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Diet composition of red-throated divers in the German Bight

1 The diet of red-throated divers (*Gavia stellata*) overwintering in the German Bight (North
2 Sea) analysed using molecular diagnostics

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16 Abstract

17 In Europe, the German Bight is one of the most important non-breeding areas for protected
18 red-throated divers (*Gavia stellata*). It is unclear what attracts the birds to this area, especially
19 as the food composition of seabirds outside the breeding season is notoriously difficult to
20 study. To obtain information on prey species composition of red-throated divers in this area,
21 faecal samples from 34 birds caught alive were analysed using DNA metabarcoding. Prey
22 DNA was detected in 85% of the samples with a mean number of 4.2 ± 0.7 taxa per sample
23 ($n=29$). Altogether we found a broad prey spectrum with 19 fish taxa from 13 families
24 dominated by five groups: clupeids, mackerel, gadoids, flatfish and sand lances with clupeids
25 being the most frequently detected prey.

26 Our results indicate that red-throated divers are generalist opportunistic feeders in the German
27 Bight, but pelagic schooling fish that aggregate at frontal zones and have a high energetic
28 value might be favoured. Atlantic mackerel appears to be a more important prey for red-
29 throated divers in this area than previously thought.

30 The precision achievable using metabarcoding has revealed a number of prey species that are
31 consumed by red-throated divers in the German Bight, which helps to explain the selection of
32 this area by divers in winter and spring.

33 Key words: Diet composition, DNA Metabarcoding, Next Generation Sequencing, North Sea,
34 Red-throated diver/loon, Site selection

35 Introduction

36 Understanding resource utilisation is fundamental for managing wildlife populations. Data on
37 diet composition and feeding strategies are essential for understanding habitat selection and
38 for predicting the ecological consequences of habitat change (Davoren et al. 2003). Predator
39 abundance is often regulated by bottom-up effects of prey abundance (Engelhard et al. 2013).
40 Thus, the availability of prey may affect not only predator distribution and abundance but also
41 foraging strategies (Fauchald et al. 2011; Lynam et al. 2017).

42 Diet composition of seabirds outside the breeding season, when they remain at sea, is
43 notoriously difficult to study. This is especially true for protected species where only non-
44 invasive methods are applicable. In the past, various techniques have been developed to
45 analyse seabird diet. These include visual observations, morphological identification of
46 regurgitates or gut contents, or biochemical methods such as the analysis of fatty acid and
47 stable isotope concentrations (Barrett et al. 2007; Meier et al. 2017; Quillfeldt et al. 2017;
48 Quinn et al. 2017). A highly efficient alternative approach is to use DNA metabarcoding
49 (Deagle et al. 2005, 2007; Pompanon et al. 2012; Vesterinen et al. 2013; Alonso et al. 2014).
50 This involves amplification of DNA from faecal material and assignment of taxonomical
51 information using Next Generation Sequencing (NGS) and DNA barcode databases.

52 Our study focused on the prey spectrum of the red-throated diver (*Gavia stellata*), a protected
53 marine bird species, in its wintering and spring staging areas in the German Bight (eastern
54 part of the North Sea). During the non-breeding season about 84,200–186,000 individuals stay
55 in the Baltic Sea, the North Sea and the NE-Atlantic (BirdLife International 2018; Dierschke
56 et al. 2012). Around 20% of the NW-European wintering population occurs in the German
57 Bight (Dierschke et al. 2012; Garthe et al. 2007; Mendel et al. 2008) classifying it as an
58 internationally important staging area for these birds, especially in spring before migration
59 starts (Garthe et al. 2012, 2015). To date three studies have been published on the prey
60 composition of non-breeding red-throated divers in the North Sea and the Baltic Sea, which
61 analysed gut contents using morphological tools (Table 1). However, information is not
62 available from the German Bight (Fig. 1). Red-throated divers feed on a wide range of fish
63 species and, given that the energy content of prey fish varies with size and season, they appear
64 to choose prey of high energetic value (Pedersen and Hislop 2001) like gadoids (Madsen
65 1957) or clupeids (Durinck et al. 1994; Guse et al. 2009). Additionally cephalopods were
66 found in one of these studies (Durinck et al. 1994) in four of eight birds. Small specimens of
67 polychaetes, crustaceans, copepods, bivalves and gastropods were reported in all studies

68 although these were considered to be secondary prey (i.e. prey in the guts of the fish eaten by
69 the divers). The German Bight is characterised by an estuarine frontal system, created by the
70 Jutland coastal current (JCC) that is primarily driven by discharges from the Elbe river and
71 other rivers further south (Skov and Prins 2001). Red-throated divers have been shown to
72 concentrate at the productive frontal zone, where prey fish aggregate (Skov and Prins 2001).
73 The area is also suitable for the development of offshore wind farms as it has extensive areas
74 of shallow waters (< 40 m). To date, 17 wind farms have been installed in German North Sea
75 waters. Thus, there is potential overlap between offshore wind farm sites and the preferred
76 habitat of non-breeding red-throated divers (Garthe et al. 2015; Heinänen et al. unpubl data).
77 Red-throated divers have been shown to strongly avoid both shipping traffic and wind farms
78 (Garthe and Hüppop 2004; Bellebaum 2006; Petersen et al. 2006; Dierschke et al. 2006, 2012;
79 Mendel et al. 2019; Heinänen et al. unpubl data; Burger et al. unpubl data). To understand the
80 environmental importance of the German Bight for red-throated divers, to assess the possible
81 impacts arising from displacing divers from substantial parts of their staging areas, and to
82 analyse whether alternative staging areas might be available, it is crucial to understand what
83 resources these birds rely on.

84 In this study we had the unique opportunity to collect a small number of faecal samples from
85 red-throated divers captured in the German North Sea in 2015 and 2016 in both winter and
86 spring. We applied DNA metabarcoding as a non-invasive technique to analyse diet
87 composition, and thus to provide a detailed overview of recent meals of these birds in the
88 German Bight. Specifically, we aimed to document the diversity of prey species eaten by the
89 birds in this particular staging area when red-throated diver abundance is highest.
90 Additionally, we aimed to compare data for two consecutive sampling years to determine if
91 the prey species consumed is consistent between years. By comparing dietary data with
92 published data on local fish distribution, we aimed to determine whether the abundance and
93 distribution of prey fish correlate with red-throated diver diet and how this may help to
94 explain red-throated diver distribution.

95 Methods

96 *Sample collection and study site*

97 This dietary study was part of a satellite telemetry project on red-throated divers. A total of 36
98 red-throated divers were captured in March and April 2015 and in February and March 2016
99 in the German Bight (Fig. 1). Sampling was focused on late winter and spring when red-

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100 throated diver abundance is highest in the German Bight (Mendel et al. 2008; Dierschke et al.
101 2012; Garthe et al. 2015). The capture area was approximately 30 km offshore in water depths
102 of around 20 m, which is approximately in the centre of the staging area for red-throated
103 divers (Fig. 1). Birds were captured from a rigid inflatable boat using a hand net and the
104 “night lighting technique”, where the sea is searched for resting divers with a spot light. If a
105 bird is sighted, it often becomes disoriented by the bright light and can be captured with a net
106 (Whitworth et al. 1997; Ronconi et al. 2010). In 2015 captured birds were kept in boxes for an
107 average time of 18.3 h (min 6.3 h, max 27 h) and in 2016 for an average time of 9.2 h (min 7
108 h, max 13 h). After release the boxes were searched for scat. The boxes were cleaned and
109 disinfected after every use with bleach (1% hypochlorite solution), water and ethanol (70%)
110 to prevent cross contamination. During the two field seasons a total of 34 faecal samples were
111 collected (2015 n = 15; 2016 n = 19, Table 2). Samples were preserved in absolute ethanol
112 and stored at -20°C until further analysis.

113 *DNA extraction*

114 Faecal DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) following the
115 manufacturers protocol with the following modifications: (i) the samples were resuspended in
116 the storage ethanol by vortexing before moving 200 µL of the ethanol-scat slurry to a new
117 clean 2 ml Eppendorf tube and centrifuging for 30 s at 4000 x g (Deagle et al. 2005); (ii) the
118 lysis step was extended by adding 1.4 mL Buffer ASL instead of 1.6 mL to each sample and
119 incubating at 70 °C for 10 min and then for 1.5 h at room temperature to improve lysis output;
120 (iii) the digestion step was extended by adding 20 µl instead of 25 µl proteinase K and
121 incubating samples at 70 °C for 30 minutes prior to an increased incubation time at a lower
122 temperature (56 °C for 1.5 h). All remaining steps followed the manufacturer’s instructions
123 except that buffer volumes were cut down to reduce risk of cross contamination by
124 minimizing the number of pipetting steps and by reducing the volume of liquid loaded into
125 spin columns and tubes (Deagle et al. 2005). The final elution step used a total elution volume
126 of 100 µl (as recommended by the manufacturer’s protocol), but was divided into two steps
127 with each elution using 50 µL Buffer AE.

128 *Primer design and preparation for sequencing*

129 Three separate PCR primer pairs were used to comprehensively target all the major potential
130 prey species of red-throated divers in this area (Table 3). These prey species are widespread in

131 the North Sea and were informed by previous diet studies on red-throated divers (Table 1;
132 Madsen 1957; Durinck et al. 1994; Guse et al. 2009).

133 Primers for each prey group were tested *in silico*, using ClustalX 2 (Larkin et al.2007) and
134 MEGA7 (Kumar et al. 2016). Conserved primer binding sites were tested against a DNA
135 barcode database of barcode-sequences extracted from GenBank. Sequences of 16S DNA of
136 28 representative fish species from 7 orders and 15 families as well as 12 cephalopod species
137 from 5 families were aligned for these tests. For crustaceans COI barcode sequences of
138 potential prey species from 6 orders and 8 families of shrimp and krill were aligned and
139 tested. Furthermore primers for each prey group were tested *in vitro* on DNA from tissue
140 samples of corresponding potential prey species occurring in the German Bight (clupeids,
141 perciformes, gadoids, flatfish, octopus, squid, cuttlefish and shrimp) to optimise PCR
142 conditions. Multiplex identifier (MID) tags were added to the primer sequences and used to
143 assign DNA sequences to their respective samples (n = 34). MID tags were added to each of
144 the three tested primer sets (fish, cephalopods and crustaceans). For each of the three primer
145 sets we used 24 forward primers/MID and 2 reverse primer/MID combinations, and all *in*
146 *vitro* testing was performed using primer pairs first without and then with the MID tags to
147 ensure amplification was not affected.

148 To amplify DNA from fish and cephalopods, we used primers targeting the 16S region
149 originally published by Waap (2015) and modified from Chord_16S_F/Chord_16S_R
150 (Deagle et al. 2009). We further modified the primer sequence to comprehensively match the
151 range of potential prey species (Table 3). To amplify fish DNA, the forward primer has
152 additional CT bases at the 3' end for NGS sequencing to improve the blocking probes (see
153 below), so that the mismatch was not located at the last base pair (Waap, pers comm.). To
154 amplify cephalopod DNA, we modified the forward primer by one base and the 5' end of the
155 reverse primer. Both primer pairs tested positive *in silico* and *in vitro* for potential prey of red-
156 throated divers.

157 To amplify crustacean DNA, a primer combination targeting the Cytochrome oxidase I region
158 (COI) was used that was likely to amplify crustaceans and molluscs (Stockdale 2018, Table
159 3). The forward primer (Leray et al. 2013) was designed to amplify arthropod DNA, including
160 crustaceans and molluscs. The reverse primer (Simon et al. 1994) was also designed to
161 amplify arthropods including crustaceans. The primers tested positive *in silico* and *in vitro* for
162 potential prey of red-throated divers and provided a good coverage of our target species and a
163 good coverage with reference sequences available in public databases. This primer set

164 amplified a product size of 332 bp and thus represents a good compromise as it is long enough
165 to provide good taxonomic information and short enough to survive digestion.

166 *Blocking primer*

167 The primers chosen to amplify fish prey were universal chordate primers that could also
168 amplify other chordates, including predator DNA. To prevent the amplification of predator
169 DNA, we developed a blocking probe using a C3 spacer (Table 3; Vestheim and Jarman
170 2008). However, the blocking probe reduced amplification success and a second amplification
171 of samples was performed excluding the blocking probe. Gel electrophoresis (see below) was
172 used to visually monitor the amplification of predator and prey DNA, assisted by the inclusion
173 of red-throated diver (300 bp) and fish (264 bp) reference samples. This differential in PCR
174 product size allowed for predator amplicons to be easily identified (Fig. 2).

175 *PCR amplification of DNA from faeces*

176 PCR amplifications were performed in single reactions using Multiplex PCR Kits (Qiagen)
177 and a 20 µL PCR reaction volume. Thermal cycling conditions for fish and cephalopod prey
178 were 95 °C for 15 min followed by 45 cycles of: 94 °C for 30 s, a primer specific annealing
179 temperature (Table 3) for 90 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 5
180 min. Thermal cycling conditions for crustaceans were 95 °C for 15 min followed by 45 cycles
181 of: 94 °C for 30 s, a primer specific annealing temperature (Table 3) for 90 s, and 72 °C for 90
182 s, followed by a final extension at 72 °C for 15 min.

183 All PCR products were visualised by gel electrophoresis on 2% agarose gels stained with
184 SYBR®Safe (ThermoFisher Scientific, Paisley, UK) and compared to a standardised 1000 bp
185 ladder. The PCR product concentration in successful reactions was quantified with a Qubit
186 fluorometer (Thermofischer) and subsequently pooled into two equimolar libraries of
187 individually tagged amplicons (PoolA using a blocking probe and PoolB without a blocking
188 probe). To remove primer dimer we ran a magnetic clean up (AMPure). Concentrations of
189 DNA and primer dimer were measured on a tape station (D1000 Screen Tape; Tape Station
190 Analysis Software A.01.05 SR1, Agilent technologies) and a Qubit before and after the
191 magnetic clean up.

192 *Next Generation Sequencing*

193 NGS library preparations were performed at the NERC Biomolecular Analysis Facility –
194 Sheffield (NBAF-S), Sheffield, UK using the NEBNext Ultra DNA Library Prep Kit for

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195 Illumina (New England Biolabs, Ipswich, MA). To characterise the diet content of the
196 individually tagged amplicons the libraries (PoolA and PoolB) were sequenced at the
197 Sheffield Diagnostics Genetics Service (Children's Hospital, Sheffield, UK) using 250 bp
198 paired-end reads on a MiSeq desktop sequencer (Illumina, San Diego, CA).

199 *Bioinformatics*

200 We performed eight steps to transform the raw Illumina sequence data into a list of molecular
201 operational taxonomic units (MOTUs) with assigned taxonomy. These steps included
202 assessing sequence quality, trimming sequences (Bolger et al. 2014), aligning paired reads
203 (Magoc et al. 2011), matching sequences to MID tags and amplicon primers (Schloss et al.
204 2009), and demultiplexing sequences into files for each amplicon. We used USEARCH
205 (Edgar 2010) to dereplicate the sequence file, to detect and to remove chimeric sequences and
206 to cluster into MOTUs based on 97% identity. Clustering is an important step in
207 metabarcoding analysis to group similar sequences into distinct taxonomic units, but remains
208 one of the central challenges. If the clustering threshold is too conservative, e.g. 5% sequence
209 divergence, the dietary richness could be underestimated due to a high mean overlap of
210 MOTUs. Conversely, a less conservative decreased threshold, e.g. 2% sequence divergence,
211 could overestimate species richness (Clare et al. 2016). Here we applied the established
212 clustering threshold of 97% similarity (Edgar 2013, 2016) using the 'cluster_fast' function in
213 USEARCH (Edgar 2010). We applied the BLASTn algorithm (Altschul et al. 1990) to match
214 MOTU sequences to reference sequences in the NCBI GenBank nucleotide database, using a
215 cut-off of 90% minimum sequence identity and a maximum e-value of 0.00001. For detailed
216 information about options, parameters and values please see Table 1 in the supplementary
217 material.

218 We subsequently manually performed further filtering steps to produce robust taxonomic
219 assignments. We discarded MOTUs (sequence clusters 97%) that corresponded to
220 contaminants that can occur regularly in faecal samples, such as bacterial, human or predator
221 DNA. MOTUs were retained in a sample only if they contained a minimum of 5 sequences.
222 Taxonomic assignment was based on the percentage similarity of the query and the reference
223 sequences. Since short fragments are less likely to contain reliable taxonomic information we
224 only retained sequences with a minimum length of 190 bp and a BLASTn assignment match
225 greater than 98%, following Deagle et al. (2009) and Vesterinen et al. (2013).

226 Finally, we combined both pools (PoolA with a blocking probe and PoolB without a blocking
227 probe) together for final analyses. To avoid overrepresentation we excluded prey species of
228 samples from PoolB that were also present in PoolA.

229 *Analysing the Blast output*

230 We used MEGAN Community Edition version 6.8.8 to visualise the accession number
231 identifiers on the NCBI taxonomy (Hudson et al. 2016). We imported the blast output and
232 used the default LCA parameters to assign a taxon name to each MOTU (Huson et al. 2007).
233 If all retained hits of a MOTU with the same quality criteria (sequence identity, sequence
234 length, e-value) matched the same species then we have a species-level assignment, otherwise
235 the MOTU was assigned to the lowest shared taxonomic level, e.g. genus or family.

236 *Statistical analysis*

237 We analysed prey range by determining the presence of prey items, their frequency of
238 occurrence (FO) (Barrett et al. 2007, Tollit et al. 2009), and species richness. FO was
239 calculated as: $FO = (n/t) \times 100$ where n was the number of samples in which the specific prey
240 item appeared and t was total number of samples containing prey. FO reveals the percentage
241 of sample units in which each prey item occurred (Barrett et al. 2007). The number of
242 MOTUs (defined by 97% clustering threshold, n = 169) assigned for each prey taxa were
243 additionally presented as percent occurrence in faecal samples (n=29) to visualise the
244 sequencing output Fig. 4. No further quantitative analyses were done with these data due to a
245 range of possible biases and as interpretation of sequence proportions generated via high-
246 throughput sequencing requires careful data analysis (Deagle and Tollit 2007; Pompanon et
247 al. 2012; Deagle et al. 2013, 2018).

248 Whether or not there is consistency in prey consumption by red-throated divers over time
249 informs our understanding of prey selection in this particular area. We tested this by
250 comparing FO of prey items in 13 samples from 2015 with FO of prey items in 16 samples
251 from 2016. Statistical tests suitable for small sample sizes were performed in Rcmdr (Fox and
252 Bouchet-Valat 2018). We used Pearson's chi squared-test to compare the frequency of
253 occurrence between years for each prey group when sample sizes fulfilled the minimum
254 requirements for this test ($n > 5$). When sample sizes were small ($n < 5$), we implemented the
255 Fisher's Exact Test for Count Data. To compare the number of prey detections per sample
256 between sampling years the T-Test for independence was used. Small sample sizes precluded
257 further analyses (e.g. comparing seasons) or to use other statistical tests. Considering the

258 sample size and the temporal scope of faecal DNA sampling only marked differences were
259 expected to be identified.

260 *Results*

261 *Overview of sample quality and prey species found*

262 Neither cephalopods nor crustaceans were detected in the diet, despite successful *in vitro* PCR
263 amplification using reference tissue samples from potential prey items from the German Bight
264 (octopus, squid, cuttlefish and shrimp samples).

265 The fish primer set produced more than 800,000 sequences from both pools combined, for
266 specific information on number of sequences during bioinformatics analysis, see Table 2 in
267 supplementary material. Of 34 screened samples 29 samples gave positive PCR
268 amplifications (PoolA: n = 21; PoolB n = 29). Both pools had ~50% of MOTUs assigned to
269 prey fish (PoolA = 56%; PoolB = 48%), plus with other MOTUs being from the predator
270 DNA (red-throated diver) and contaminants such as bacteria and human DNA (Fig. 3). Using
271 the blocking probe, we still amplified predator DNA but the amount of MOTUs assigned to
272 the predator was slightly lower in PoolA (9%) than in PoolB (17%).

273 After filtering for contaminants, sequence length and mapping to reference sequences, 20 and
274 24 faecal samples remained for PoolA and B respectively. After merging both pools, the final
275 sample set consisted of 29 samples (PoolA n = 20, PoolB n = 9) which corresponds to 85% of
276 all samples collected (Table 2). Four samples were discarded (PoolB) as they contained only
277 contaminants and predator DNA, and two samples were discarded as the amplicon length
278 criteria were not met (1x PoolB, 1x PoolA).

279 Clustering the sequences by 97% similarity to each other and subsequent filtering resulted in
280 169 MOTUs that were used for further analyses. A list of a representative query sequences of
281 each MOTU and its quality criteria is listed for each prey assignment in Appendices (Table
282 A1) and for all MOTUS in Table 3, supplementary material. For the two sampling periods 19
283 taxa from 13 families were identified in 29 faecal samples (Fig. 4, Table 4). In 2015 we
284 detected a slightly higher number of taxa in comparison to 2016 (18 and 13 taxa assigned to
285 species, respectively; Table 4). The prey species spectrum was similar between the two years
286 with 12 matching taxa and no significant differences ($\chi^2 = 1.004$, $p = 0.316$). European
287 anchovy (*Engraulis encrasicolus*), turbot (*Scophthalmus maximus*), European pollock
288 (*Pollachius pollachius*), cod (*Gadus sp.*), European bass (*Dicentrarchus labrax*) and sand

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289 lances of the genus *Ammodytes* were detected only in 2015, and whiting (*Merlangius*
290 *merlangus*) only in 2016 (Table 4).

291 *Prey detection*

292 Of the samples where prey were detected, the mean number of taxa found was 4.2 ± 0.7 per
293 sample (n=29) with minimum and maximum values of 1 and 16 respectively. There was no
294 significant difference ($t = 1.58$, $p = 0.135$) between the number of prey items detected in 2015
295 (mean = 5.3) and 2016 (mean = 3.1).

296 Clupeids were the most frequently detected prey group (FO of 65.5%, Table 4). Within this
297 group, Atlantic herring (*Clupea harengus*) and European sprat (*Sprattus sprattus*) occurred
298 most frequently (FO of 55.2% and 58.6%, respectively). No significant differences were
299 found between years for clupeids ($\chi^2 = 0.030$, $p = 0.863$), European sprat ($\chi^2 = 0.283$, $p =$
300 0.595), or for Atlantic herring ($\chi^2 = 0.005$, $p = 0.945$).

301 The Atlantic mackerel (*Scomber scombrus*) was the only species of mackerel detected (Table
302 4), with a total FO of 55.2% and no significant differences between the two sampling years
303 (FO 53.8% in 2015, FO 56.3% in 2016; $\chi^2 = 0.005$, $p = 0.945$).

304 Flatfish were recorded with a total FO of 51.7% (Table 4) and no significant difference
305 between the two sampling years (61.5% in 2015, 43.8% in 2016; $\chi^2 = 0.287$, $p = 0.592$). Most
306 taxonomic assignments were at the family or genus levels. Righteye flounders
307 (Pleuronectidae) were dominant and where MOTUs were assigned at the species level the
308 common dab (*Limanda limanda*) was the most frequent species detected.

309 Gadoids (Gadidae) were recorded with a total FO of 37.9% and high similarity between
310 sampling years (38.5% in 2015, 37.5% in 2016; $\chi^2 = 0.001$, $p = 0.972$, Fishers exact test $p =$
311 0.976). Most MOTUs could only be assigned to the family level, but of those assigned to
312 species cod (*Gadus sp.*), European Pollock (*Pollachius pollachius*), whiting (*Merlangius*
313 *merlangus*) and haddock (*Melanogrammus aeglefinus*) were detected at least once. Detections
314 of these species varied between years but sample sizes were too small for statistical tests.

315 Sand lances had a total FO of 31%, with a similar proportion of greater sand eel (*Hyperoplus*
316 *lanceolatus*; FO of 13.7%) and sand lances of the genus *Ammodytes* (FO of 20.7%). There
317 were significantly more sand lances detected in 2015 (61.5%) in comparison to 2016 (6.3%;
318 $\chi^2 = 5.394$, $p = 0.020$; Fishers exact test $p = 0.026$).

319 Other prey species infrequently occurred and are detailed in Table 4 and Figure 4.

320 *Discussion*

321 The aim of this study was to analyse prey species composition in faecal samples from red-
322 throated divers caught in the German Bight, using high throughput sequencing. In our data set
323 we found an exclusively piscivorous diet, with no evidence of cephalopod or crustacean
324 consumption and a similar prey spectrum between two consecutive sampling years.

325 Application of high throughput sequencing to study diver diets

326 The DNA metabarcoding methodologies utilised in this study have previously been applied in
327 diet studies on other marine predators (Deagle et al. 2005, 2007; Pompanon et al. 2012).

328 However, this study is the first application of this approach to analyse the diet of red-throated
329 divers in the German Bight or elsewhere. Using reference sequences, we found high
330 taxonomic coverage for both the COI and 16S barcode primers. Because of their commercial
331 importance in the German Bight many fish species (e.g. Atlantic herring), alongside some
332 cephalopod species, are well studied and the majority of these species appear in the Genbank
333 database (Dickey-Collas et al. 2010, Engelhardt et al. 2013).

334 Sequences were clustered at 97% identity and represented consistent taxonomical units
335 (MOTUs). Some prey species were represented by multiple MOTUs, suggesting that the
336 clustering threshold could have been lower. However, a lower threshold would have increased
337 the risk of clustering two closely related species into a single MOTU and thus reduced
338 taxonomic discrimination. In practice, it is difficult to apply an ‘average’ threshold when diet
339 is diverse and the prey are likely to have differing evolutionary rates. On balance, we deem
340 the clustering threshold applied as appropriate and this method provided a good estimate of
341 species richness with distinct taxonomic units.

342 We obtained sufficient sequencing data from 85% of the analysed faecal samples using
343 universal primers. The species richness was higher in 2015 but individual variances may be
344 due to sampling conditions, sample quality and amplification success. The use of a blocking
345 probe proved to be of little advantage, with sufficient prey DNA amplified using both
346 approaches (Fig. 3). The use of a blocking probe reduced the amplification of predator DNA
347 but also amplification success in general since the output of prey-positive samples was higher
348 when the blocking probe was omitted.

349 The detection rate of prey species can be biased by the method applied. For example, Tollit et
350 al. (2009) found some prey (Ammodytidae, Cottidae and Gadidae) were more reliably
351 detected with morphological tools, whereas other prey (Salmonidae, Pleuronectidae,
352 Elasmobranchii and cephalopods) were only detected with molecular tools. However, the
353 overall results did not dramatically differ. In general molecular methods have been shown to
354 identify more trophic links (number of taxa identified) with higher rates of taxonomic
355 discrimination in comparison to morphology (e.g. Soininen et al. 2009; Alonso et al. 2014;
356 Berry et al. 2015; Waap et al. 2017). Using molecular methods, we found a similar prey
357 composition to conventional morphological methods applied in previous studies on red-
358 throated diver diet. Using faecal samples coupled with DNA metabarcoding is now an
359 established non-invasive approach for dietary studies. However, it is debatable whether or not
360 this method can provide quantitative (read number) in addition to qualitative (presence and
361 absence) estimates of diet (Deagle and Tollit 2007; Pompanon et al. 2012; Deagle et al. 2013;
362 2018). In this study we applied a conservative approach of using only qualitative data.
363 However, if quantitative data are required we recommend combining DNA metabarcoding
364 and morphological methodologies, where the latter can provide quantitative information as in
365 Alonso et al. (2014) and Waap et al. (2017).

366 A faecal sample, for most species, will represent an individual's most recent meals. Other
367 methods, including fatty acid composition and stable isotope analyses, can provide
368 information over a longer time frame (Meier et al. 2017). Although our sample size is small,
369 samples were collected from birds caught in two consecutive years at dispersed intervals
370 encompassing late winter and spring (February – April); when red-throated diver abundance
371 is highest in the German Bight. Thus, this dataset provides dietary information from a time
372 period when this area is particularly attractive to these birds. Wintering home ranges of red-
373 throated divers can cover several connected sites, including sites outside the German Bight,
374 such as the Baltic Sea (Kleinschmidt et al. unpub data). The German Bight also represents an
375 important staging area in spring when some birds have already started migration (Garthe et al.
376 2015) and the availability of suitable prey types is probably one of the main determinants of
377 habitat quality for these birds. In this context the time frame over which a faecal sample
378 provides dietary information helps to reflect the situation in the particular area of interest for
379 this study.

380 Fish availability in the German Bight, red-throated diver diet and comparison to previous
381 studies

382 Potential prey availability is an important factor affecting habitat choice and diet selection.
383 We searched the species factsheets (ICES 2006 a,b), reports and publications (ICES 2008,
384 2011, 2016, 2017a, 2017b, 2018; DFS 2016) to compare fish distribution (a proxy for
385 potential prey availability) with the diet of red-throated divers in our study in addition to
386 previous studies. In our dataset red-throated divers consumed a wide range of fish prey
387 species consisting of both a pelagic and a benthic component. We found mainly clupeids,
388 mackerels, flatfish, gadoids and sand lances in the diet of red-throated divers but no clear
389 dominance of a single species or species group could be identified. A similarly wide, although
390 slightly different range of prey species was found in previous studies on red-throated diver
391 diet. For example, Madsen (1957) found a broad prey spectrum but the majority of analysed
392 birds (82%) fed exclusively on cod, gobies, sticklebacks and herring with varying intensities.
393 Guse et al. (2009) found 11 species from 9 families with clupeids, zander, European smelt,
394 ruffe, lesser sandeel, three spined stickleback and common goby being dominant species.
395 Similarly, Durinck et al. (1994) identified clupeids and gadoids as the most frequent prey
396 items.

397 Clupeids, specifically sprat and herring occurred most frequently in both sampling years of
398 our study. These species are typically high in lipid content and energy density (Pedersen and
399 Hislop 2001; Ball et al. 2007). Sprat and juvenile herring are also two of the most abundant
400 pelagic species in the German Bight in spring (ICES 2006 a,b), which coincides with our
401 sampling period. The size of available prey fish is also important for prey selection. In
402 general, herring occurs in the North Sea with a size of 20-30 cm but in our sampling period
403 smaller (juvenile) herring with a size <20 cm are the most abundant and widely distributed in
404 the German Bight and the Kattegat (ICES 2006 a; Trueman et al. 2017). Sprat is a pelagic
405 species abundant in frontal areas of the North Sea with a size of <16 cm (Kanstinger and Peck
406 2009). We also found European sardine (*Sardina pilchardus*) and European anchovy
407 (*Engraulis encrasicolus*) in the diver diet but less frequently, which is consistent with the
408 distribution of both these clupeid species. They originate from the Mediterranean Sea (Motos
409 et al. 1996) and since 2003 are expanding into the North Sea (Kanstinger and Peck 2009).
410 Like sprat, sardine occurs in frontal areas whereas anchovy is primarily found in near-shore
411 areas. The distribution of clupeids is in good agreement with red-throated diver distribution,
412 which appear to be attracted by frontal zones (Skov and Prins 2001; Goyert et al. 2016;
413 Heinänen et al. unpubl data). Hence these areas provide a source of energetically valuable
414 species for red-throated divers. The high detection rate of clupeids is in line with two earlier

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415 studies on red-throated diver diet and reinforces their importance as red-throated diver prey
416 (Durinck et al. 1994; Guse et al. 2009).

417 Atlantic mackerel is widespread throughout the North Sea and is one of the most commonly
418 exploited species (ICES 2011, 2016, 2017). Due to its high energetic value, mackerel is an
419 attractive fish for seabirds (Montevecchi et al. 1984, 1988; Garthe et al. 2014). Overfishing
420 triggered a population collapse in the North Sea in the 1970s but since 2000 the stock has
421 increasing again (ICES 2011; Jansen 2014; Jansen and Gislason 2013; Jansen et al. 2012a,
422 2012b; 2014; Kooij et al. 2016). These changes in mackerel availability may explain why
423 both Madsen (1957) and the current study detected mackerel in the diet, while Durinck et al.
424 (1994) did not. Mackerel appeared in our samples in considerable numbers indicating that it
425 may now be a more important prey than previously thought.

426 Most flatfish were identified to family level, but of those identified to species level, common
427 dab was the most common in both years. Flatfish have been recorded in low numbers in red-
428 throated diver diet (Madsen 1957; Durinck et al. 1994; Guse 2009), possibly due to their
429 wide-bodied shape making adult flatfish an unfavourable prey item (Reimchen and Douglas
430 1984; Guse et al. 2009). Dietary studies in the adjacent Wadden Sea have shown that juvenile
431 flatfish are selected as important food items by other water birds such as benthic feeding
432 cormorants (Nehls and Gienapp 1997). The Wadden Sea and adjacent waters are an important
433 nursery ground for several flatfish species (DFS 2016) and juvenile common dab is highly
434 abundant in spring within the German Bight over a wide depth range (Beek et al. 1989; Bolle
435 et al. 1994; Campos et al. 1994; Hufnagl et al. 2013; DFS 2016; ICES 2017a,b). Prey size
436 cannot be deduced from metabarcoding but red-throated divers may be preying on juvenile
437 flatfish. Although flatfish are considered to have a low energy content (Ball et al. 2007), the
438 probable high encounter rate may explain the high detection rate in our samples.

439 Gadoids, particularly cod, were described by Madsen (1957) as the most important prey group
440 for red-throated divers in the Kattegat and Belt Sea. In the current study, gadoids were
441 infrequently present in the diet. This is in line with findings of Durinck et al. (1994) from the
442 south-western part of the Skagerrak. Juvenile gadoids (<20 cm) are more likely than adults to
443 be prey for red-throated divers. Recordings of this size class of gadoids are mostly restricted
444 to the eastern inshore water of the Skagerrak and Kattegat, with low abundances in the
445 German Bight (Munk et al. 1999, Munk 2014; André et al. 2016). Thus, gadoids may be a
446 favoured prey item but low availability at the study site limits feeding on these species.

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447 Sand lances are an important prey for seabirds in general, particularly in the North Sea (Harris
448 and Wanless 1991; 2013; Mendel et al. 2008; ICES 2011; Engelhardt et al. 2013; ICES 2016).
449 Sand lances appeared at a high frequency in 2015 but were less common in 2016 in our data
450 set. This pattern is reflected in commercial catch rates for sand lances in the central and south-
451 eastern North Sea ecoregion (Division 4b-c): average catch rates and a low recruitment in
452 2015 and low catch rates and high recruitment in 2016 (ICES 2018a,b). Previously, sand
453 lances have been recorded at both high (Guse et al. 2009) and low (Madsen 1957, Durinck et
454 al.1994) frequencies in red-throated diver diet. These patterns suggest that the frequency of
455 sand lances in the diet is determined by their availability.

456 Smelt (*Osmerus eperlanus*) was not detected in this study but has been highlighted as an
457 important prey species for red-throated divers in the Baltic Sea (Žydelis 2002; Guse et al.
458 2009). Smelt occurs in parts of the Wadden Sea with low salinity and close to the coast. Here
459 it forms dense spawning aggregations in estuaries and anadromous migrations in late winter
460 and early spring (DFS 2016). The German Bight is further away from river mouths, the lack
461 of smelt in our dataset could probably be explained by the low abundance of this species here.

462 Sea trout (*Salmo trutta*), European hake (*Merluccius merluccius*), sticklebacks (*Gasterosteus*
463 *sp.*), European bass (*Dicentrarchus labrax*) and sand goby (*Pomatoschistus minutus*) were
464 recorded in our dataset at low frequencies. These species are widely distributed in the North
465 Sea with varying densities. Some, such as gobies, are known to be important prey items for
466 other marine predators (Haelters et al. 2012; Méheust et al. 2015; Andreasen et al. 2017) and
467 were previously recorded as prey items of red-throated divers (Madsen 1957, Durinck et al.
468 1994, Guse et al. 2009). Sticklebacks were frequently found in all previous studies. However,
469 the current study suggest that these species are of low importance for red-throated divers in
470 the German Bight.

471 In contrast to our study, Guse et al. (2009) found zander as one of the most important prey
472 items of red-throated divers wintering in the Baltic Sea. This fish species prefers freshwater or
473 brackish habitats, and therefore is almost absent in the saline waters of the German Bight.

474 Non-fish prey such as insects, polychaetes, molluscs or crustaceans were detected in small
475 amounts in all previous studies. Cephalopods were detected in a single previous study
476 (Durinck et al. 1994). We found no evidence that non-fish prey were consumed by red-
477 throated divers in the German Bight and thus our results reinforce previous conclusions that
478 these taxa are not an important part of the diet.

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479 In summary, prey species of red-throated divers identified in this study occur in the study area
480 as both adult (e.g., clupeids, sand lances) and juvenile fish (e.g., gadoids, flatfish, mackerels).
481 Thus the area seems to be a good foraging ground for red-throated divers. There is an overlap
482 between the prey fish of red-throated divers and commercial fish species, like herring and
483 mackerel (ICES 2011, 2016, 2017). This overlap increases the risk of gill-net mortality, which
484 is a conservation issue in other regions such as the Baltic Sea. In the German Bight, there is a
485 lower potential for such conflicts because trawls are more commonly used to fish as opposed
486 to gill-nets. The oceanographic conditions (sea surface temperature (SST), salinity and
487 chlorophyll a, NAO) were similar between the two sampling years and no important changes
488 in prey community can be expected within such short timeframe, with the exception of the
489 observed fluctuations in sand lance abundance. For this prey group, detections in the diet and
490 reported catch rates (ICES 2018a,b) showed a similar trend. Reasons for this are unclear but
491 sand lance productivity in the North Sea is known to fluctuate. Such fluctuations depend on a
492 combination of several regulating factors including fishing, climate effects, density
493 dependence and food availability (Wright et al. 2017; Lindegren et al. 2018). Although we
494 present data from only two sampling years, the consistent pattern of prey species suggests a
495 relatively stable diet that is likely to reflect the availability of these fish species in the study
496 area. There are long-term increases in sea temperature and species usually associated with
497 warmer waters are expanding their range to include the North Sea. Such species include
498 European sardine and European anchovy (Kanstinger and Peck 2009). The diet of red-
499 throated divers in the German Bight includes these expanding species and also recovering
500 species like mackerel, indicating that the dietary data may reflect changes in the fish
501 community and some flexibility in prey consumption. However, a larger sample size across a
502 broader temporal scale is required to fully support this conclusion.

503 The samples analysed here were collected in late winter and early spring, shortly before the
504 migration to the breeding grounds. For non-breeding red-throated divers little is known about
505 energy expenditure, resource partitioning and energy requirements during wintering, staging
506 and migration. Schmutz (2014) suggested that marine conditions could affect adult survival of
507 red-throated divers with indications of a higher risk of mortality during the non-breeding
508 season. Red-throated divers are medium sized birds with weight varying between 1400g –
509 2000g (own observations), and with high wing loading (Storer 1958; Lovvorn and Jones
510 1994). Despite this, these birds often need to cover long distances to their breeding grounds
511 (www.divertracking.com; McCloskey et al. 2018), with some individuals travelling as far as
512 850km or 1300km in a single flight (Kleinschmidt et al. unpubl data). Weber et al. (1997)

513 showed the importance of resting sites for refuelling. Consequently, migration represents
514 periods of high energetic demand and adequate energy reserves seem to be essential. If prey
515 of rich calorific value becomes unavailable due to displacement effects, red-throated divers
516 may fail to balance their energy budgets. In general, these birds winter in temperate marine
517 waters with low ambient temperatures, consequently reliable and sufficient energy intake is
518 likely to be a necessity and influences prey consumption.

519 Conclusion

520 Overall, our results demonstrate that the use of faecal samples coupled with DNA
521 metabarcoding and NGS is a valid and appropriate approach to non-invasively study the diet
522 composition of red-throated divers.

523 Our results provide important dietary data for red-throated divers in the German Bight, which
524 is needed for a good understanding of their habitat preferences during wintering and spring
525 staging. This baseline information can be used to evaluate changes associated with human
526 developments in the offshore environment, changes in oceanography, or population declines.
527 The results for the German Bight complement other dietary studies on red-throated divers that
528 show a somewhat different composition of fish species, reflecting regional differences in fish
529 fauna. Among a generalised prey spectrum, benthic-pelagic schooling fish seem to dominate
530 the diet of red-throated divers (Cramp and Simmons 2004; Guse et al. 2009). In our study five
531 species groups are concluded to be major dietary components for red-throated divers in the
532 German Bight. We found clupeids, mackerels, flatfish, and gadoids occurring in substantial
533 proportions in both sampling years, and the frequency of sand lances varied between the two
534 sampling years. Hence the diet consistently includes some common species with a high
535 nutritional value (Hislop et al. 1991; Ball et al. 2007), indicating the importance of these fish
536 groups as prey items for red-throated divers in the German Bight. Red-throated divers stage in
537 a specific habitat, mostly influenced by frontal zones in coastal areas in the German Bight
538 (Skov and Prins 2001; Heinänen et al. unpubl data). The preferred feeding at frontal zones
539 may also explain the higher abundance of pelagic fish among the red-throated diver prey,
540 where these species aggregate, while demersal species depend mainly on suitable sediments.
541 Considering the effects of disturbance, displacement or barrier effects arising from
542 anthropogenic activities such as ship traffic and offshore wind farms (Mendel et al. 2019), the
543 broad prey spectrum that we found could indicate resilience of red-throated divers against
544 changes in community composition of available fish or resilience against displacement from
545 suitable habitat. However, if alternative sites of high-quality habitat are not sufficiently

546 available, displacement may result in a decreased energy intake and subsequently poorer body
547 condition. Thus, altered food accessibility as a result of disturbance or displacement could
548 have severe effects on red-throated divers. In general, the availability of some prey species
549 may explain, at least to some extent, the preference of this area as wintering and staging
550 habitat. Further studies could aim to discern whether the birds use this area because of a high
551 abundance of suitable and energy rich prey or if they simply feed on the most abundant prey.

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566 Compliance with ethical standards

567 Conflict of interest: The authors explicitly declare that they have no conflict of interest.

568 Ethical approval: We herewith assure that the ethical rules as well as the legal requirements
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