model A and B assumed higher

268   TEFs than Model0, but they were relevant for marine ducks (McCutchan et al., 2003; Hobson et

269   al., 2009; Federer et al., 2010). ModelC was run with mean TEFs for carbon, as also suggested by

270   Caut et al. (2009). According to Monte Carlo simulations for a priori evaluation of mixing models,

271   we omitted ModelA and ModelB as unsuitable for estimation of Velvet Scoter winter diets (Smith

272   et al., 2013). In the cases of Model0 and Model C, one of eight ducks was eliminated from further

273   diet analysis due to possible non-equilibrium of δ^{34}S ratios to local food sources in the Lithuanian

274   coastal ecosystem. We also applied external information about gut content compositions which

275   were collected during this study using ducks that had been caught by fishermen. As their gut

276   contents were assessed according to the proportions of the wet weights and AFDW of the prey

277   items, we used both these estimations as prior information for the SI mixing models. Moreover,

278   even though some potential prey items (e.g. polychaetes) were not detected during gut content

279   analysis, their high frequency of occurrence in guts of marine ducks had been documented

280   previously (Žydelis, 2002), so we still considered them as source material in our SI mixing models

281   for the evaluation of the Velvet Scoter diet within the Lithuanian coastal zone.

282   Estimates of food source proportions were very similar between Model0 and ModelC, from

283   which the maximum difference for the proportions of the food sources was 2% (see Table 5 and

284   Annex 5). The difference between the mixing model results was negligible due to the relatively

285   low variability of carbon TEFs found among the different food sources. Thus, we conclude that

286   even we apply the varying TEF for carbon (according to Caut et al., 2009), this variation was

287   sufficiently low (from -0.2 to 0.4‰), that it did not affect mixing model results.

288   As SIA analysis is based on previously known and potential diet estimations, gut content

289   analysis is assumed to be crucial for the taxonomic identification of prey objects. In this study, one

290 single individual of the invasive *Rangia cuneata* bivalve species was found within the guts of a

291 Velvet Scoter. In Lithuanian waters, the first case involving the identification of finding the

292 presence of this bivalve was reported in 2013 (Solovjova, 2017), and so current study has

293 confirmed that *R. cuneata* plays a role in the local marine food web.

294 **Application of δ^{34}S ratios**

295 This study showed that analysis of the δ^{34}S ratios increased the capacity to discriminate a

296 higher number of macrozoobenthos taxa for modelling the food source contributions in the diet of

297 benthivorous Velvet Scoter. Benthic invertebrates obtain their sulphur from either sediments, the

298 below sediment-water interface, or the water column, and this could be the reason for taxa-specific

299 δ^{34}S values (Croisetière et al., 2009; Karube et al., 2012). Unfortunately, the homogenous SI values

300 found in *M. arenaria* and *C. glaucum* did not allow for further discrimination, and therefore, they

301 were aggregated for further use in the SI mixing model. However, in using δ^{34}S values, we could

302 distinguish polychaetes and *M. balthica* from the other bivalves and crustaceans, which might be

303 explained by their different use of organic material. *M. balthica* might be attributed to switches

304 between suspension- and deposit-feeding (Zwarts and Wanink, 1989; Lin and Hines, 1994) and

305 this might be reflected in their sulphur isotopic composition. We have found that facultative

306 suspension feeders, such as *M. balthica* and polychaetes, had approximately 5.5‰ lower δ^{34}S

307 values than the obligatory suspension feeders such as *C. glaucum* and *M. arenaria*. Moreover, in

308 this study, polychaetes had much higher δ^{15}N values than *M. balthica* (the difference was 3.5‰),

309 which reflected their higher trophic position in the food web relative to the primary sources of

310 organic matter available. Therefore, the triple isotope approach allowed the relatively precise

311 discrimination of the main macrozoobenthos organisms as food sources for the Velvet Scoter.

312 **Estimation of the winter diet of the Velvet Scoter**

313 The results concerning the winter diet composition of Velvet Scoters, which were estimated

314 using both the triple SI approach and the gut content analysis, were comparable and

315 complementary. Both methods revealed the preference by Velvet Scoters for the *M. arenaria* and

316 *C. glaucum*, while the proportions of other food sources varied. The joint contribution of *C.

317 glaucum* and *M. arenaria* comprised approximately half of Velvet Scoter's diet (Table 5), while

318 *M. balthica* was only responsible for 7 to 16% of their diet. This result differed from a previous

319 study during 1996-2002, which showed the dominance of *M. arenaria* for 82% of the total wet

320 weight content found in the gut of Velvet Scoter (Žydelis, 2002). Although previously, *C. glaucum*

321 had not been reported as prey items for scoters in the Lithuanian coastal zone (Žydelis, 2002), it

322 was consumed by 92% of total number of Velvet Scoters analysed in this study (Table 3).

323 Moreover, *C. glaucum* has been reported as one of the dominant prey items in their diet along the

324 Danish, English, Polish, and German Baltic coasts (a review by Fox, 2003).

325   As the number of certain prey species might vary temporally, the diet composition of Velvet

326 Scoters reflects this variability (Fox, 2003). The biomass of *C. glaucum* increased from 0 gm^{-2} in

327 1996-2002 to more than 18 gm^{-2} in 2012-2016 (while more than 100 gm^{-2} in 2014) within a depth

328 range of 13-15 m at Juodkrantė, Lithuania (State monitoring data of the Marine Research

329 Department under the Environmental Protection Agency; Solovjova, 2017.). Therefore, the

330 differences in the diet compositions of the Velvet Scoter estimated by Žydelis (2002) and this

331 study could be explained by possible shifts in the biomasses and proportions of the prey species

332 available to Velvet Scoters between the two periods.

333   The results of our SI mixing model revealed that the *S. entomon* contributed 9% towards the

334 diet of the Velvet Scoter, while the gut content analysis revealed a contribution of 36% by wet

335 weight and 29% by AFDW. Using data from gut content analyses as prior information, SI mixing

336 models revealed the higher importance of this crustacean to the Velvet Scoter diet (by 35% by wet

337 weight and 26% by AFDW; Table 5). Previous gut content analyses only showed a small

338 contribution of *S. entomon* to the Velvet Scoter's diet (3% of total wet weight; Žydelis, 2002), but

339 it was an important prey item for the Long-tailed duck over the same sandy bottom habitat (74 %

340 of total wet weight) (Žydelis & Ruškytė, 2005). *S. entomon* is abundant in deeper areas compared

341 to the inshore coastal zone, so ducks that feed on this prey might do so in deeper waters; this may

342 be especially so in the northern part of Lithuanian marine waters, where a marine protected area

343 was established due to high and regular marine bird concentrations, including Velvet Scoters

344 (Daunys et al., 2015). Therefore, the number of *S. entomon* in the coastal zone and its importance

345 to the feeding of marine ducks might differ during the course of winter when they come closer to

346 the coast and among other years, depending on the hydrological conditions (e.g. Bacevičius, 2013).

347   As bird gut content analysis is based on the weights of objects found in the gizzard and

348 esophagus, it is common to overestimate indigestible items or those that are more difficult to digest

349   which could contribute to the total weight of prey items. Conversely, soft-bodied prey as
350   polychaetes are often underestimated because of their rapid digestion in the foregut and lower
351   detection probability, which is further influenced by the proficiency of the researchers (review of
352   Žydelis and Richman, 2015). In this study, we did not find polychaetes in any ducks examined,
353   but the SI mixing models, without prior information, estimated their contribution of 18% to the
354   diet. Their inferred importance declined considerably to 2% when using gut-based information in
355   the process of mixing modelling (Table 5). Moreover, polychaetes have been mentioned as
356   common foods for marine ducks by other authors; e.g., Žydelis (2002) reported that polychaetes
357   were taken by 83% of all the Velvet Scoters studied, but contributed only 3% to the total wet
358   weight.

359   The energy/caloric value of the prey is important determinant of their nutritional value for
360   marine ducks in winter. Bivalves are of low caloric value with a high inorganic indigestible content
361   (Fox, 2003). Moreover, crushing the hard shell of *C. glaucum* might require more energy in
362   comparison to the lighter shell of *M. balthica* and *M. arenaria* (Rumohr et al., 1987). Scarcer but
363   more easily digestible prey items such as polychaetes or fish could provide a greater energy/caloric
364   value than bivalves (review of Žydelis and Richman, 2015). This might account for the apparent
365   differences in diet estimates provided by SI mixing models vs. gut content analysis in this study.
366   Moreover, the SIA provides information on assimilated (not only ingested) food items and
367   assumptions on the importance of other prey items, as soft-bodied prey which are usually
368   underestimated during gut content analysis. This is important because the food items of
369   benthivorous ducks differ from each other by energy/caloric values and may have already
370   undergone temporal physiological changes (Waldeck and Larsson, 2013).

371   This study was based on a relatively low sample size of live bird blood samples due to inherent
372   difficulties of catching live birds in their marine wintering grounds in the open coastal zone. A
373   larger sample of Velvet Scoters for SIA analysis of blood would likely improve precision of
374   estimates and permit a comparison of diets inferred for different sex and age groups, as well as
375   uncover potentially important temporal and spatial variation in winter diets.

Conclusions

377   In this study, we demonstrated how information concerning diet composition can be obtained
378   using non-lethal blood sampling from live ducks, gut content analysis of bycaught individuals, and

379   triple SI mixing modelling, including discussions of TEF selection. Moreover, we also illustrate

380   the benefits of the application of the δ^{34}S ratio as complementary to the δ^{13}C and δ^{15}N ratios in

381   discriminating sandy bottom macrozoobenthos organisms with obligatory and facultative

382   suspension feeding in the Baltic Sea.

383   The results revealed the main contribution of the group of *M. arenaria* and *C. glaucum* to be

384   46-54% of the Velvet Scoter's diet. The *S. entomon* contributed one third towards the diet, while

385   other food sources accounted for the rest. We also discussed possible diet shifts by Velvet Scoters

386   from changes in feeding habitats. Questions on methods to study the diet composition and its

387   temporal changes should be taken into account when analysing the strong decline in the number

388   of wintering marine ducks in the Baltic Sea.

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Figure 1

Sampling locations of alive velvet scoters and prey items for the stable isotope analysis and bycaught velvet scoters for gut content analysis.

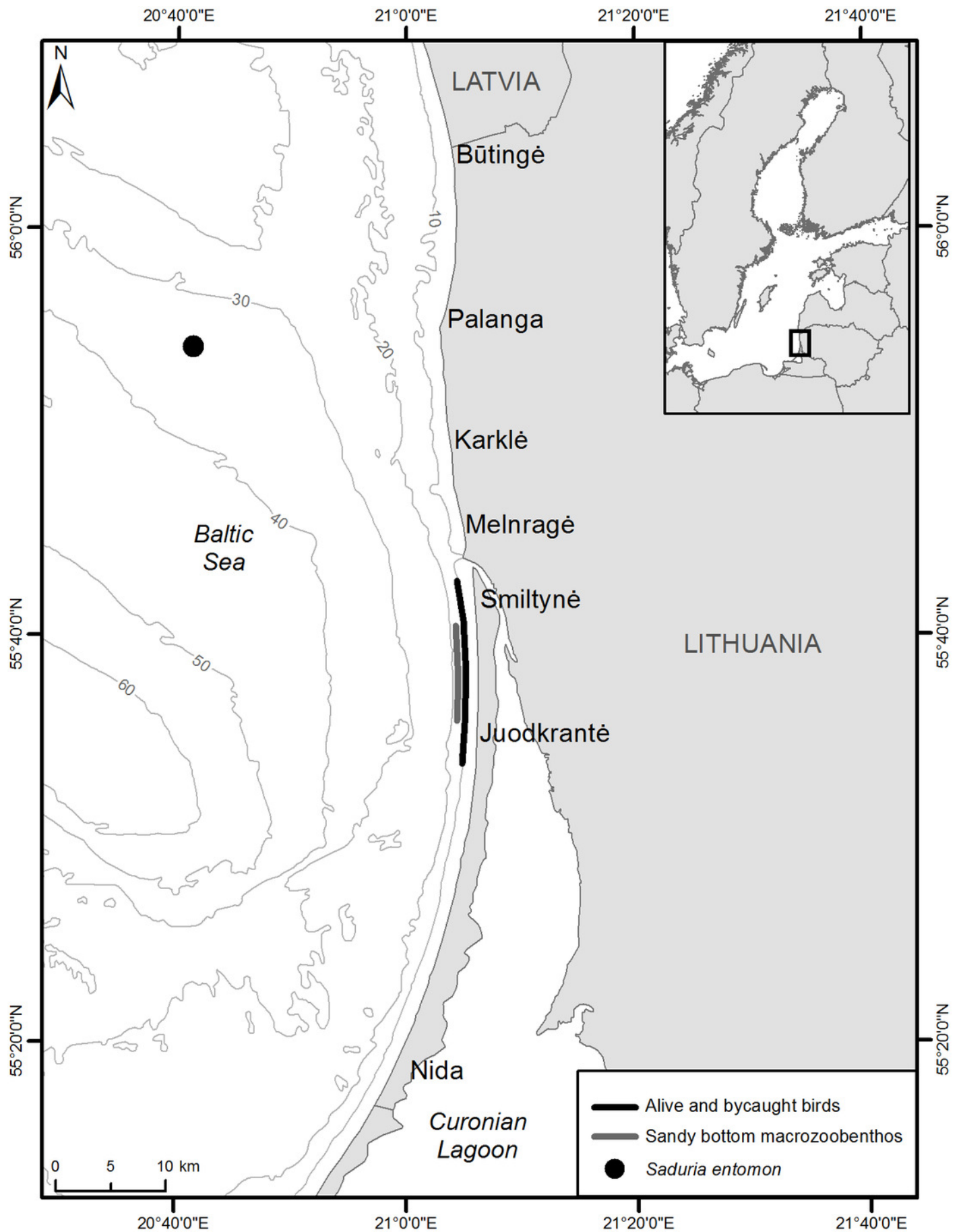



Figure 2

The mean δ^{34}S, δ^{15}N, and δ^{13}C values (± SD) in the Velvet Scoters and potential food sources.

Open circles denote the Velvet Scoters. Triangles denote bivalves: Mac – *Macoma balthica*, Mya – *Mya arenaria*, Cer – *Cerastoderma glaucum*. Grey circles mark crustaceans: Sad – *Saduria entomon*, Cra – *Crangon crangon*. Black circle denotes polychaetes.

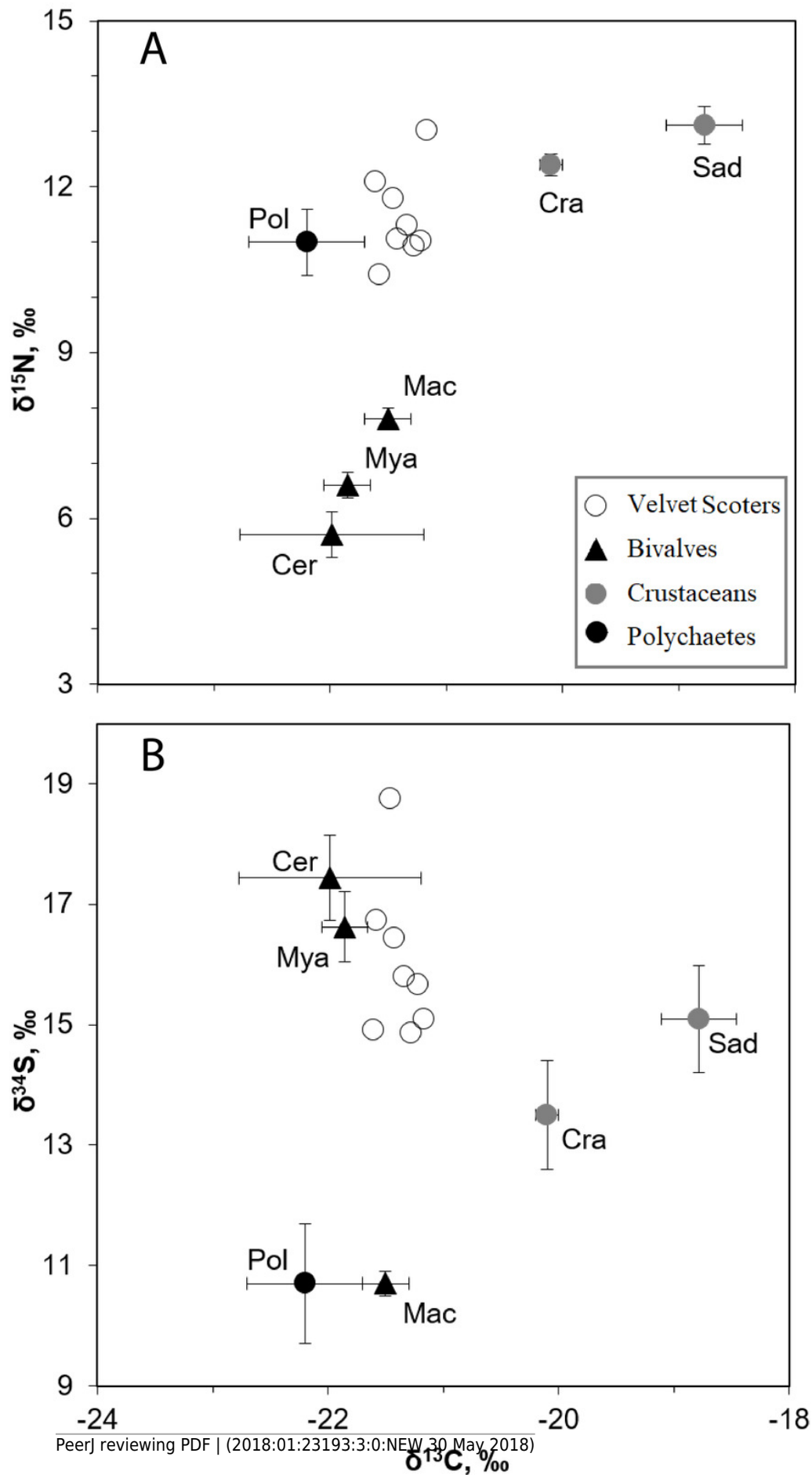



Figure 3

A, C, E. The five-source mixing model biplots with $\delta^{34}S$, $\delta^{15}N$, and $\delta^{13}C$ values after the TEF corrections in potential food sources and the Velvet Scoters (Model0). B, D, F. The simulated mixing polygons for the biplots.

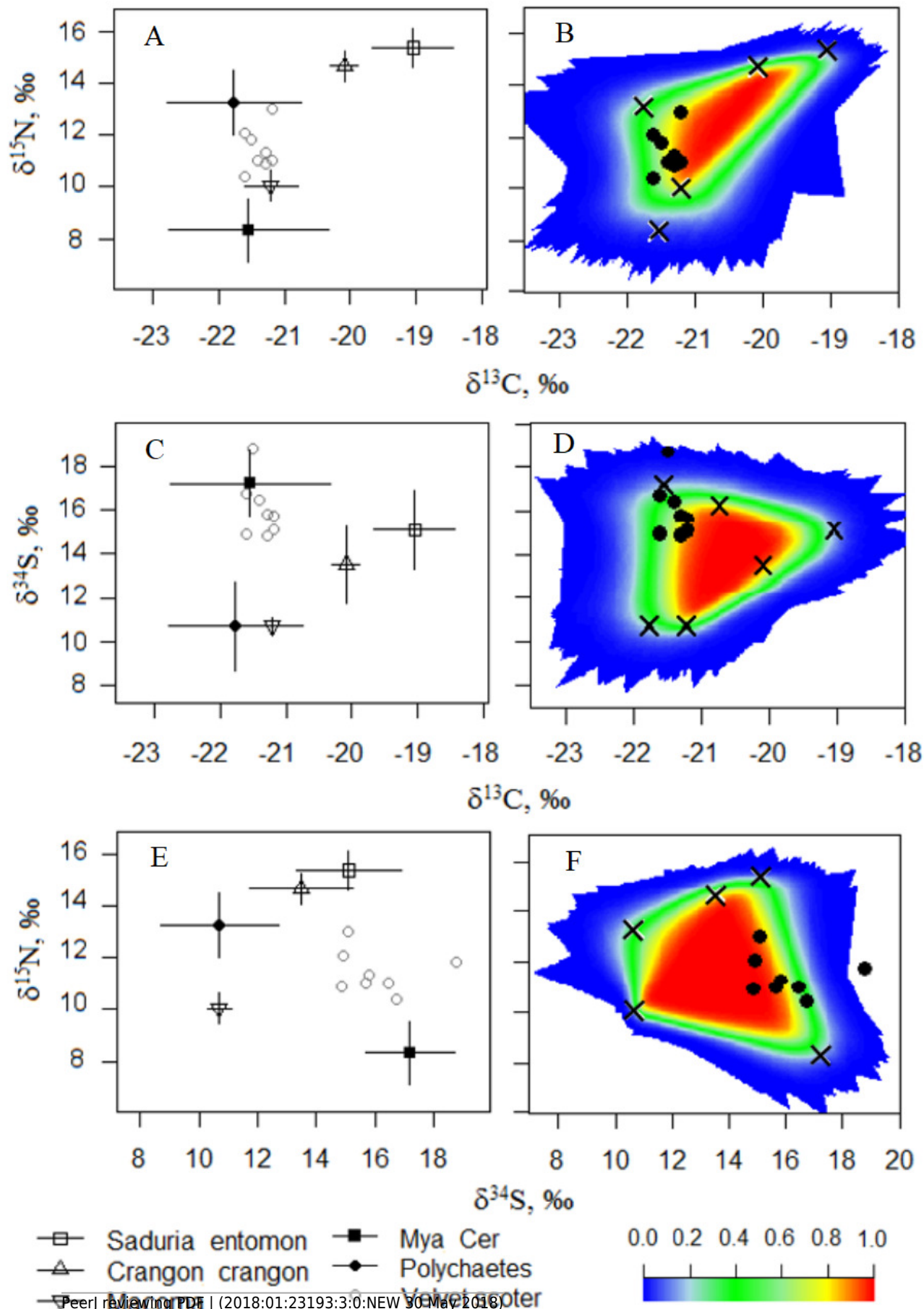



Figure 4

Density histograms showing estimated contribution of food sources for seven velvet scoters (Model0).

A) The model without prior information on diet. B) The model with organic matter weight (ash free dry weight; AFDW) and C) the model with wet weight (WW) of different food objects from gut contents analysis as prior information.

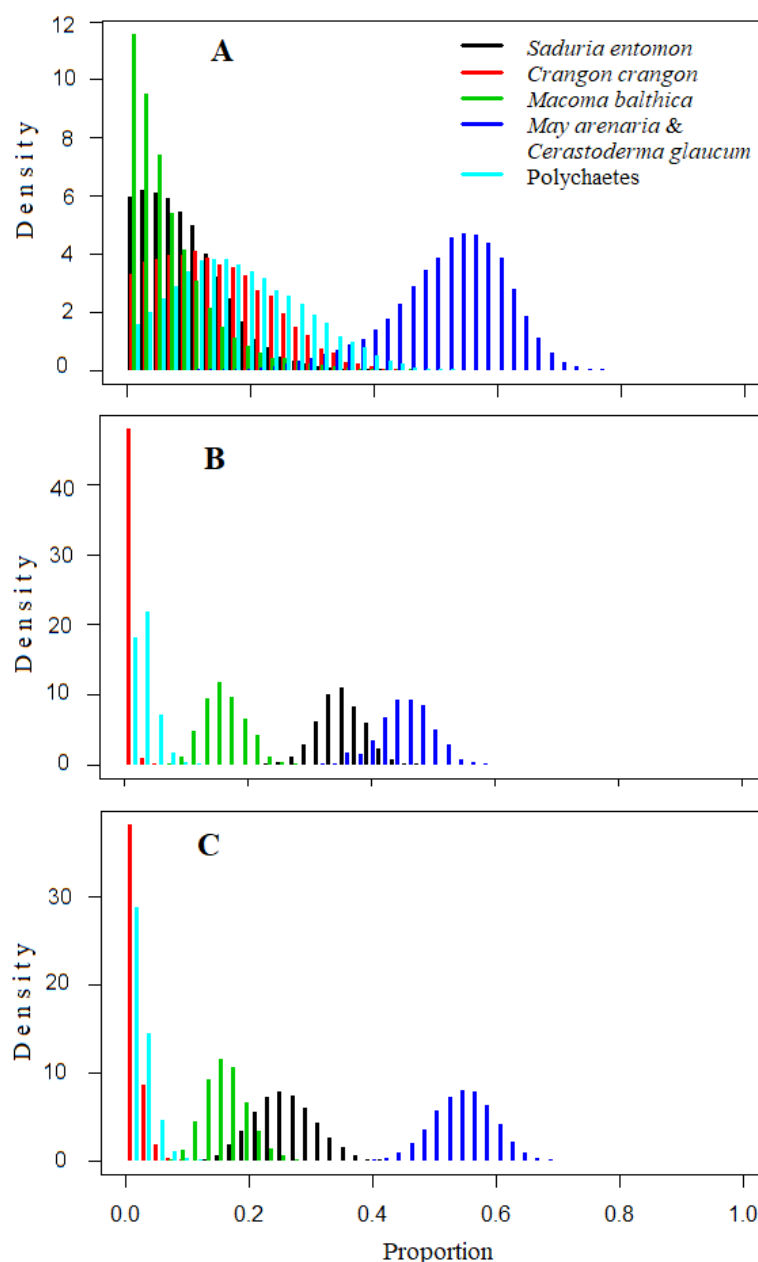
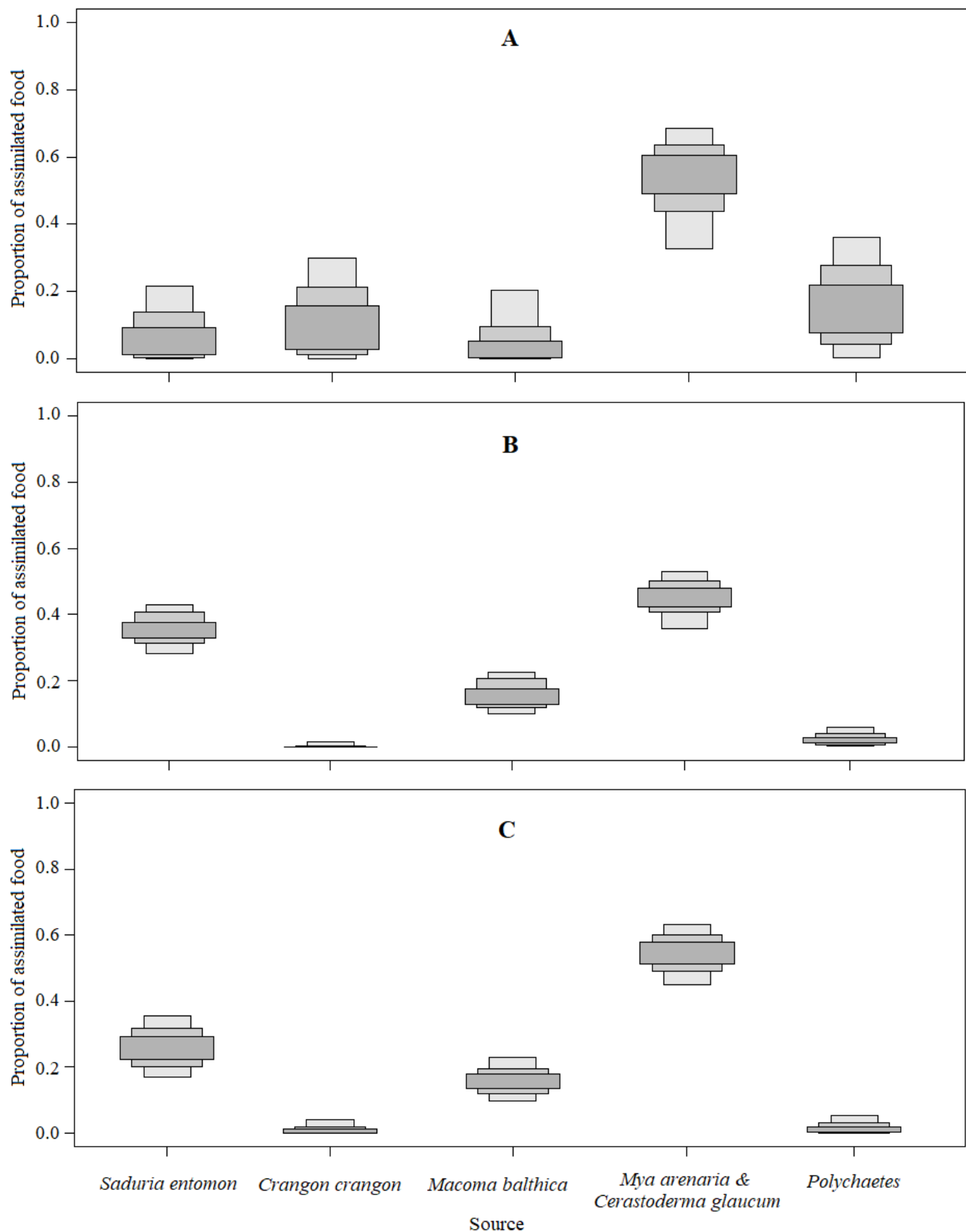



Figure 5

The estimated relative contributions of food sources (Model0)

Each plot shows 50% (dark grey), 75% (medium grey), and 95% (light grey) Bayesian credibility intervals of contributions of each source. A) The model without prior information on diet. B) The model with organic matter weight (ash free dry weight; AFDW) and C) the model with wet weight (WW) of different food objects from gut contents analysis as prior information.

Macrozoobenthos organisms as the food sources for the mixing models of the Velvet Scoters.

Sources	Sample size for $\delta^{13}C\&\delta^{15}N$ / $\delta^{34}S$	C:N	$\delta^{13}C_{untreated}$, ‰	$\delta^{13}C$, ‰	$\delta^{15}N$, ‰	$\delta^{34}S$, ‰
Saduria entomon	6/6	6.1±0.4	-21.5±0.3	-18.8± 0.3	13.1±0.3	15.1±0.9
Crangon crangon	6/6	3.4±0.0	-20.1±0.1	-20.1± 0.1	12.4±0.2	13.5±0.9
Macoma balthica	6/6	4.8±0.1	-22.8±0.1	-21.5±0.2	7.8±0.2	10.7±0.2
Mya arenaria	9/5	4.1±0.1	-22.6±0.3	-21.9±0.2	6.6±0.2	16.6±0.6
Cerastoderma glaucum	12/12	5.0±0.2	-23.6± 0.7	-22.0±0.8	5.7±0.4	17.4±0.7
Polychaetes	9/4	4.3±0.2	-23.1±0.4	-22.2±0.5	11.0±0.6	10.7±1.0

Information about the models and applied simulations in the study.

Model sets	Applied trophic enrichment factors			Validation by Monte Carlo simulations	Mixing model results	Prior information for mixing models
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$			
Model0	-0.2 to 0.4‰ by [1] (see Annex 1)	2.25±0.01 ‰ [1]	not applied (used as 0±0 ‰)	Yes, but one individual was removed from further analysis	Yes	No
						WW*
						AFDW**
ModelA	1.0±0.2 ‰ [2]	4.5±0.2 ‰ [2]		No; too many individuals lied outside the 95% mixing region or on its limit, and that requires alternative models to explain their isotopic signatures.	No	No
ModelB	0.4±0.17 ‰ [3]	2.67±0.7 ‰ [4]			No	No
ModelC	0.17±0.01 ‰ (as a mean of calculated TEFs based [1]	2.25±0.01 ‰ [1]		Yes; one individual was removed from further analysis (the same one as in Model0)	Yes	No
						WW*
						AFDW**

2   [1] Using formula for C values and stated values for N (Caut et al., 2009)

3   [2] Federer et al., 2010 as an average between cellular blood and plasma)

4   [3] McCutchan et al., 2003

5   [4] Hobson et al., 2009.

6   * Wet weight (WW) of different food objects from gut contents analysis

7   ** Organic matter weight (ash free dry weight; AFDW) of different food objects from gut contents analysis

Diet composition of velvet scoters (N=66).

Wet weight (WW) and organic matter weight (AFDW) of different food objects in grams (g)
and %. Frequency of occurrence (FO) of prey objects by number of individuals (ind.) and % of
duck specimens which consumed particular prey

Taxa of prey objects	WW, g	WW, %	AFDW, g	AFDW, %	FO, n	FO, %
Mollusca						
Mya arenaria	57.48	8.90	8.31	9.48	33	50.00
Macoma balthica	59.01	9.14	7.56	8.62	32	48.48
Cerastoderma glaucum	117.56	18.20	9.71	11.08	61	92.42
Rangia cuneata	<0.01	<0.01	<0.01	<0.01	1	1.52
Hydrobia ulvae	<0.01	<0.01	<0.01	<0.01	1	1.52
Unident. Mollusca	232.90	36.06	25.46	29.04	31	46.97
Crustacea						
Crangon crangon	0.79	0.12	0.21	0.24	2	3.03
Saduria entomon	175.82	27.22	34.22	39.04	23	34.85
Pisces					2	
Ammodytes tobianus	2.25	0.35	2.19	2.50	2	3.03

Blood samples characteristics of the Velvet Scoters.

Number of analysed individuals	Body weight, g	C:N mass ratio	δ¹³C, ‰		δ¹⁵N, ‰		δ³⁴S, ‰	
			Min-Max	Mean	Min-Max	Mean	Min-Max	Mean
8	1574±128	3.5±0.04	-21.6-(-21.2)	-21.4±0.2	10.4-13.0	11.5±0.8	14.9-18.8	16.3±1.3

Contributions of food sources to the diet of the Velvet Scoters, which were calculated by the five-source mixing Model0.

Different sets of prior information as the wet weight (WW) or the organic matter weight (AFDW) of food objects from gut contents analysis were used for the mixing models.

Sources	Proportions, % as Mean±SD (CI$_{95}$)		
	No prior information	WW	AFDW
Saduria entomon	9 ± 7 (0-21)	35 ± 4 (28-43)	26 ± 5 (17-35)
Crangon crangon	13 ± 9 (0-30)	0,3 ± 0,5 (0-2)	1 ± 1 (0-4)
Mya arenaria & Cerastoderma glaucum	52 ± 9 (32-68)	46 ± 4 (37-54)	54 ± 5 (45-64)
Macoma balthica	7 ± 6 (0-21)	16 ± 3 (10-22)	16 ± 3 (10-23)
Polychaetes	18 ± 10 (0-36)	3 ± 2 (0-6)	2 ± 2 (0-5)