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

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Abstract

This study presents a quantification of the contributions of different food sources in the winter diet of the Velvet Scoter (*Melanitta fusca*) in the 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 *Saduria entomon* crustacean composed 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 the 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.

Alterations in the availability of feeding resources or the extent of feeding habitat degradation are mentioned as important issues contributing substantially to the decline in the number of wintering ducks in the Baltic Sea (Skov et al., 2011). However, they have not been directly reported for the Velvet Scoter (*Melanitta fusca*), although regular observations of the winter diet composition and foraging grounds of this species might be important for an analysis of declines and conservation management.

The Velvet Scoter has the status of a vulnerable species in the entire distribution range (BirdLife International, 2016). In the Baltic Sea, the total number of its wintering population was reported as having decreased by 60% over the last two decades (Skov et al., 2011). According to mid-winter surveys in the Lithuanian coastal zone of the south-eastern Baltic Sea, the decline of wintering individuals by 80% (from 40,000 to 8,000 individuals) could be stated too (Švažas, 2001; Šniaukšta, 2012, 2014, 2015, 2016). However, at the same time numerous duck concentrations could be present offshore (at a depth up to 35 meters; Daunys et al., 2015) and were not taken into account for the mid-winter estimations. However, a lack of studies, including trophic ecology research, limits an analysis on the main changes in the number of wintering Velvet Scoters.

Outside its breeding period, the Velvet Scoter mainly feed upon marine bivalve molluscs that live on the surface or within the upper sandy substrates less than 20 m deep. Crustaceans, including isopods and amphipods, annelids, echinoderms and fish had been also found in the oesophagus contents (Žydelis, 2002; Fox, 2003). Since a single species often dominates the scoter's diet, the dominant food items depend on local sufficient quantity and the availability of certain benthic organisms that are enough to fulfil the nutritional needs of ducks. Regarding the diving depth, it is assumed that scoters feed in shallow areas, which is consistent with the highest amount of available suitable prey biomass. Moreover, scoter flocks fly daily among coastal areas in order to monitor the possibilities of the best feeding habitats. Because research based on direct observations are very limited in a marine environment, the ducks' dietary studies have been mostly based on the gut content analysis of bycaught specimens (Duffy and Jackson, 1986; Fox, 2003; Barrett et al., 2007).

The decreased numbers of wintering populations have led to lower number of bycaught birds available for the dietary studies. Moreover, insufficient fishery regulations and a protection status targeted towards a zero bycatch mortality led to an unwillingness of fisherman to deliver

specimens they have caught for scientific studies. This has resulted in a search for alternative non-lethal methods to investigate the feeding habits of marine birds. Stable isotope analysis (SIA) of blood samples from living birds has opened up opportunities for non-lethal dietary studies, which is important for the protection of threatened species and ethical reasons (e.g. Jardine et al., 2003; Cherel et al., 2008; Morkūnė et al., 2016). The stable isotope (SI) approach has been widely applied to estimate energy flows and food web interactions. However, this method has been particularly powerful when isotopic patterns ('isoscapes') in a study ecosystem are known and the appropriate food sources differ isotopically among each other (Phillips et al., 2005). In the Baltic Sea, with present riverine discharge and nitrogen-fixing cyanobacteria blooms, the isotopic differentiation between carbon ($^{13}C/^{12}C$, $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$, $\delta^{15}N$) isotopes might be complicated, due to highly variable SI values in the primary organic matter sources (Rolff and Elmgren, 2000; Antonio et al., 2012; Lesutienė et al., 2014). However, our previous study on the application of additional sulphur ($^{34}S/^{32}S$, $\delta^{34}S$) isotope analysis in the Baltic Sea (Morkūnė et al., 2016) revealed a possibility to distinguish food sources that were either derived from benthic production influenced by sulphur reduction, or pelagic well-oxygenated water layers (Connolly et al., 2004; Croisetiere et al., 2009, Fry and Chumchal, 2011).

This study aims to quantify the contributions of different food sources in the winter diet of the Velvet Scoter based on triple SIA in blood samples in the Baltic Sea. It highlights the application of $\delta^{34}S$ as complementary to $\delta^{13}C$ and $\delta^{15}N$ ratios to discriminate sandy bottom macrozoobenthos organisms as potential food sources for the Velvet Scoter. The gut content analysis of Velvet Scoters that have been caught was used to verify the SI mixing model results and complement SI mixing models by prior diet information.

Methods

Study site

The study site is located in the Lithuanian coastal zone of the south-eastern Baltic Sea. It is an open coastal area with dominant sandy benthic habitats which serve as important wintering grounds for Western Palearctic concentrations of the Velvet Scoter. Due to the permanent sand transfer, wave and current actions, as well as the absence of macrophytes and boulders, the benthic species biomass in the shallow mobile sand habitat to ~6 m depth is low and dominated by

burrowing infaunal (p*olychaetes, bivalves Macoma balthica*) and actively swimming nectobenthic common shrimps (*Crangon crangon*). The deeper (up to 30 meters in depth) benthic community is mostly represented by *M. balthica*, *Mya arenaria, Cerastoderma glaucum,* polychaetes, and nectobenthic isopods *(Saduria entomon)* (Olenin and Daunys, 2004).

Collection of the ducks caught and gut content analysis

The diet composition was estimated for 71 Velvet Scoters. These birds drowned in gillnets during regular fishery activities throughout March and November of 2012 and from November 2015 to April 2016 at depths ranging from 2 to 22 meters above the sandy habitat (Fig. 1). The carcasses were collected from coastal fishermen. In a laboratory, the contents of their esophagi and gizzards were treated by sorting material, along with the identification of each prey object. A majority of the collected birds contained some pebbles, which were not considered to be prey items and were excluded from further calculations. The diet composition was assessed according to the total wet weight of prey in grams and the proportion of the total wet weight (%), including mollusc shells. The ash-free dry weight (AFDW) of the prey in grams and % represented a measurement of the weight of organic material and was calculated according to Rumohr et al. (1987) and Timberg et al. (2001). The frequency of the occurrence of the various prey items found in their guts was expressed in numbers of duck specimens and as a proportion (%) of the total number used for the diet analysis.

Sample collection for stable isotope analysis and measurements

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

Macrozoobenthic organisms were collected for SIA in two foraging areas important for velvet scoters in December 2012 (Fig. 1). A Van Veen sampler was used to collect macrozoobenthos (bivalves, polychaetes) in the coastal sandy bottom area at a depth range from 10 to 15 m.

115 However, as crustaceans *S. entomon* were not found in the samplers, they were collected from a
116 scientific bottom trawl on the sandy Klaipeda-Ventspils Plateau at a depth of 35 m in the northern
117 part of the study site. Information about the distribution and biomass of *S. entomon* is not extensive
118 for the Lithuanian coastal zone because the species prefers deeper habitats in the Baltic Sea.
119 However, it is known that after disruption of the thermocline during the second part of winter, *S.*
120 *entomon* come to the coastal areas (Bacevičius, 2013) and become available for coastal predators
121 such as benthivorous ducks and fishes (based on preliminary stomach analysis; Žydelis, 2002;
122 Šiaulys et al., 2012), including the area where birds were caught for this study. Moreover, the area
123 where *S. entomon* were sampled, has been designated as an important marine area for marine birds,
124 particularly due to their stable numerous concentrations during winter time (Daunys et al, 2015).
125 As we assume that *S. entomon* must be available prey on the coastal zone at least at the second
126 part of winter, and that Velvet Scoters could move between main coastal areas and deeper sandy
127 Klaipeda-Ventspils Plateau, the *S. entomon* sampling site were representative for this study.

128 The entire bodies of polychaetes, muscle tissue of crustaceans, and soft tissues of bivalves
129 were taken for the analysis. The sampled material was dried at 60°C for 48 hours and then was
130 stored frozen until analysis. Unfrozen samples were ground into a fine powder in an agate mortar,
131 weighed and placed into tin capsules (0.5-0.7 mg for $\delta^{13}C$ and $\delta^{15}N$ analysis and 1.1-2.3 mg for
132 $\delta^{34}S$ analysis).

133 Isotope-ratio analysis involved precise measurement by mass spectrometry of the less
134 abundant heavy isotope relative to the more abundant light isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$)
135 of the carbon dioxide (CO_2), nitrogen gas (N_2), or sulphur dioxide gas (SO_2) generated from the
136 combustion of the sample material. The $\delta^{13}C$ and $\delta^{15}N$ ratios in the samples were determined using
137 a Thermo Scientific Delta V Advantage mass spectrometer coupled to a Flash EA 1112 elemental
138 analyser at the State Research Institute Center for Physical Sciences and Technology, Lithuania.
139 The $\delta^{34}S$ values were determined using a SerCon elemental analyser and custom cryofocusing
140 system interfaced to a SerCon 20-22 IRMS (Sercon Ltd., Cheshire, UK) at the Stable Isotope
141 Facility, University of California, USA.

142 The results of the isotopic ratios were compared to conventional standards, i.e., Vienna Peedee
143 Belemnite (VPDB), for carbon, atmospheric N_2 for nitrogen, and Vienna Canyon Diablo troilite
144 (VCDT) for sulphur, defined as δ values: $\delta X = [(R_{sample}/R_{standard}) - 1)] \times 10^3$ (‰), where $X = {}^{13}C$, ^{15}N

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 international standards from the International Atomic Energy Agency (Vienna) were used: IAEA-600 (Caffeine, $\delta^{13}C$= -27.771 ± 0.043‰$_{VPDB}$) and NBS-22 (Oil $\delta^{13}C$= -30.031 ± 0.043‰$_{VPDB}$) were used for ^{13}C and IAEA-600 (Caffeine, $\delta^{15}N$=1 ± 0.2‰$_{air\ N2}$) for ^{15}N. Repeated analyses of the homogeneous material yielded standard deviations of less than 0.08‰ for carbon and 0.2‰ for nitrogen. For calibration of the SO_2 reference gases, three laboratory standards were calibrated directly against IAEA-S-1 (Silver Sulphide, $\delta^{34}S$= -0.30‰$_{VCDT}$), IAEA-S-2 (Silver Sulphide, $\delta^{34}S$= 22.7 ± 0.2‰$_{VCDT}$), and IAEA-S-3 (Silver Sulphide, $\delta^{34}S$= -32.3 ± 0.2‰$_{VCDT}$) were used. Repeated analysis of the three laboratory standards yielded standard deviations of less than 0.3‰. The long-term reproducibility of $\delta^{34}S$ measurements is ± 0.4‰.

Lipid removal in the benthic samples was not performed in order to keep the $\delta^{15}N$ values unaffected by treatment (Post et al., 2007). The C:N ratios the in majority of the benthos samples were higher than the recommended limit for aquatic organisms (C:N>3.5), at which a lipid correction should be performed (Table 1). Therefore, we corrected their $\delta^{13}C$ values using an arithmetic lipid normalization equation proposed by Post et al. (2007): $\delta^{13}C = \delta^{13}C_{untreated}$ - 3.32 + 0.99 × C:N. Lipid correction for bird blood was not applied (Cherel et al., 2005).

Analysis of stable isotope ratios

The SPSS statistical software (SPSS/7.0) and R software (R Core Team, 2013) were used for the calculations and presentations of the results.

The food sources were defined when a significantly different isotopic composition of at least one isotope existed. The differences of SI ratios among species were compared using a multivariate analysis of variance (MANOVA). Tukey's Honestly Significant Difference (HSD) test was used to detect significantly different means. Levene's test was used to test the homogeneity of variances.

Selection of trophic enrichment factors

Different sets of trophic enrichment factors (TEFs) for carbon and nitrogen were used in a number of SI models (Table 2). For the Model0, carbon TEF was calculated for each food source individually by applying a function of -0.199 × $\delta^{13}C_{source}$ – 3.986 as suggested by Caut et al. (2009); the values ranged from -0.2 to 0.4‰ for individual species and/or combined sources. The standard error for the carbon TEF of the combined sources was determined by first-order error propagation

of uncertainties (Annex 1). The nitrogen TEF for bird blood was set at 2.25 ± 0.20‰ (Caut et al., 2009). However, these TEF values come from the study of Caut et al. (2009), who suggested the method to adjust isotope discrimination values for different consumer groups and their tissues according to the isotope composition of diet sources. As this method was criticized by Perga and Grey (2010) due to an inapplicable use of a variable TEF without specific knowledge of the predator-prey fractionation dynamics, we applied more sets of TEFs (Table 2) to provide their effects to model the final outcomes. In ModelA and ModelB, TEFs of carbon and nitrogen were used in order to prove the selection of the TEF values for Model0. In ModelC, we applied the TEF values obtained from Caut et al. (2009), but used averaged single values (Table 2).

The mean reported trophic shift for sulphur (0.5 ± 0.56‰) is not significantly different from zero (Peterson and Fry, 1987; McCutchan et al., 2003). Thus, we did not apply any TEF for sulphur in any of the SI models of this study.

A Monte Carlo simulation of mixing polygons

A Monte Carlo simulation of mixing polygons (Smith et al., 2013) was used to apply the point-in-polygon assumption to the models. Convex hulls (*mixing polygons*) were iterated using the distributions of the dietary sources (Fig. 2) and different sets of TEFs (Table 2), and the probabilities for consumers being in the mixing polygons were calculated. It provided a quantitative basis for consumer exclusion (those outside the 95% mixing region) or model rejection/validation.

In Model0, one individual Velvet Scoter was excluded from further analysis (Fig. 3A-F). Regarding the $\delta^{34}S$ values, that individual had higher $\delta^{34}S$ values which were outside the 95% mixing region of the food sources. Consequently, the Bayesian mixing models were only calculated for the seven Velvet Scoters that were determined to be within the 95% mixing region of the sources by three analysed isotopes. As the TEF for carbon in ModelC differed only slightly from the one in Model0, the fitness of both models to the mixing polygons were very similar. Thus, further Bayesian mixing modelling for ModelC were used for the seven Velvet Scoters (see mixing polygons at Annex 4).

In ModelA and ModelB, relatively high TEFs effected the extents of mixing polygons which did not validate these models. A majority of consumers were characterized with very low probabilities to occur within the mixing polygons (Annex 2 and 3). Thus, we rejected these models

as unsuitable for diet estimation for the Velvet Scoters with the current food sources available within the Lithuanian coastal zone.

Stable isotope mixing models

Models, which were validated by Monte Carlo simulations of mixing polygons (i.e. Model0 and ModelC), were used for mixing modelling in the package SIAR (Stable Isotope Analysis in R; Parnell et al., 2010). The triple $\delta^{34}S\&\delta^{15}N\&\delta^{13}C$ values were applied to estimate multiple food source contributions to the diet. Additionally, we used three different information sets for mixing models: A) no prior data, prey proportions based on both B) ash free dry weight and C) wet weight as prior data from gut content analysis. The mean percentage with standard deviation (SD) and the 95% credibility interval (CI_{95}) were the outputs of the isotopic mixing models.

Results

Diet composition by gut content analysis

Individuals that had been caught with at least some food remains in their esophagi and gizzards accounted for 94 % of all the individuals analysed. Five species of soft bottom molluscs, two species of crustaceans, and benthic fish species were identified in the guts (Table 3). Regarding the wet weights of prey items, soft bottom molluscs dominated in the diet. *C. glaucum* bivalves dominated among the identified molluscs by wet weight, while the estimation of AFDW revealed that all three bivalve species were equally important in the diet. *S. entomon* were identified as important prey objects by estimations of both wet weight and AFDW. Fish only accounted for a negligible portion of the prey items found in the gut content (Table 3). Regarding the frequency of occurrence, *C. glaucum* was the most frequent item, while half of the ducks also had other bivalves in their guts. *S. entomon* was consumed by the one third of ducks analysed.

Stable isotope ratios of Velvet Scoters and their food sources

The SI ratios found within the blood samples of the eight Velvet Scoter individuals ranged by 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 sandy bottom macrozoobenthos which significantly differed in isotopic composition (MANOVA, $F_{15, 86}=107.6$, $p<0.05$; Fig. 2; Table 1). Regarding the similar values of three SI bivalves, *C. glaucum,* and *M. arenaria* were pooled into one homogeneous group (HSD, $p>0.05$). The polychaetes and *M. balthica* had similar $\delta^{34}S$ values (HSD, $p>0.05$), but might still be separated

by $\delta^{15}N$ values (HSD, $p<0.001$). The *C. crangon* and *S. entomon* crustaceans differed significantly between each other by $\delta^{13}C$ values (HSD, $p<0.05$).

According to the defined SI values for the homogeneous groups, five benthic food sources could be distinguished: 1) *S. entomon*, 2) *C. crangon,* 3) *M. balthica,* 4) *M. arenaria* and *C. glaucum*, 5) polychaetes. These groups could be included as separate end-points into the mixing model.

Mixing model results

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

In comparing the results of the models, which were based on different prior information (Table 5), it is clear that application of AFDW decreased 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

In this study, triple SI measurements and gut content analysis provided relevant estimations on the Velvet Scoter's diet in the wintering grounds of the Lithuanian Baltic Sea coastal zone. However, as the applied methods have specific limitations and require some assumptions, diet estimations might differ. Velvet Scoters, as other marine ducks, are mobile consumers and even in winter, when their forage is mainly available in the marine environment, they move large distances within shifts of hydrological conditions (Cherel et al., 2008). As the tissues of newly arrived individuals might be isotopically acquired in previous feeding habitats (Phillips and Gregg, 2001),

261 the SIA results should be interpreted with the assumption that the tissues analysed have reached
262 an isotopic equilibrium before sampling at any particular wintering site. The isotopic half-life of
263 the bird blood was estimated being approximately two weeks, while complete equilibrium could
264 take longer (Vander Zanden et al., 2015). Therefore, in this study, we checked the isotopic
265 equilibrium in the blood of Velvet Scoters, according to SI ratios in food sources and different sets
266 of TEFs (Fig. 3; according to Smith et al., 2013).

267 The selection of the most suitable TEFs for this particular study was a very important
268 conjecture. It is known that TEFs may vary depending on a consumer's type, its nutritional status,
269 diet quality, size, age, dietary ontogeny, tissue, elemental composition, and the isotopic value of
270 their diet objects (e.g. McCutchan et al., 2003). We used a method by Caut et al. (2009) to calculate
271 TEFs for carbon and nitrogen from the SI ratios of food sources, depending on the consumer
272 classes and types of tissue. As this method was found to be contradictory (Perga and Grey, 2010),
273 we also showed the effects of different sets of TEFs to final estimations about the winter diet for
274 Velvet Scoters. The Model0 used TEFs from Caut et al. (2009) (i.e. varied TEFs for carbon
275 according SI values of the selected food sources; Table 2). Model A and B included higher TEFs
276 than Model0, but they were relevant for marine ducks (McCutchan et al., 2003; Hobson et al.,
277 2009; Federer et al., 2010). ModelC was run with mean TEFs for carbon, which came from Caut
278 et al. (2009) calculations. According the Monte Carlo simulations as a priori evaluation of mixing
279 models, we omitted ModelA and ModelB as unsuitable for an estimation of the diet of the Velvet
280 Scoter during the wintering period (Smith et al., 2013). In the cases of Model0 and Model C, one
281 of eight duck individuals was eliminated from the further diet analysis due to possible non-
282 equilibrium $\delta^{34}S$ ratios to local food sources of the Lithuanian coastal ecosystem. We also applied
283 external information about gut content compositions which were collected during this study using
284 ducks that had been caught by fishermen. As their gut contents were assessed according to the
285 proportions of the wet weights and AFDW of the prey items, we used both these estimations as
286 prior information for the SI mixing models. Moreover, even some potential preys were not find in
287 their guts during this study (e.g. polychaetes), but their importance to marine ducks was
288 documented previously, they were still used as potential prey items in our SI mixing models for
289 the evaluation of the Velvet Scoter diet within the Lithuanian coastal zone.

290 The estimations of the food source proportions were very similar between Model0 and
291 ModelC, as the maximum difference for the proportions of the food sources was 2% (see Table 5

and Annex 5). The difference between the mixing model results was negligible due to the relatively low variability of carbon TEFs found among the different food sources. Thus, we conclude that even we apply the varying TEF for carbon (according to Caut et al., 2009), the variability is low (from -0.2 to 0.4‰), it has not affected the mixing model results.

As SIA analysis is based on previously known and potential diet estimations, gut content analysis is assumed to be crucial for the taxonomic identification of prey objects. In this study, one single individual of the invasive *Rangia cuneata* bivalve species was found within the guts of a Velvet Scoter. In Lithuanian waters, the first case involving the identification of finding the presence of this bivalve was reported in 2013 (Solovjova, 2017), and thus, this study has proven the role of *R. cuneata* in the food web.

Application of $\delta^{34}S$ ratios

This study showed that analysis of the $\delta^{34}S$ ratios increased the capacity to discriminate a higher number of macrozoobenthos taxa for modelling the food source contributions in the diet of benthivorous Velvet Scoter. Benthic invertebrates obtain their sulphur from either sediments, the below sediment-water interface, or the water column, and this could be the reason for taxa-specific $\delta^{34}S$ values (Croisetière et al., 2009; Karube et al., 2012). Unfortunately, the homogenous SI values found in *M. arenaria* and *C. glaucum* did not allow for further discrimination, and therefore, they were aggregated for further use in the SI mixing model. However, in using $\delta^{34}S$ values, we could distinguish polychaetes and *M. balthica* from the other bivalves and crustaceans, which might be explained by their different use of organic material. *M. balthica* might be attributed to switches between suspension- and deposit-feeding (Zwarts and Wanink, 1989; Lin and Hines, 1994) and this might be reflected in their sulphur isotopic composition. We have found that facultative suspension feeders, such as *M. balthica* and polychaetes, had approximately 5.5‰ lower $\delta^{34}S$ values than the obligatory suspension feeders such as *C. glaucum* and *M. arenaria*. Moreover, in this study, polychaetes had much higher $\delta^{15}N$ values than *M. balthica* (the difference was 3.5‰), which reflected their higher trophic position in the food web relative to the primary sources of organic matter available. Therefore, the triple isotope approach allowed the relatively precise discrimination of the main macrozoobenthos organisms as food sources for the Velvet Scoter.

Estimation of the winter diet of the Velvet Scoter

The results concerning the winter diet composition of Velvet Scoters, which were estimated using both the triple SI approach and the gut content analysis, were comparable and complementary. Both methods revealed the preference of the Velvet Scoters for the *M. arenaria* and *C. glaucum*, while the proportions of other food sources varied. The joint contribution of *C. glaucum* and *M. arenaria* comprised approximately half of Velvet Scoter's diet (Table 5), while *M. balthica* was only responsible for 7 to 16% of their diet. This result differs from a previous study for a period of 1996-2002, which showed the dominance of *M. arenaria* for 82% of the total wet weight content found in the gut of Velvet Scoter (Žydelis, 2002). Although previously, *C. glaucum* had not been reported as prey items for scoters in the Lithuanian coastal zone (Žydelis, 2002), it was consumed by 92% of total number of Velvet Scoters analysed in this study (Table 3). Moreover, *C. glaucum* has been reported as one of the dominant prey items in their diet along the Danish, English, Polish, and German Baltic coasts (a review by Fox, 2003).

As the number of certain prey species might vary temporally, the diet composition of Velvet Scoters reflects this variability (Fox, 2003). The biomass of *C. glaucum* increased from 0 gm^{-2} in 1996-2002 to more than 18 gm^{-2} in 2012-2016 (while more than 100 gm^{-2} in 2014) within a depth range of 13-15 m at the Juodkrantė (State monitoring data of the Marine Research Department under the Environmental Protection Agency; Solovjova, 2017.). Therefore, the differences in the diet compositions of the Velvet Scoter between the periods of 1996-2002 (Žydelis, 2002) and 2012-2016 (this study) could be explained by possible shifts in the biomasses and proportions of the prey species among different years.

The results of our SI mixing model revealed that the *S. entomon* contributed 9% towards the diet of the Velvet Scoter, while the gut content analysis revealed a contribution of 36% by wet weight and 29% by AFDW. Using data from the gut content as prior information, the SI mixing models revealed the higher importance of this crustacean to the Velvet Scoter diet (by 35% by wet weight and 26% by AFDW; Table 5). Previous gut content analyses only showed a small contribution of *S. entomon* to the Velvet Scoter's diet (3% of total wet weight; Žydelis, 2002), but it was an important prey item for the Long-tailed duck over the same sandy bottom habitat (74 % of total wet weight) (Žydelis & Ruškytė, 2005). *S. entomon* is abundant in deeper areas than the coastal zone, so ducks that feed on this prey might do so in deeper waters, especially in the northern part of the Lithuanian marine waters, where the marine protected area was established due to high and regular marine bird concentrations, including Velvet Scoters (Daunys et al., 2015). Therefore,

the number of *S. entomon* in the coastal zone and its importance to the feeding of marine ducks might differ during the course of winter when they come closer to the coast and among other years, depending on the hydrological conditions present (e.g. Bacevičius, 2013).

As bird gut content analysis is based on the weights of objects found in the gizzard and esophagus, it is common to overestimate indigestible items or those that are more difficult to digest which could contribute to the total weight of prey items. Conversely, soft-bodied prey as polychaetes are often underestimated because of their rapid digestion in the foregut and rare detection, which also depends on the proficiency of the researchers (review of Žydelis and Richman, 2015). In this study, we did not find polychaetes in duck individuals examined, but the SI mixing models, without prior information, estimated their contribution of 18% to the diet. Their importance considerably decreased to 2% when using gut-based information in the process of mixing modelling (Table 5). Moreover, polychaetes have been mentioned as common food items for marine ducks by other authors, e.g. Žydelis (2002) reported that polychaetes were taken by 83% of all the Velvet Scoters studied, but the taxon only contributed 3% to the total weight.

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

This study was based on a relatively low sample size of live bird blood samples due to the complicated approach to catching live birds in their marine wintering grounds in the open coastal zone. While the number of bycaught birds for gut content analysis was much higher than for the SIA, it should still be increased in order to make a more detailed analysis of the diet variability in

different sex and age groups, as well as the temporal and spatial diet differences that may exist throughout the winter season.

Conclusions

In this study, we demonstrated how information concerning diet composition can be obtained using non-lethal blood sampling from live ducks, gut content analysis of bycaught individuals, and triple SI mixing modelling, including discussions of TEF selection. Moreover, we also proved the benefits of the application of the $\delta^{34}S$ ratio as complementary to the $\delta^{13}C$ and $\delta^{15}N$ ratios in discriminating sandy bottom macrozoobenthos organisms with obligatory and facultative suspension feeding in the Baltic Sea.

The results revealed the main contribution of the group of *M. arenaria* and *C. glaucum* to be 46-54% of the Velvet Scoter's diet. The *S. entomon* contributed one third towards the diet, while other food sources accounted for the rest. This study contributes as one of continuous trophic studies which provide more accurate estimations on diet composition using different approaches. We also discussed possible diet alterations according to changes in feeding habitats. Questions on methods to study the diet composition and its temporal changes should be taken into account when analysing the strong decline in the number 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.

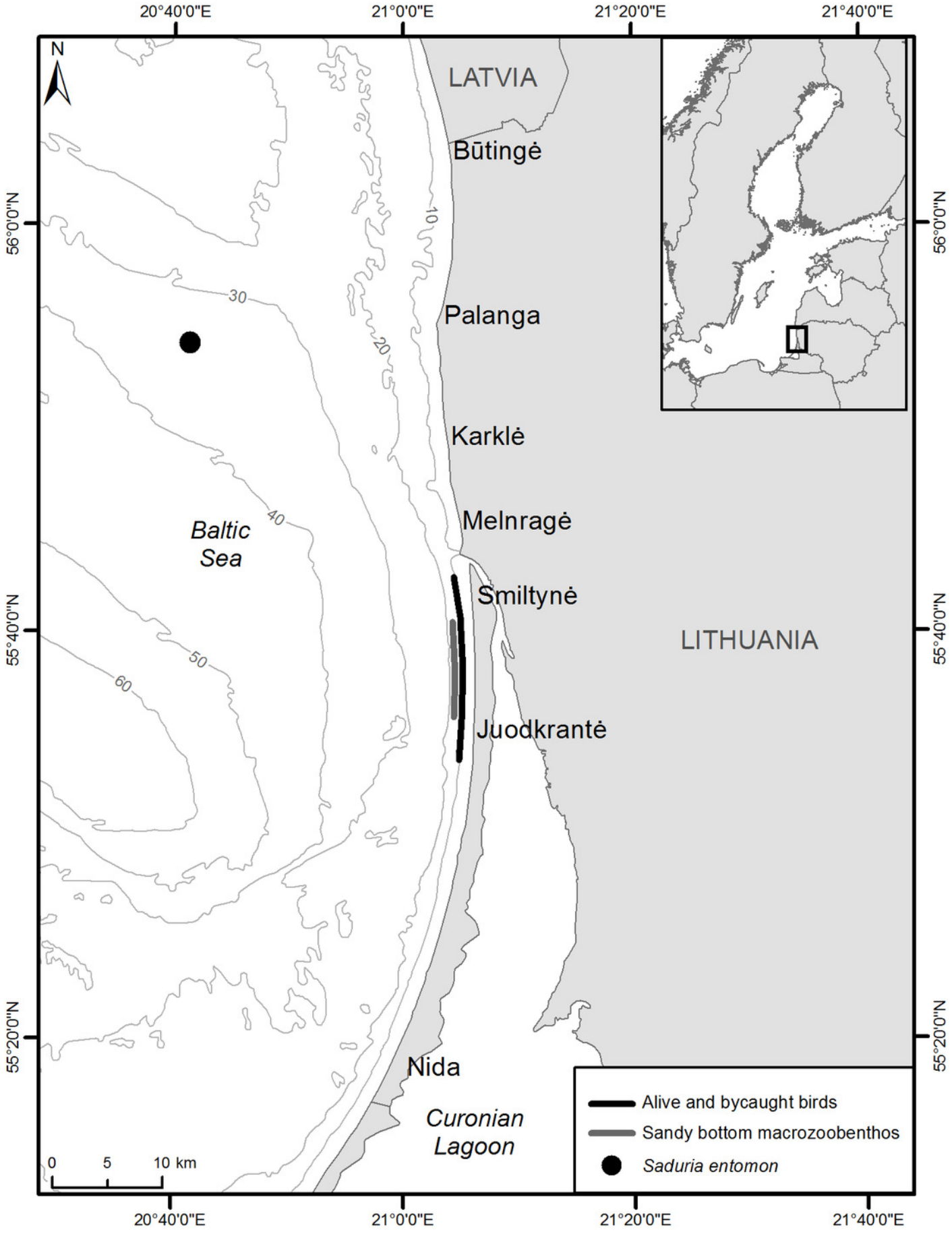
20°40'0"E
21°0'0"E
21°20'0"E
21°40'0"E
N
LATVIA
Būtingė
Palanga
Karklė
Melnragė
Smiltynė
LITHUANIA
Juodkrantė
Nida
Baltic Sea
Curonian Lagoon
10
20
30
40
50
60
56°0'0"N
55°40'0"N
55°20'0"N
0 5 10 km
Alive and bycaught birds
Sandy bottom macrozoobenthos
Saduria entomon

Figure 2

The mean $\delta^{34}S$, $\delta^{15}N$, and $\delta^{13}C$ 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.

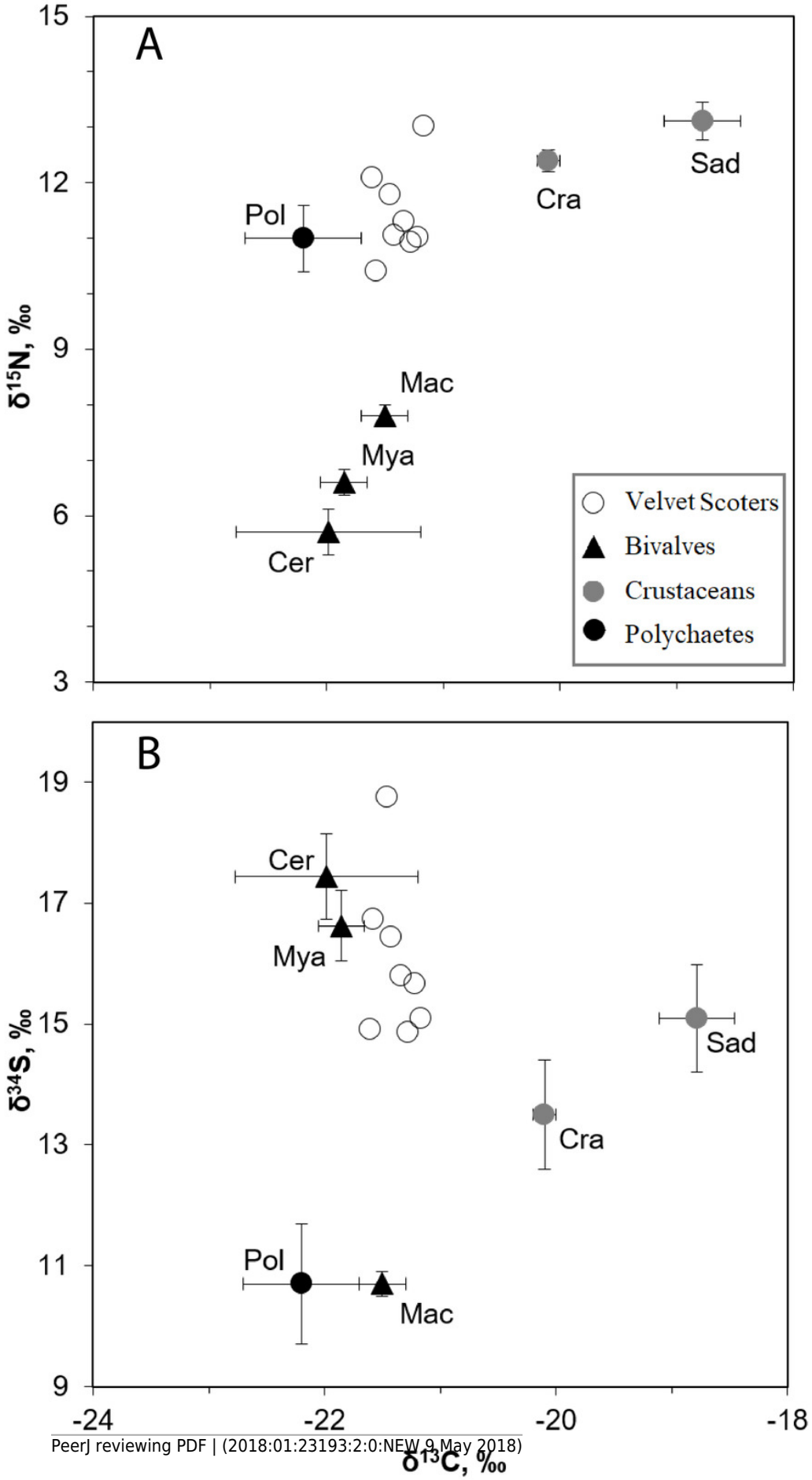
A
15
12
9
6
3
δ15N, ‰
Pol
Cra
Sad
Mac
Mya
Cer
Velvet Scoters
Bivalves
Crustaceans
Polychaetes
B
19
17
15
13
11
9
δ34S, ‰
Cer
Mya
Sad
Cra
Pol
Mac
-24
-22
-20
-18
δ13C, ‰

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 in A.

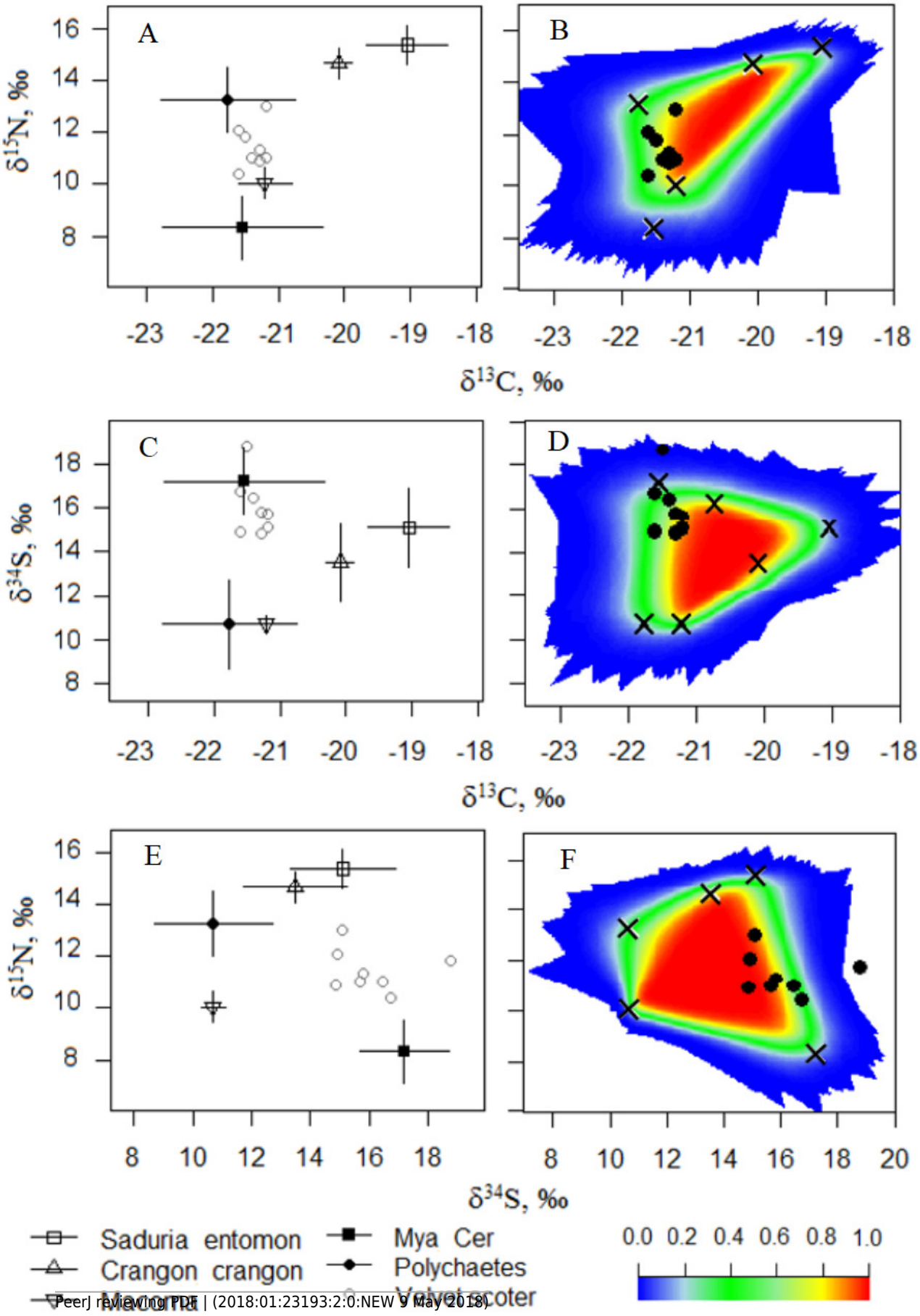
A
B
C
D
E
F
δ15N, ‰
δ34S, ‰
δ13C, ‰
16
14
12
10
8
18
-23
-22
-21
-20
-19
-18
20
Saduria entomon
Crangon crangon
Macoma
Mya Cer
Polychaetes
Velvet scoter
0.0 0.2 0.4 0.6 0.8 1.0

Figure 4

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.

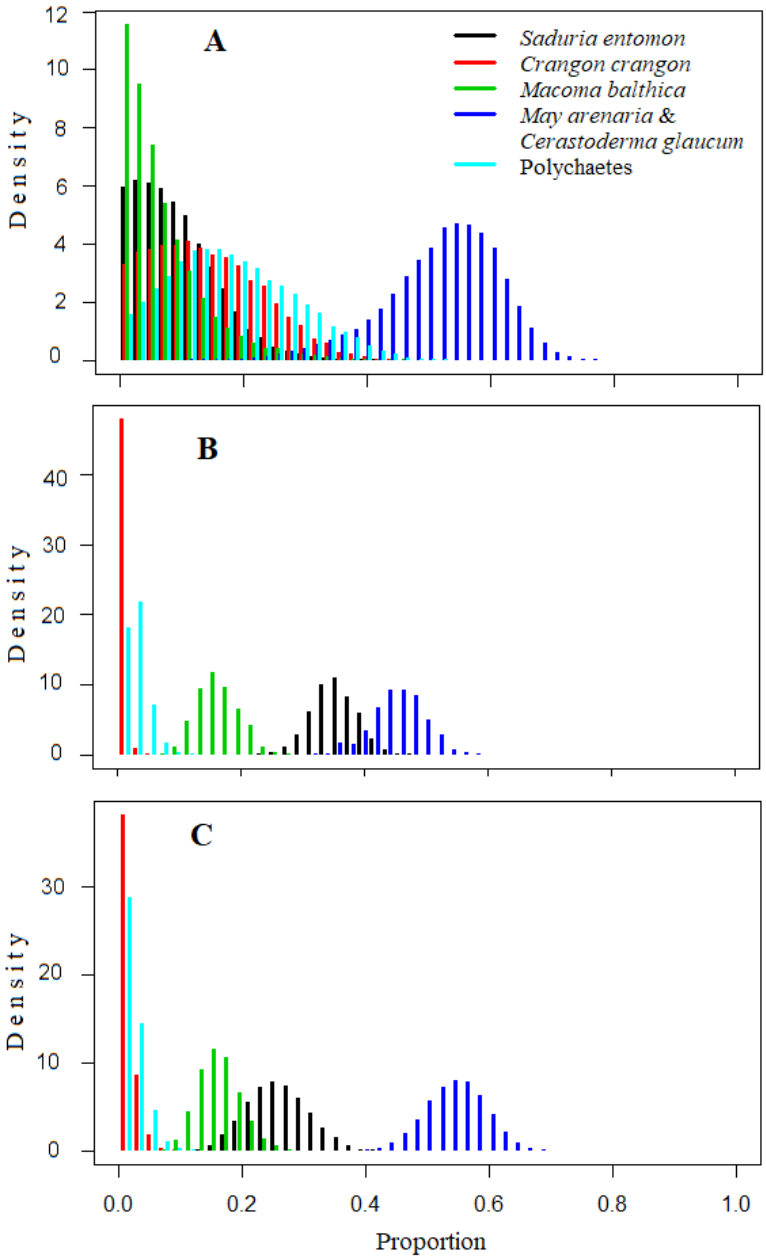
A
B
C
Saduria entomon
Crangon crangon
Macoma balthica
May arenaria &
Cerastoderma glaucum
Polychaetes
Density
Proportion
0
2
4
6
8
10
12
10
20
30
40
0.0
0.2
0.4
0.6
0.8
1.0

Figure 5

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.

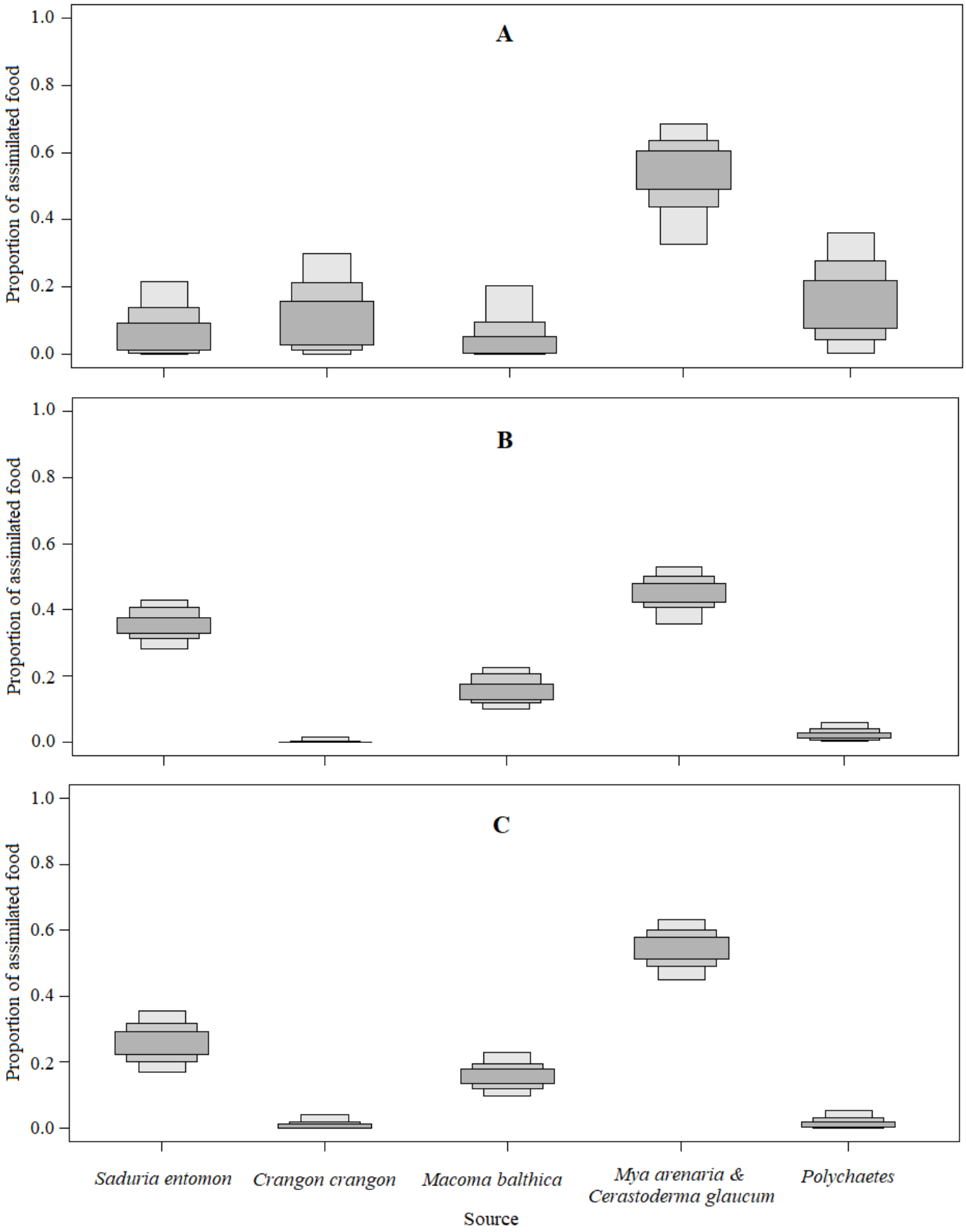
A
B
C
Proportion of assimilated food
1.0
0.8
0.6
0.4
0.2
0.0
Saduria entomon
Crangon crangon
Macoma balthica
Mya arenaria &
Cerastoderma glaucum
Polychaetes
Source

Table 1(on next page)

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

1

Table 2(on next page)

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**

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

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

[3] McCutchan et al., 2003

[4] Hobson et al., 2009.

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

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

Table 3(on next page)

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

1

Table 4(on next page)

Blood samples characteristics of the Velvet Scoters.

Number of analysed individuals	Body weight, g	C:N mass ratio	$\delta^{13}C$, ‰		$\delta^{15}N$, ‰		$\delta^{34}S$, ‰	
			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

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Table 5(on next page)

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)

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