DNA metabarcoding unveils multi-scale trophic variation in a widespread coastal opportunist

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Running title: Brown shrimp diet metabarcoding

Keywords: Community Ecology; Crustaceans; Diet Analysis; Metabarcoding; Foodwebs; Environmental DNA

Abstract

A thorough understanding of ecological networks relies on comprehensive information on trophic relationships among species. Since unpicking the diet of many organisms is unattainable using traditional morphology-based approaches, the application of high-throughput sequencing methods represents a rapid and powerful way forward. Here, we assessed the application of DNA-metabarcoding with nearly universal primers for the mitochondrial marker cytochrome c oxidase I (COI) in defining the trophic ecology of adult brown shrimp, *Crangon crangon*, in six European estuaries. The exact trophic role of this abundant and widespread coastal benthic species is somewhat controversial, while information on geographical variation remains scant. Results revealed a highly opportunistic behaviour. Shrimp stomach contents contained hundreds of taxa (>1000 molecular operational taxonomic units), of which 291 were identified as distinct species, belonging to 35 phyla. Only twenty ascertained species had a mean relative abundance of more than 0.5 %. Predominant species included other abundant coastal and estuarine taxa, including the shore crab *Carcinus maenas* and the amphipod *Corophium volutator*. Jacobs' selectivity index estimates based on DNA extracted from both shrimp stomachs and sediment samples were used to assess the shrimp's trophic niche indicating a generalist diet, dominated by crustaceans, polychaetes and fish. Spatial variation in diet composition, at regional and local scales, confirmed the highly flexible nature of this trophic opportunist. Furthermore, the detection of a prevalent, possibly endoparasitic fungus (*Purpureocillium lilacinum*) in the shrimp's stomach demonstrates the wide range of questions that can be addressed using metabarcoding, towards a more robust reconstruction of ecological networks.

Introduction

Trophic interactions provide important insights on a wide range of ecological dynamics, ranging from individual to ecosystem levels, which include animal behaviour, predator-prey interactions, food web structure and community ecology (e.g. Leray *et al.* 2015; Pinol *et al.* 2014). The feeding strategy of key consumers can have pronounced influences on ecosystem dynamics (Hanski *et al.* 1991; Holling 1965) and their stomach contents can reveal essential information on food item distribution and prey assemblage structure (Lasley-Rasher *et al.* 2015). Crustaceans are a key component in marine/estuarine soft bottom habitats (Evans 1983, 1984) and evaluating their diet is very challenging due to the complexity of direct observations on predation rates and the limitations associated with the identification of partially digested food items (Asahida *et al.* 1997; Feller 2006; Symondson 2002).

The recent application of high-throughput sequencing (HTS) tools, such as metabarcoding, promises to revolutionise the way prey diversity and composition are estimated from gut contents or faeces of consumers (Kartzinel & Pringle 2015; Leray *et al.* 2015). Metabarcoding refers to the identification of multiple taxa based on the screening of bulk DNA extracted from community or environmental samples (i.e. water, soil, faeces; Barnes & Turner 2016), by means of massive parallel sequencing of PCR amplicons (Barnes & Turner 2016; Taberlet *et al.* 2012a). Metabarcoding has proven to be highly effective for the identification of prey remains with improved taxonomic resolution, accuracy and speed of analysis, compared to traditional morphological methods (Berry *et al.* 2015; Casper *et al.* 2007; Symondson 2002). Yet, some challenges remain, such as fragmentation of partially-digested DNA, variability in taxon-specific digestion rates, secondary predation, and, typically, the presence of high proportion of DNA from the study organisms itself, which may reduce sequencing depth and render cannibalism undetectable (Barnes & Turner 2016; Berry *et al.* 2015; Pinol *et al.* 2014). Furthermore, due the sensitivity of these methods, in some cases, it might be difficult to discriminate between contaminant DNA and target DNA.

76 The brown shrimp, *Crangon crangon* (L.) is a key crustacean species in European coastal waters. Its

77 wide distribution (i.e. from the White Sea to Morocco), year round occurrence, and high abundance

78 (> 100 ind. m^{-2}; van der Veer *et al.* 1998) make it an essential part of the coastal benthic food web

79 (Ansell *et al.* 1999; Campos & van der Veer 2008; Evans 1984), a major prey item for birds and fish

80 (Evans 1984; Walter & Becker 1997), and an important target for fisheries, with recorded catches in

81 2011 up to 35,000 tons and more than 500 fishing vessels employed in the North Sea (Aviat *et al.* 2011;

82 Campos & van der Veer 2008). The trophic position of *C. crangon* is still being discussed, being

83 described as trophic generalist (Evans 1983), carnivorous opportunist (Pihl & Rosenberg 1984)

84 omnivorous (Ansell *et al.* 1999; Raffaelli *et al.* 1989; Tiews 1970) and probable scavenger (Ansell *et al.*

85 1999). As a juvenile, it relies mostly on the consumption of meiofaunal prey items while it switches to

86 larger demersal organisms as an adult, including conspecifics and juvenile stages of several

87 commercially important teleosts and bivalves (Evans 1984; Oh *et al.* 2001; Pihl & Rosenberg 1984; van

88 der Veer & Bergman 1987; van der Veer *et al.* 1998). Previous studies showed considerable variation

89 in prey item consumption, partly due to the brown shrimp's inherent trophic flexibility and nice

90 breath, but also because studies have relied on microscopic identification of prey remains (e.g.

91 Boddeke *et al.* 1986; Oh *et al.* 2001; but see also Nordström *et al.* 2009); yet, prey items are usually

92 macerated to a fine degree by *C. crangon*, and a high proportion of its stomach content is,

93 consequentially, impossible to identify through morphological examination (Asahida *et al.* 1997;

94 Wilcox & Jeffries 1974). Furthermore, most studies on *C. crangon*'s diet to date have focused on a

95 limited number of locations and relatively small spatial scales (e.g. Evans 1984; Oh *et al.* 2001; Pihl &

96 Rosenberg 1984) while large scale studies are required to assess geographical variation in the shrimp's

97 diet and to understand the relative importance of different prey items.

98 The degree to which food items are actively selected or passively ingested by consumers is an essential

99 consideration in assessing the relative importance of different prey categories and understanding the

100 trophic niche of consumers. Traditionally, indices are used to infer the predator's preference for prey

101 based on the relative abundance of prey in the predator's diet and the prey's relative abundance in

4

102   the environment (e.g. Peterson & Ausubel 1984). Examples of commonly used indices are the Ivlev's

103   Electivity Index (Ivlev 1961) and the Jacob's Index Of Selectivity (Jacobs 1974), which also corrects for

104   item depletion (Jacobs 1974). Although some attempts have been made to link diet metabarcoding

105   data with food availability in managed forests (Kowalczyk *et al.* 2011) and artificial mesocosms (Ray *et*

106   *al.* 2016), no examples exist for wild marine animal trophic studies contrasted with whole-community

107   environmental DNA data.

108

109   Here, we report on a large-scale analysis of the trophic ecology of *C. crangon,* which reveals its

110   ecological role in estuarine systems and provides a key for the reconstruction of ecological networks

111   of European coastal marine communities. By using nearly universal primers for mitochondrial

112   cytochrome c oxidase I, we used metabarcoding to describe the diet of the shrimp, alongside the soft

113   bottom communities on which they feed, over a European scale. We were expecting a wide variety of

114   prey items, reflecting variation in environmental conditions and prey availability across European

115   coasts. More specifically, we tested whether metabarcoding can (a) provide a detailed overview of *C.*

116   *crangon's* diet, including prey selectivity, using DNA extracted from stomach and environmental

117   samples; (b) identify geographical patterns in its trophic ecology, at both local and regional scales and

118   (c) assess consistent and general trophic patterns in order to better define the ecological role of this

119   widespread species.

120

Methods

Sample collection and processing

Brown shrimp and sediment samples were collected from 24 sites distributed over 6 estuaries in the

Netherlands, Portugal and the United Kingdom (Fig. 1). Adult shrimp (>20 mm total length, TL; tip of

the rostrum to tip of the telson) were captured in the intertidal zone (0-1m depth) by push-net at low

tide (±3 h). Shrimp (30-50 per site) were placed on ice and transported to the lab to be stored at -20

°C. Sediment was collected for the extraction of environmental DNA to characterise the biological

community present at each site. Sediment was sampled from the upper 2 cm surface layer, which

represent the most recent DNA deposits and the habitat where the shrimp live and feed (Pinn & Ansell

1993; Turner *et al.* 2015), with a PVC corer (3.2 mm Ø). Per site, three sediment subsamples were

collected at several meters distance from each other and combined to reduce the influence of local

heterogeneity (Taberlet *et al.* 2012b). The sediment was stored in 96 % ethanol, transported on ice

and kept at -20 °C. At each site, temperature, salinity (Fisher Scientific Traceable Salinity Meter), pH

(Hanna HI 98129), dissolved oxygen (OxyGuard Handy Mk I) and turbidity (Eutech TN-100) were

measured in triplicates. Extra sediment was collected, in triplicates, from each site for granulometric

analyses (Horiba LA-950 Particle size analyser) and Total Organic Matter (TOM) determination by

means of ashing (550 °C, 6 h). One site (Mersey 3) was not included for analysis because high-

throughput sequencing of its stomach samples did not result in sufficient read depth (< 1000 reads;

see results).

DNA extraction

Overall, 1025 shrimp (20-50 mm TL) were caught and 494 full stomachs (visual determination) were

dissected using flame-sterilised tools to avoid cross contamination. Stomachs were pooled in batches

of 8 (from shrimp collected at the same site) prior to DNA extraction (Ray *et al.* 2016). Though the

pooling of samples increases the number of stomachs analysed per sequencing run, pooling might

146 reduce the detected MOTU richness since large, recently ingested diet items in single stomachs may

147 obscure the stomach contents of other individuals. Nevertheless, this potentially negative effect of

148 pooling was deemed to be negligible, due to the pool replication conducted within location, and the

149 population-emphasis of the study. Three replicate pools were extracted per site. However, due to a

150 high percentage of empty stomachs in natural populations (20-60 %; Feller 2006; Oh *et al.* 2001; Pihl

151 & Rosenberg 1984), some sites contained only 2 replicates and some replicates contained less than 8

152 full stomachs (see Supplementary table S1): the latter were still included in the analyses as variation

153 in number of stomachs pooled did not affect the patterns observed (see results). In total, 66 pooled

154 samples were extracted, divided over 24 sites. In addition to the full stomach samples, 3 pooled

155 samples of 8 visually empty stomachs were included for comparative purposes.

156 DNA was extracted from 0.25 g of homogenized pooled stomach contents (N = 66) using the

157 PowerSoil® DNA Isolation Kit (Mo-Bio laboratories), whereas DNA from sediment (10 g; N = 24) was

158 extracted using the PowerMax® DNA Soil Kit (Mo-Bio laboratories). A Qubit fluorometer (Thermo

159 Fisher Scientific) was used to assess DNA concentrations of purified extracts. DNA extraction and pre-

160 PCR preparations were performed in separate labs from post-PCR procedures to reduce

161 contamination.

162

163 **DNA amplification and high-throughput sequencing**

164 Amplification of DNA, for both stomach and sediment samples, was achieved using a single set of

165 versatile, highly degenerated PCR primers targeting the 313-bp Leray fragment (Leray *et al.* 2013) of

166 the mitochondrial cytochrome c. oxidase subunit I (COI) region. The mICOIintF-XT primer (5'-

167 GGWACWRGWTGRACWITITAYCCYCC-3') was used as forward primer. This modified version

168 (Wangensteen *et al.* 2018) of the mlCOIintF primer (Leray *et al.* 2013) included 2 extra degenerate

169 bases (equimolar mixtures of 2 different bases at a given position) and 2 inosine nucleotides (that can

170   match any nucleotide) to enhance its eukaryotic universality. The reverse primer was jgHCO2198 (5'-

171   TAIACYTCIGGRTGICCRAARAAYCA-3'; Geller *et al.* 2013). The Leray fragment has already been

172   successfully applied for both the characterisation of marine communities and marine fish gut contents

173   (Leray & Knowlton 2015; Leray *et al.* 2015; Leray *et al.* 2013). Eight-base oligo-tags (Coissac *et al.* 2012)

174   attached to the metabarcoding primers were added to the amplicons during a single PCR step, in order

175   to label different samples in a multiplexed library. Also, a variable number (2, 3 or 4) of fully

176   degenerate positions (Ns) was added at the beginning of each primer, in order to increase variability

177   of the amplicon sequences (Guardiola *et al.* 2015). The PCR mix recipe included 10 µl AmpliTaq gold

178   360Master mix (Applied Biosystems), 3.2 µg Bovine Serum Albumin (Thermo Scientific), 1 µl of each

179   of the 5 µM forward and reverse tagged-primers, 5.84 µl H_2O and 2 µl extracted DNA template (~ 5 ng

180   $µl^{-1}$). The PCR profile included an initial denaturing step of 95 °C for 10 min, 35 cycles of 94 °C for 1

181   min, 45 °C for 1 min and 72 °C for 1 min and a final extension step of 72 °C for 5 minutes. After quality

182   check of all amplicons by electrophoresis, the tagged PCR products (including 2 PCR negative controls)

183   were pooled at equimolar concentration into 2 multiplexed sample pools (sediment and stomach) and

184   purified using MinElute columns (Qiagen). Two Illumina libraries were subsequently built from these

185   pools, using the NextFlex PCR-free library preparation kit (BIOO Scientific). Libraries were quantified

186   using the NEBNext qPCR quantification kit (New England Biolabs) and pooled in a 1:4

187   sediment:stomach molar concentration ratio (similar to the sediment:stomach sample ratio) along

188   with 0.7% PhiX (v3, Illumina) serving as a positive sequencing quality control. The libraries with a final

189   molarity of 8 pM were sequenced on an Illumina MiSeq platform using v2 chemistry (2x250 bp paired-

190   ends).

191   Preliminary analyses of the sequencing data revealed a substantial number of reads belonging to one

192   Molecular Taxonomic Unit (MOTU) in the fungal order Hypocreales (Ascomycota). For further

193   identification, the ITS fragment was amplified from 5 samples with a high number (>90% read

194   abundance) of reads of this MOTU, with the primer combination ITS1f (5'-

195   CTTGGTCATTTAGAGGAAGTAA-3'; Gardes & Bruns 1993) and ITS4ASCO (5'-

8

196 CGTTACTRRGGCAATCCCTGTTG-3'; Nikolcheva & Bärlocher 2004), specific for Ascomycota. The PCR

197 mix recipe was the same as the one used for the Leray fragment described above and the PCR profile

198 included an initial denaturing step of 95 ºC for 5 min, 32 cycles of 95 ºC for 30 sec, 55 ºC for 30 sec

199 and 72 °C for 1 min and a final extension step of 72 ºC for 10 minutes (Manter & Vivanco 2007). After

200 electrophoresis check, the amplicons of these 5 samples were cleaned and Sanger sequenced by

201 Source Bioscience Sequencing UK.

202

203 **Bioinformatic and data analyses**

204 Bioinformatic analyses were performed using the OBITools metabarcoding software suite (Boyer *et al.*

205 2016). Read quality assessment was performed with FastQC and paired-end read alignment using

206 illuminapairedend, retaining reads with an aligment quality score > 40. Demultiplexing and primer

207 removal was achieved using ngsfilter with the default options. Obigrep was applied to select all aligned

208 reads with a length between 303-323 bp and free of ambiguous bases. Obiuniq was used to

209 dereplicate the reads and the uchime-denovo algorithm (Edgar *et al.* 2011) (implemented in VSEARCH;

210 Rognes *et al.* 2016) was used to remove chimeras. Amplicon clustering was performed using the

211 SWARM algorithm (Mahé *et al.* 2014, 2015) with a d value of 13, which offers a conservative solution

212 to the high variability of the COI gene (Wangensteen & Turon 2017). After removal of singletons,

213 taxonomic assignment of the representative sequences for each MOTU was performed using the

214 ecotag algorithm (Boyer *et al.* 2016), using a local reference database (Wangensteen *et al.* 2018)

215 containing filtered COI sequences retrieved from the BOLD database (Ratnasingham & Hebert 2007)

216 and the EMBL repository (Kulikova *et al.* 2004). This algorithm uses a phylogenetic approach to assign

217 sequences to the most reliable monophyletic unit, based on the density of the reference database.

218 The data was refined by clustering MOTUs assigned to the same species, abundance renormalization

219 (to remove false positives due to tag-switching; Wangensteen & Turon 2017) and by removing

220 bacterial reads and contaminations of human or terrestrial origin. MOTUs with a maximum of four or

221 less reads per sample were removed on a sample by sample basis to avoid false positives and low

222   frequency noise (De Barba *et al.* 2014; Wangensteen *et al.* 2018). All MOTUs for which the abundance

223   in the PCR negative controls was higher than 10 % of the total reads of that MOTU were removed

224   (Wangensteen & Turon 2017). Samples with a low read depth (< 1000) following removal of predator,

225   parasite and contaminant reads were removed prior to analysis. All statistical analyses were

226   performed in R v3.1.3 (https://www.R-project.org/) with the vegan (v2.3-5) and BiodiversityR (v2.5-3)

227   packages (Kindt & Coe 2005; Oksanen *et al.* 2016). Only MOTUs showing mean relative abundance ≥

228   0.5% in the full stomach samples were considered (Albaina *et al.* 2016) for non-metric

229   multidimensional scaling (nMDS), canonical correspondence analysis (CCA) and PERMANOVA

230   analyses. Correlation between sediment and stomach community composition was tested with a

231   Mantel test (Bray-Curtis dissimilarities; Pearson's product-moment correlation; 999 permutations).

232   The influence of environmental variables (mean temperature, salinity, pH, oxygen saturation,

233   turbidity, median sediment grain size and TOM) on the full stomach contents were tested by means

234   of CCA and PERMANOVA. PERMANOVAs were calculated using the function Adonis (vegan) with Bray-

235   Curtis dissimilarities and 1000 permutations. Prior to CCA and PERMANOVA, model selection was

236   performed using the function ordistep (vegan). Prey MOTU richness for each estuary was represented

237   as MOTU accumulation curves after rarefaction for the number of reads (1000 reads, 500

238   permutations) and the number of samples (9-15 samples, 1000 permutations). The Jacobs' Selectivity

239   Index was calculated based on the relative read abundances of the MOTUs extracted from sediment

240   and stomach samples in accordance to Jacobs (1974). Trophic significance of individual MOTU was

241   determined based on the relative read abundance, fraction of samples with MOTU presence and

242   Jacobs' Selectivity Index as follows: Trophic significance = (relative abundance) * (fraction of samples)

243   * (Jacobs' Selectivity Index + 1). Trophic significance was represented in categorical terms based on

244   the relative trophic significance of each MOTU (high: > 10 %, medium 1 % - 9 %, low < 1 %) instead of

245   exact values since the relative abundances of individual taxa should be considered with caution

246   (Deagle *et al.* 2005).

Results

Collection statistics

A total of 1025 *C. crangon* were caught with a 1:8 male:female sex ratio (based on 767 shrimp which could be sexed morphologically). About 7.5 % of the females were ovigerous. Mean (± SD) wet weight was 0.40 ± 0.26 gram; mean (± SD) TL was 35.1 ± 7.6 mm and mean (± SD) carapace length (CL) was 7.4 ± 1.6 mm (CL = 0.214*TL; r^2 = 0.81, N = 1025). TL varied significantly between sites (Supplementary table S2; One-way ANOVA: Df = 23, F = 47.95, P <0.001). Overall, the proportion of *C. crangon* with a full stomach was 57.9%. Mean proportion of full stomachs per site (58.9 ±19.3%) was not correlated with the time of sampling (Pearson's correlation: R^2 = 0.07, P = 0.754, N = 24).

High-throughput DNA sequencing

A total number of 8,895,448 reads were obtained from an Illumina MiSeq run of pooled amplicon libraries built from 24 sediment samples, 69 pooled *C. crangon* full stomach samples (from now on referred to as stomach samples), 3 pooled *C. crangon* empty stomach samples (comprising of stomach tissue and clear liquid) and 2 PCR negative controls. Variation in the number of pooled stomachs did not affect the patterns of diet composition (PERMANOVA: pseudo-$F_{1,50}$ = 1.0, P = 0.453) or MOTU richness (rarefied to 1000 reads) per sample (generalized linear model with quasipoisson distribution: t = 1.08, P = 0.650). In total, 5,704,471 reads remained after sample demultiplexing, quality and sequence-length filtering, and removal of bacterial reads, contaminations and false-positives due to tag-switching (sediment samples: 742,286; stomach samples: 4,828,136; empty stomach samples: 134,049). After taxonomic assignment, a total of 39 MOTUs (16 Metazoa, 7 Rhodophyta, 5 Stramenopiles, 11 unassigned) were removed because their abundance in the PCR negative control was > 10 % of the total reads of those MOTUs. Mean (± SD) proportion of *C. crangon* reads was 28 ± 29 % (range: 0.2-97.6 %) in the stomach samples and 47 ± 46 % (range: 10.6-99.1 %) in the empty

271   stomach samples. Mean proportion of *C. crangon* reads was 1 ± 4 % in the sediment samples (range:

272   0.0-21.0 %). Remaining number of reads per sample ranged 179-203,808 in full stomach, 7-332 in

273   empty stomach and 5,114-71,770 in sediment samples. A high number of reads (4,828,136 reads)

274   belonging to a fungus of the species *Purpureocillium lilacinum* (Ascomicota: Hypocreales) was

275   detected in almost all (95 %) stomach samples and identified using both COI (100 % identity) and ITS

276   markers (100 % identity; Supplementary table S3). Mean (± SD) proportion of *P. lilacinum* reads was

277   36 ± 37 % (range: 0.0-97.4 %) in full stomach, 53 ± 47 % (range: 0.1-89.4 %) in empty stomach and 0.1

278   ± 0.2 % (range: 0.0-0.8 %) in sediment samples. No *P. lilacinum* were detected in the PCR negative

279   controls. All *C. crangon* and *P. lilacinum* reads were removed from the database prior to further

280   analyses on diet, resulting in a total of 2,687,877 reads divided over 66 pooled stomach samples (Fig.

281   2) and 24 sediment samples (Fig. 3). A total of 14 pooled stomach samples were removed, prior to

282   further analyses, because they contained less than 1000 diet-related reads (Supplementary table S4).

283   One sediment sample (Mersey 3) was, consequently, also removed since no stomach samples were

284   included for that site. The final dataset consisted of a total of 8,321 MOTUs, of which 6,299 MOTUs

285   belonging to 40 phyla were detected in the sediment samples, 2,342 (35 phyla) in the stomach

286   samples, and 14 (7 phyla) in the empty stomach samples. A total of 502 MOTUs were detected both

287   in the sediment and stomach samples and only 2 (an unassigned Rhodophyta and an unassigned

288   Eukaryota) were detected exclusively in the empty stomach samples. Of the total number of MOTUs

289   detected, 370 could be assigned to the species level of which 291 were detected in the stomach

290   samples. Twenty taxa showed a relative abundance greater than 5 % in any given sample while 85 taxa

291   showed an abundance greater than 1%. The final number of diet-related reads per stomach sample

292   varied randomly, without systematic trends across estuaries (One-way ANOVA: Estuary: $F_{5,29}$: = 2.017,

293   P = 0.106; Sites nested in estuary: $F_{17,29}$: = 0.811, P = 0.669). Rarefaction curves (Supplementary Fig.

294   S1) showed that a plateau in the number of MOTUs was achieved in almost all cases, indicating an

295   overall sequencing depth adequate to capture the number of MOTUs present. Empty stomach

296   samples contained a very low number of MOTUs and reads, and were, therefore, not taken into

297   account for any further analyses (Fig. 4A).

298

299   **Description of *Crangon crangon* diet**

300   Analysis of *C. crangon* stomach contents showed large variation in relative MOTU abundances

301   between samples (Fig. 2). Notable patterns are: the lack of a dominant MOTU detected in stomachs

302   from the Aveiro estuary; a relatively high (10-25 %) contribution of the decapod crabs *Carcinus maenas*

303   and *Pisidia longicornis* in the Minho estuary; the detection of the introduced barnacle *Austrominius*

304   *modestus* in the Scheldt and Mersey estuaries; high amounts (~25 %) of the polychaete *Pista cristata*

305   in the Eastern Scheldt; the substantial proportion (~10 %) of the mysid *Neomysis integer* reads in the

306   Mersey estuary, and the dipteran *Chironomus salinarius* also, in the most inland sampling site of this

307   estuary (> 75 %); the large contribution (~ 50 %) of the amphipod *Corophium volutator* in the Kent

308   estuary. In general, the shore crab *C. maenas* and the amphipod *C. volutator* were the trophically

309   preponderant prey items for *C. crangon* (Table 1). Other important MOTUs included annelids (*Hediste*

310   *diversicolor* and *P. cristata*), other amphipods (*Bathyporeia sarsi*), other decapods (*P. longicornis*),

311   chironomids (unassigned), mysids (*N. integer*) and barnacles (*A. modestus*). Fish reads were detected

312   in all estuaries with a total of 22 species present in 27 stomach samples. Five fish species were

313   relatively abundant (≥ 5 %; Table 1) but were generally only present in a small number of stomach

314   samples. One other noteworthy observation is the presence of low abundances of known parasitic

315   taxa in several stomach samples (*Hematodinium* sp., 3 stomach samples; Apicomplexa, 6 stomach

316   samples).

317

318   **Selectivity in *Crangon crangon* diet**

319 MOTU diversity within phyla was generally higher in the sediment than in the stomach samples, except

320 for Arthropoda, Annelida, Mollusca and Chordata (Fig. 4A). The proportion of MOTUs that could not

321 be assigned at the phylum level was higher in the sediment (73 %) than in the stomach samples (58

322 %) and many abundant taxa in the sediment could not be identified at lower taxonomic ranks (Fig. 3).

323 Data combined per sample type (sediment/stomach) and MOTUs pooled at the phylum level showed

324 that sediment samples contained high relative read abundances of Bacillariophyta (20 ± 3 %), Discosea

325 (10 ± 2 %), Dinoflagellata (6 ± 2 %) and Arthropoda (5 ± 1 %) while *C. crangon* stomach samples

326 contained a high mean (± SE) relative read abundance (%) for Arthropoda (53 ± 5 %), Annelida (12 ± 3

327 %) and Chordata (5 ± 2 %; Fig. 4B). Mantel test results showed a significant correlation between the

328 community structure detected in the stomach and sediment samples (r: 0.43, P < 0.01), indicating an

329 association between the shrimp's diet and its prey abundance in the environment. Analyses of DNA

330 extracted from both sediment and *C. crangon* pooled stomach samples showed, based on all MOTUs

331 detected, significant differences between sample types and estuaries (Fig. 5 and 6A; PERMANOVA:

332 sample type: pseudo-$F_{1,68}$ = 7.8, P < 0.001; estuary: pseudo-$F_{5,68}$ = 2.5, P < 0.001). Visual inspection of

333 the relative abundances of the most important MOTUs also showed a high discrepancy between the

334 abundances in the stomach (Fig. 2) and sediment samples (Fig. 3). MOTUs abundant in the stomach

335 samples (≥ 1% abundance) showed, furthermore, a low read abundance in the sediment samples in

336 all estuaries (Fig. 6A). These differences in relative abundances, resulted in many MOTUs having a

337 maximum Jacobs' selectivity index value of 1 which indicates that prey items were highly selected

338 (Table 1). Phylum composition differed significantly between sediment and full stomach samples

339 (PERMANOVA: pseudo-$F_{1,44}$ = 34.1, P < 0.001). Apart from Cnidaria and Rhodophyta, all phyla with ≥ 1

340 % abundance in either sediment or stomach samples showed significant differences (based on paired

341 Wilcoxon signed-rank tests) in relative read abundances between the sediment and stomach samples

342 (Supplementary table S5). Visualisation of the importance of the phyla detected in the stomach

343 samples based on the mean relative abundance (%), presence (%) and Jacobs' selectivity index (D) is

344 shown in Fig. 4C.

Variation between estuaries

Multivariate analysis on the stomach contents (MOTUS ≥ 0.5 % abundance) showed significant differences between estuaries (Fig. 6B; PERMANOVA: pseudo-$F_{5,29}$ = 2.7, P < 0.001) and sites nested within estuaries (PERMANOVA: pseudo-$F_{17,29}$ = 0.6, P < 0.001). Bonferroni-corrected pairwise comparisons showed similarity in consumed community structure among the Eastern Scheldt, Western Scheldt and Mersey estuaries. Minho differed significantly from the Mersey and Eastern Scheldt estuaries. Aveiro or Kent significantly differed from all other estuaries (See Supplementary table S6 for details). Step-wise model selection (both forward and reverse) and CCA (Fig. 6C) showed significant influences of salinity (P < 0.01), median grain size (P < 0.01) and TOM (P < 0.05; see table S7 for means per estuary) on MOTU composition in *C. crangon* stomach samples (≥ 0.5 % abundant MOTUs). The environmental variables (constrained CCA axes) explained 34 % of the variance in the dataset. Temperature, turbidity and oxygen saturation did not have a significant influence on the model and pH was strongly correlated with salinity (r^2 = 0.75, P < 0.001, N = 23). These factors were, therefore, not included in the final model. MOTU richness (rarefied to 1000 reads) in *C. crangon* stomach contents also showed differences between estuaries, with the Aveiro and Eastern Scheldt estuaries showing a higher number of MOTUs than the others (Fig. 7). The slopes of the MOTU accumulation curves, however, did not approach an asymptote, offering a glimpse of the vast amount of marine biodiversity yet to be uncovered.

Discussion

Evaluation of *C. crangon* diet

This study provides a detailed overview of the brown shrimp's trophic ecology, focusing on dietary variations at multiple geographical scales. Adult brown shrimp were caught in a variety of sandy estuarine intertidal habitats. Mainly females were captured, probably due to the spatial sex-specific segregation of *C. crangon* during the summer-autumn period (Bamber & Henderson 1994; Henderson & Holmes 1987). The results confirm *C. crangon* as a generalist consumer feeding on a broad variety of food items but preferring larger mobile epifaunal prey items such as crustaceans, annelids and fish. The present investigation uncovered a great diet contribution of decapods and teleosts, while these were usually not considered to be important contributors to the shrimp diet in previous studies (e.g. Ansell *et al.* 1999; Oh *et al.* 2001; Plagmann 1939; Raffaelli *et al.* 1989). Although comparisons of prey contribution should be made cautiously due to diversity of quantification methods used, the observed trend could be partly explained by scavenging behaviour on large organisms, previously not recorded in crangonid shrimps. Crangonid shrimps generally macerate and eat the soft body parts of larger preys (Asahida *et al.* 1997; Gibson *et al.* 1995; Seikai *et al.* 1993; Wilcox & Jeffries 1974). Smaller food items, on the other hand, are often ingested as a whole, including their hard body parts (Tiews 1970) and are thus more easily identified by morphological methods. This discrepancy in detectability might possibly have played a role in studies that have detected low amount of fish and decapods but considerable amounts of unidentified soft tissue (e.g. Oh *et al.* 2001; Raffaelli *et al.* 1989). Metabarcoding methods can detect and taxonomically identify such soft tissues, thus highlighting the enhanced suitability of molecular approaches to present a more realistic picture of trophic ecology in marine invertebrates.

The diet of *C. crangon* showed a high MOTU richness, including previously described food items (Table 1). The number of COI MOTUs (2,342) detected in the shrimp's stomachs may be an overestimation

390   of the total number of real species (e.g. due to detection of pseudogenes; Tang *et al.* 2012; Vamos *et*

391   *al.* 2017) and includes protists and microalgae (Wangensteen *et al.* 2018), which are unlikely to be

392   prey items of *C. crangon*. Nonetheless, even just the 306 ascertained or 20 most abundant (> 0.5 %

393   relative abundance) species in the shrimp's diet was remarkably higher than the number found in

394   previous studies based on morphological identification (see Table 1). Furthermore, twenty taxa

395   showed a high abundance (>5 %) in any given sample, probably representing important prey items at

396   some locations or times.

397

398   Two species were predominant in our study: the shore crab *Carcinus maenas* across the overall

399   geographic distribution, and the amphipod *Corophium volutator* in UK localities (characterised by

400   muddy sediments and high organic matter content)*.* Both species are well-known prey of *C. crangon*

401   (Evans 1984; Moksnes *et al.* 1998; Pihl & Rosenberg 1984) and can occur at high densities in soft-

402   bottom estuarine habitats (Meadows & Reid 1966; Moksnes 2002). Consumption of *C. maenas* could

403   be the result of scavenging, although juvenile crabs could be captured, while *C. volutator* are likely to

404   be predated, as these amphipods are small (up to 11 mm TL). Overall, the local distribution of the

405   detected food items followed environmental gradients reflecting their ecology. Euryhaline deposit

406   feeders such as *C. volutator* and *H. diversicolor, N. integer* and *Chironomus salinarius* (larval stage)

407   were mainly associated with muddy, brackish sites with high organic matter content, commonly

408   inhabited by these species (Anderson 1972; Drake & Arias 1995; Mauchline 1971; Meadows 1964;

409   Mees *et al.* 1993; Ólafsson & Persson 1986). Stomach samples taken from sites with larger grain size

410   contained species adapted to coarser sands, such as *P. longicornis* and *Talitrus saltator* (Fanini *et al.*

411   2007; Pallas *et al.* 2006). Detection of fish DNA could reflect a combination of direct predation on

412   juveniles of species which use the estuaries as nurseries (e.g., *Platichthys flesus* and *Dicentrarchus*

413   *labrax*) and scavenging on dead bodies of species which do not regularly use estuaries as a nursery

414   (e.g., *Scomber scombrus* and *Labrus bergylta*; Elliott & Dewailly 1995). The high presence of the

415   invasive barnacle *A. modestus* DNA at several locations was likely due to the capture of cypris or nauplii

416   larvae (Ansell *et al.* 1999; Boddeke *et al.* 1986).

417

418   This is also the first study showing a high occurrence of *P. lilacinum* (Ascomycota: Hypocreales) in the

419   digestive system of *C. crangon*. *Purpureocillium lilacinum* is a well-studied fungus, being abundant in

420   terrestrial soils (Cham Thi Mai *et al.* 2016) and detected in the marine environment (Redou *et al.* 2015;

421   Yue *et al.* 2015). It is a known pathogen of nematodes and therefore of commercial importance as a

422   biological control agent to manage pests of several crops (Castillo Lopez *et al.* 2014; Singh *et al.* 2013).

423   This fungus is even considered to be of medical importance since it can infect humans and other

424   vertebrates with compromised immune systems (Luangsa-Ard *et al.* 2011). As *P. lilacinum* has been

425   successfully cultured (for the production of chitosanase) using farmed marine shrimp by-products as

426   substrate (*Penaeus* sp.; Nidheesh *et al.* 2015) and is closely related to known parasites of crabs (Smith

427   *et al.* 2013), it might be postulated that it has a symbiotic relationship with *C. crangon*, although more

428   research is required to test this hypothesis. Its occurrence and high relative abundance (although

429   possibly overestimated since its DNA was extracted from a living community, as opposed to digested

430   food) in *C. crangon* stomach samples over a large geographical area are clear indicators that this

431   species might be important for the brown shrimp's ecology and/or physiology. Alongside *P. lilacinum*,

432   several other known parasitic taxa have been detected in the shrimp's stomachs, including

433   *Hematodinium* sp. and Apicomplexa (Molnar *et al.* 2012; Rueckert *et al.* 2011; Stentiford & Shields

434   2005).

435

436   **The application of metabarcoding in crustacean trophic studies**

437   Metabarcoding using universal primers is generally considered as a simple, rapid and relatively

438   inexpensive method to define in detail the feeding ecology of organisms (Berry *et al.* 2015; Kartzinel

439 & Pringle 2015; Pinol *et al.* 2014). The fraction of the brown shrimp DNA detected in its own gut was

440 low allowing for the detection of prey items without using predator-specific blocking primers (average:

441 28%; compared to e.g. Olmos-Perez *et al.* 2017; Pinol *et al.* 2014). Metabarcoding has several clear

442 advantages over traditional trophic methods including the better detection of soft-bodied, small and

443 cryptic taxa, higher speed of analysis (Berry *et al.* 2015; Casper *et al.* 2007; Chariton *et al.* 2015;

444 Symondson 2002), and traceability of identifications, which do not rely on the availability of

445 morphological taxonomic expertise. Furthermore, the application of metabarcoding even allows for

446 the detection of prey items in empty guts (Harms-Tuohy *et al.* 2016), albeit the DNA extracted from

447 visually empty *C. crangon* stomachs was too low in prey read number and MOTU richness for robust

448 comparisons.

449

450 Both traditional morphological examination and DNA-metabarcoding of food items suffer from

451 limitations in providing quantitative descriptions of the diet of consumers (Casper *et al.* 2007). For

452 metabarcoding, errors can occur due to technical artefacts specific to DNA amplification and

453 sequencing (Barnes & Turner 2016; Pompanon *et al.* 2012), and biological limitations such as species-

454 specific digestion and DNA degradation rates (Deagle *et al.* 2010; Murray *et al.* 2011; Pinol *et al.* 2014;

455 Sakaguchi *et al.* 2017). Furthermore, some of the DNA detected might come from secondary predation

456 (taxa present in the stomach of preyed organisms; Berry *et al.* 2015; Kartzinel & Pringle 2015).

457 Cannibalism also imposes a specific problem in trophic molecular studies since it cannot be identified

458 by means of metabarcoding (Berry *et al.* 2015; Ray *et al.* 2016). Large brown shrimp*s* are known to be

459 cannibalistic (Evans 1984; Pihl & Rosenberg 1984) but the removal of *C. crangon* sequence reads from

460 our data set makes it impossible to gauge insights into the extent of cannibalism in this species. Due

461 to the restrictions in the quantification of consumed prey volume, many trophic studies only use

462 presence/absence data (e.g. Deagle *et al.* 2010; Harms-Tuohy *et al.* 2016; Pinol *et al.* 2015). This might,

463 however, result in an overestimation of small taxa that are abundant in the sediment, but with low

464   trophic relevance, as they could, in the case of *C. crangon*, be passively acquired when shrimp ingest

465   sediment to crush food in their stomach (Ansell *et al.* 1999; Deagle *et al.* 2018; Tiews 1970). Multiple

466   stomachs were pooled prior to analysis and data was subjected to rigorous filtering to allow for a semi-

467   quantitative estimation of proportions of prey DNA (Deagle *et al.* 2005; Lejzerowicz *et al.* 2015;

468   Pompanon *et al.* 2012; Thomas *et al.* 2016). Relative abundances of individual taxa should, however,

469   be considered with caution and viewed more in categorical terms (low or high trophic significance)

470   than exact proportions (Deagle *et al.* 2005). This study provides a significant addition to a growing

471   body of studies in showing the applicability of semi-quantitative estimations in molecular trophic

472   ecology (e.g. Albaina *et al.* 2016; Deagle *et al.* 2018; Ray *et al.* 2016; Sakaguchi *et al.* 2017; Soininen *et*

473   *al.* 2013).

474

475   Finally, the results presented draw a close link between prey distribution in estuarine habitats and

476   ingested prey item abundance. The use of eDNA from sediments to assess community composition

477   and to relate this to the shrimp's diet is a novel contribution to the fields of molecular trophic analysis

478   and eDNA, which goes beyond the taxon studied. It should be noted, however, that a correct

479   assessment of the predator's trophic niche by means of prey selectivity determination relies on a

480   correct assessment of prey abundance, both in the stomach and in the environment. Issues with

481   incorrect abundance estimations, for example due to species-specific detection rates (e.g. due to

482   different rates of DNA sequestering in the environment; Barnes & Turner 2016), are not specific to

483   molecular studies (Strauss 1979) and the constant work on improving the reliability of relative

484   abundance estimations from eDNA (Deagle *et al.* 2018; Thomas *et al.* 2016; Ushio *et al.* 2018) should

485   substantially enhance the applicability of selectivity indices in molecular research.

486

487   **Geographic variation in *C. crangon* trophic ecology**

488   This study also assesses for the first time a large geographical variation in the brown shrimp's trophic

489   ecology at multiple spatial scales. Previous studies have shown local variability in *C. crangon* diet

490   (Evans 1984; Oh *et al.* 2001; Pihl & Rosenberg 1984) but no studies have been performed across

491   multiple European estuaries. The results indicate that the consumed prey community can vary at local

492   (within estuary, as discussed above) and regional (between estuaries) scales. The seasonal and tidal

493   migratory behaviour of *C. crangon* (Al-Adhub & Naylor 1975; Henderson & Holmes 1987) may

494   complicate localized diet assessments since their stomach contents might also contain food consumed

495   at distant locations. Yet, this effect is considered to be minimal since the brown shrimp's relatively

496   fast gut passage time ensures that their stomach contents mainly contain recently consumed items

497   (4-20h; Feller 2006; Pihl & Rosenberg 1984; van der Veer & Bergman 1987). Large scale assessment of

498   *C. crangon*'s trophic ecology showed high similarity between the Eastern Scheldt, Western Scheldt

499   and Mersey estuaries and distinct diets in the Aveiro, Minho and Kent estuaries. The Aveiro estuary

500   forms a large, saline lagoon with a wide variety of different habitats incorporating euhaline/polyhaline

501   areas with relatively high species richness (Rodrigues *et al.* 2011). On the other hand, the Minho

502   estuary is characterized by high water discharge, salinity variations (Costa-Dias *et al.* 2010) and

503   significantly larger sediment grain size, factors which determine significantly divergent biodiversity

504   features. The Kent estuary has a low species diversity caused by its fine sediments and low salinity

505   (Anderson 1972). The Mersey estuary also showed a relatively low species richness detected in the

506   stomach contents of *C. crangon*, probably related to its history of anthropogenic stress (Jones 2000).

507   Overall, trophic variation in *C. crangon* depends on patterns in the local abundance and distribution

508   of its prey (in line with: Oh *et al.* 2001; Pihl 1985; Pihl & Rosenberg 1984). In order to evaluate this

509   variation more exhaustively, knowledge on the ecology and seasonality of the local macrozoobenthic

510   community is required.

511

512   ***Crangon crangon's* ecological role**

513   Based on the results of this study, *C. crangon* can best be described as a highly opportunistic carnivore

514   and scavenger. Despite its broad dietary range (Fig. 8), it shows, nevertheless, a prominent level of

515   selectivity to larger motile mobile epifaunal prey items. This high level of flexibility in its trophic

516   ecology might contribute to its very wide distribution on European coasts (Campos *et al.* 2009). In

517   order to feed on diverse prey taxa, adult *C. crangon* are capable of employing a variety of methods

518   (Fig. 8) including ambush predation (Gibson *et al.* 1995; Pinn & Ansell 1993), gulping behaviour (Tiews

519   1970) and scavenging (Fig. 8; Ansell *et al.* 1999; Price 1962). Since meiofaunal and protist phyla were

520   not selected as prey items based on Jacobs' selectivity index (present but not abundant; in line with:

521   Evans 1983; Feller 2006), it is possible that these taxa were passively consumed during the ingestion

522   of sand to aid digestion (Ansell *et al.* 1999; Tiews 1970) or through secondary predation. Several

523   studies classify *C. crangon* as an omnivore (Ansell *et al.* 1999; Raffaelli *et al.* 1989; Tiews 1970), but we

524   cannot confirm this classification, because the primers used during this study have a very low affinity

525   for chlorophytes resulting in many algal taxa not being detected (Wangensteen *et al.* 2018).

526   Nevertheless, the algal phyla that can be detected with these primers (e.g. Rhodophyta, Phaeophyta

527   and Bacillariophyta) had a low selectivity, indicating a negligible trophic importance for *C. crangon*.

528   More research is required with plant-specific primers to assess the actual contribution of herbivory to

529   the diet of the brown shrimp. Overall, the results of this study yield a level understanding of the trophic

530   ecology of this species that would not have been possible through traditional morphological analysis,

531   and which is key to providing essential insights into coastal community interactions.

532

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Data Accessibility

The dataset, including sequences, taxonomic assignment and abundances for all MOTUs in every sample, have been deposited in Dryad (doi:10.5061/dryad.sk2155m). Custom R scripts are publicly available from https://github.com/Andjin/Crangon-diet-analysis and https://github.com/metabarpark for scripts related to the bioinformatics pipeline.

Author Contributions

A.S. participated in the study design, carried out the field, laboratory and molecular work, performed the data and statistical analyses, and wrote the manuscript; O.S.W. participated in the molecular work, designed the bioinformatics pipeline and assisted in the statistical analyses; C.B. participated in the study design; J.C. assisted in the collection of the samples; S.M. conceived and designed the study. All authors provided critical comments on the manuscript and gave final approval for publication.

Tables and figures
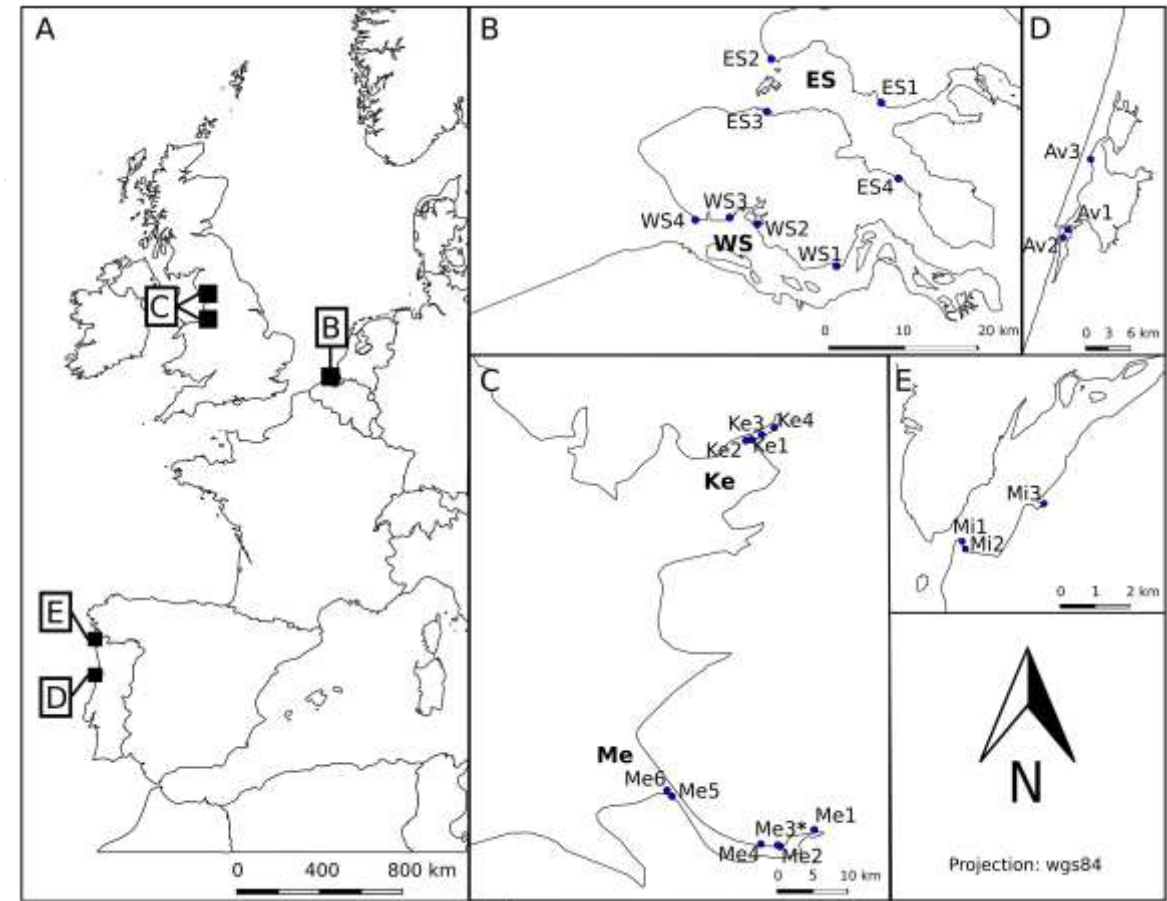
Figure 1. Overview of sample locations, illustrating (A) the overall western European scale; (B) the Dutch estuaries, Western Scheldt (WS) and Eastern Scheldt (ES); (C) the British estuaries, Mersey (Me) and Kent (Ke); the Aveiro (D) and Minho (E) estuaries in Portugal. Small dots within estuaries represent individual collection points for shrimp and sediment samples. *Site removed prior to molecular analysis. Source map: OpenStreetMap.
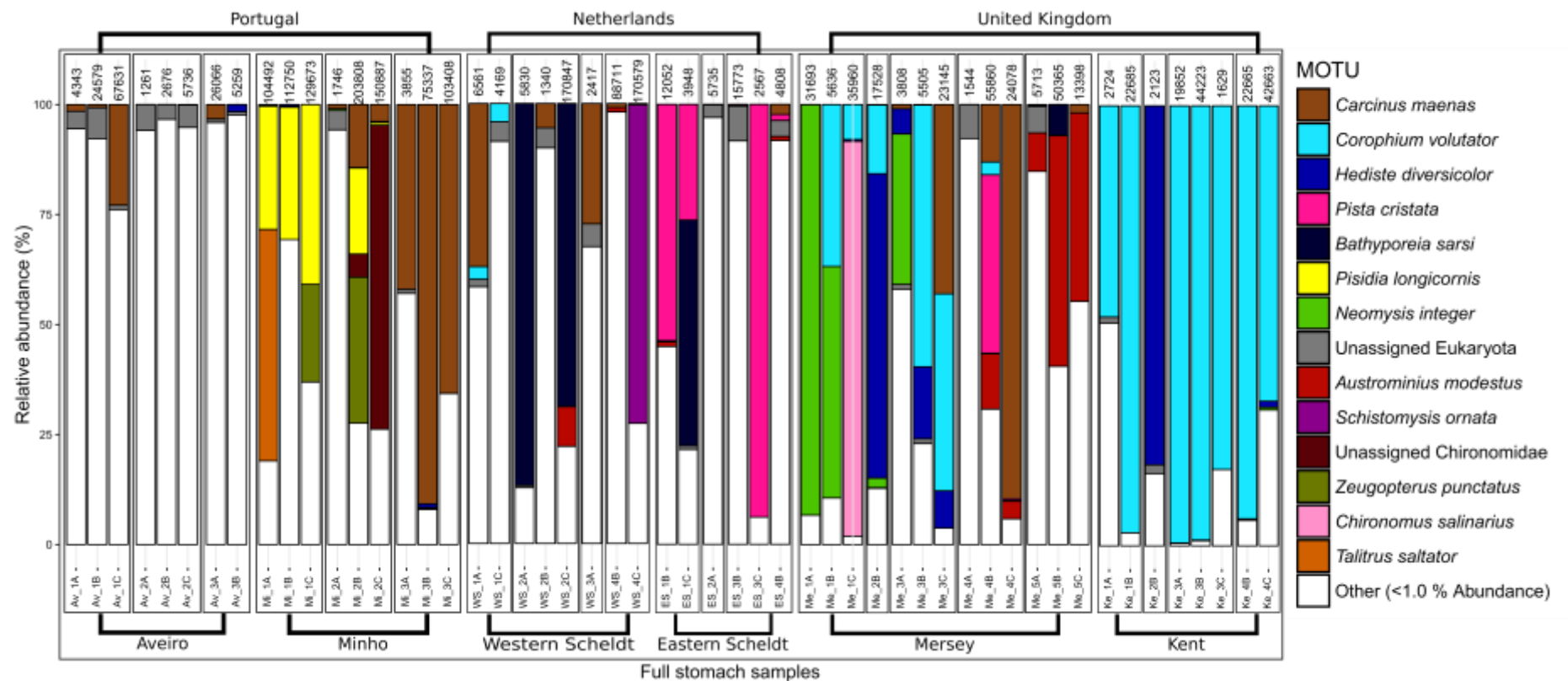
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838 Figure 2. Relative abundances of MOTUs detected in *Crangon crangon* stomach samples by COI metabarcoding, after removal of *C. crangon* and *P. lilacinum*

839 reads. Each bar represents one sample. Countries are shown on top of the graph, estuaries below and boxes contain the individual sites. The number on top

840 of each sample represents the number of diet-related COI reads. The category other is comprised of MOTUs with < 1.0 % COI reads.

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Table 1. Trophic significance of *Crangon crangon* prey items. MOTUs shown (≥ 0.5 % mean relative abundance in the stomach samples) are assigned to the family level or lower.

Phylum	Order	Family	Species	Best identity	Presence (%)	Mean (±SE) abundance (%)	Mean (±SE) selectivity (D)	Trophic significance	Literature	Source
Annelida	Phyllodocida	Nereididae	*Hediste diversicolor*	0.98	30.8	7.1±4.5	0.9±0.0	Medium	Sto+	Lloyd and Yonge (1947); Pihl and Rosenberg (1984)
	Spionida	Spionidae	*Scolelepis foliosa*	1.00	5.8	0.7±0.6	1.0±0.0	Low	Exp[2] Sto-[2]	Van Tomme et al. (2014); Ansell et al. (1999)
	Terebellida	Terebellidae	*Pista cristata*	0.99	21.2	4.5±2.6	0.6±0.2	Medium	Sto-[2]	Ansell et al. (1999)
Arthropoda	**Amphipoda**	**Corophiidae**	***Corophium volutator***	**0.99**	**28.8**	**13.8±5.9**	**1.0±0.0**	**High**	Sto+	Pihl and Rosenberg (1984); Evans (1984)
		Pontoporeiidae	*Bathyporeia sarsi*	1.00	7.7	3.5±2.5	1.0±0.0	Medium	Exp	Van Tomme et al. (2014)
		Talitridae	*Talitrus saltator*	0.99	1.9	0.8±0.8	1.0±0.0	Low		
	Calanoida	Centropagidae	*Centropages typicus*	1.00	5.8	0.7±0.7	1.0±0.0	**Low**		
	Decapoda	**Carcinidae**	***Carcinus maenas***	**1.00**	**55.8**	**8.0±3.3**	**1.0±0.0**	**High**	Exp[1] Sto-	Moksnes et al. (1998); Raffaelli et al. (1989); Pihl and Rosenberg (1984)
		Porcellanidae	*Pisidia longicornis*	1.00	9.6	1.7±1.5	1.0±0.0	Medium		
	Diptera	Chironomidae	Unassigned	0.88	53.8	0.9±0.4	0.8±0.1	Medium	CA	Nordström et al. (2009)
		Chironomidae	*Chironomus salinarius*	1.00	1.9	1.3±1.3	1.0±0.0	Low		
	Mysida	Mysidae	*Mesopodopsis slabberi*	0.99	3.8	0.6±0.6	1.0±0.0	Low		
		Mysidae	*Neomysis integer*	0.98	13.5	2.7±2.1	1.0±0.0	Medium	Sto-	Raffaelli et al. (1989)
		Mysidae	*Schistomysis ornata*	0.98	1.9	1.6±1.6	1.0±0.0	Low	Sto+[2]	Oh et al. (2001)
	Sessilia	Austrobalanidae	*Austrominius modestus*	1.00	17.3	2.0±1.5	1.0±0.0	Medium		
Chordata	Atheriniformes	Atherinidae	*Atherina presbyter*	1.00	1.9	0.6±0.6	1.0±0.0	Low		
	Clupeiformes	Clupeidae	*Sardina pilchardus*	1.00	3.8	0.6±0.6	1.0±0.0	Low		
	Pleuronectiformes	Scophthalmidae	*Zeugopterus punctatus*	0.99	3.8	0.8±0.6	1.0±0.0	Low		
	Scombriformes	Scombridae	*Scomber scombrus*	1.00	1.9	1.0±1.0	1.0±0.0	Low		
	Spariformes	Sparidae	*Spondyliosoma cantharus*	1.00	7.7	0.6±0.4	1.0±0.0	Low		
Cnidaria	Actiniaria	Actiniidae	*Anthopleura elegantissima*	0.99	3.8	0.6±0.6	1.0±0.0	Low		

Exp: Experimental study; Sto+: Major contributor based on stomach analysis; Sto-: Minor contributor based on stomach analysis; CA: Contribution assumed by source; 1: Larvae; 2: Related taxa (same family). In bold: High trophic significant taxa. Best identity: Alignment score of the best match in the reference database.
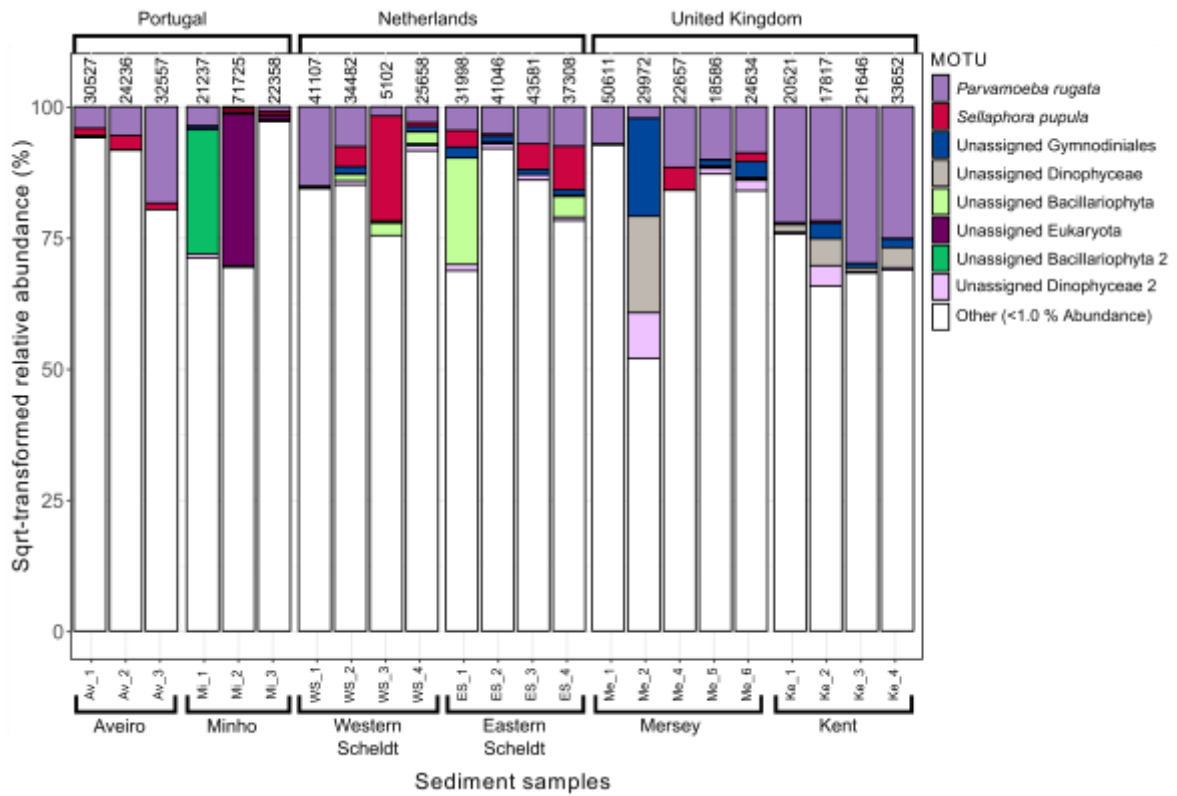
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Figure 3. Relative abundances of MOTUs detected in sediment samples by COI metabarcoding. Each bar represents one sample. Countries are shown on top of the graph, estuaries below. The number on top of each sample represents the number of COI reads. The category other is comprised of MOTUs with < 1.0 % COI reads.
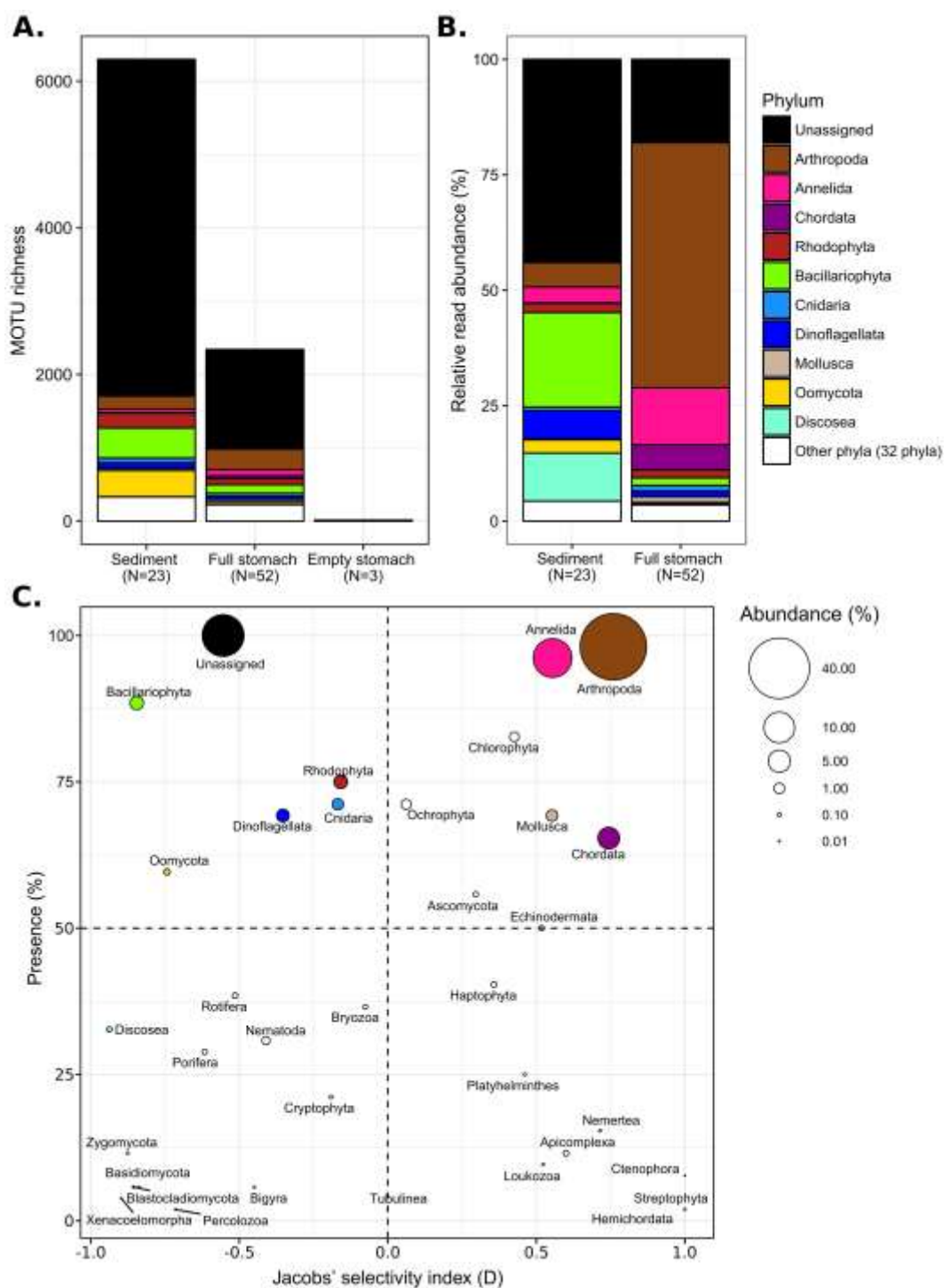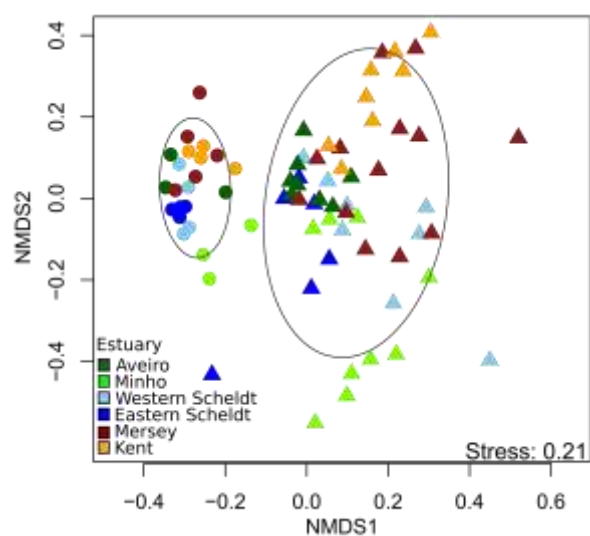
Figure 4. Phyla detected in sediment and *Crangon crangon* stomach samples by COI metabarcoding. (A) Total number of MOTU detected per phylum in sediment, full stomachs and visually empty stomachs. (B) Mean relative read abundance of phyla detected in sediment and *C. crangon* full stomach samples, after removal of *C. crangon* and *P. lilacinum* reads. (C) Phylum trophic significance based on presence (%), mean relative abundance (%) in full stomach samples and Jacobs' selectivity index. Stomach samples consisted of a pool of up to 8 stomachs. The category "other phyla" (represented in white) contains phyla with < 1% COI reads in both the sediment and full stomach samples.

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Figure 5. Multidimensional scaling analysis of MOTUs detected in sediment (dots) and *Crangon crangon* stomach samples (triangles), based on square-root transformed Bray-Curtis dissimilarities. 75% confidence ellipses are shown per sample type. WS = Western Scheldt; ES = Eastern Scheldt.
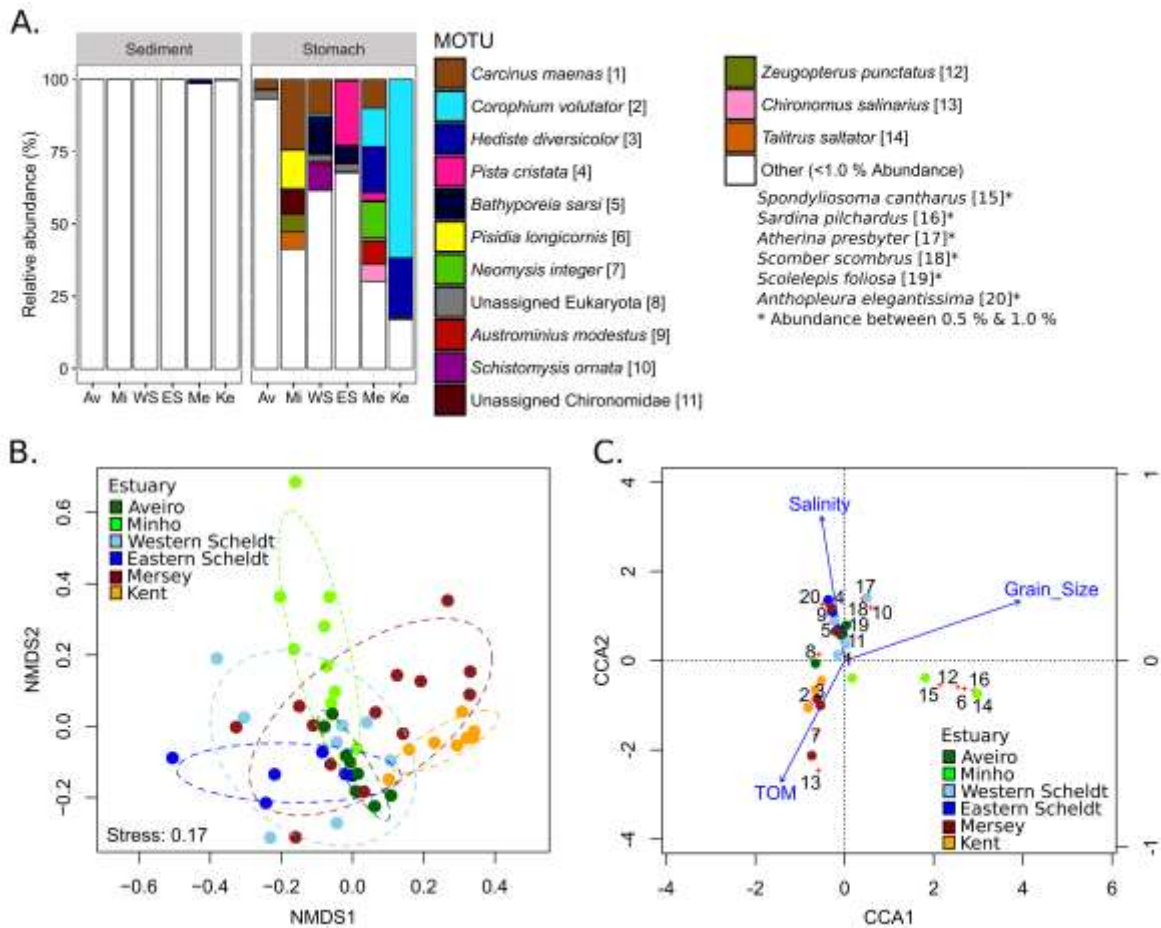
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Figure 6. Multivariate analysis of *Crangon crangon* diet in six estuaries determined by COI metabarcoding based on MOTUs (N = 20) over all stomach samples (N = 53). (A) Mean relative read abundance of each MOTU per estuary based on DNA extracted from sediment and stomach samples (after removal of *C. crangon* and *P. lilacinum* reads). MOTUs are identified for ≥ 1.0% average read abundance in the stomach samples, otherwise are referred as "Other" (B) Non-metric multidimensional scaling (nMDS) analysis based on Bray-Curtis dissimilarities of square-root transformed relative abundances in *C. crangon* stomach samples. Each dot represents one pooled stomach sample, estuaries are identified by colours (see below) and ellipses show 75% confidence intervals. (C) Canonical correspondence analysis (CCA) of square-root transformed relative read abundances in relation to salinity, total organic matter (TOM) and median grain size. Reads were averaged per site (displayed as dots) and estuaries are identified by colour (see below). Red crosses represent the MOTU scores and numbers refer to the MOTU names given in panel A.
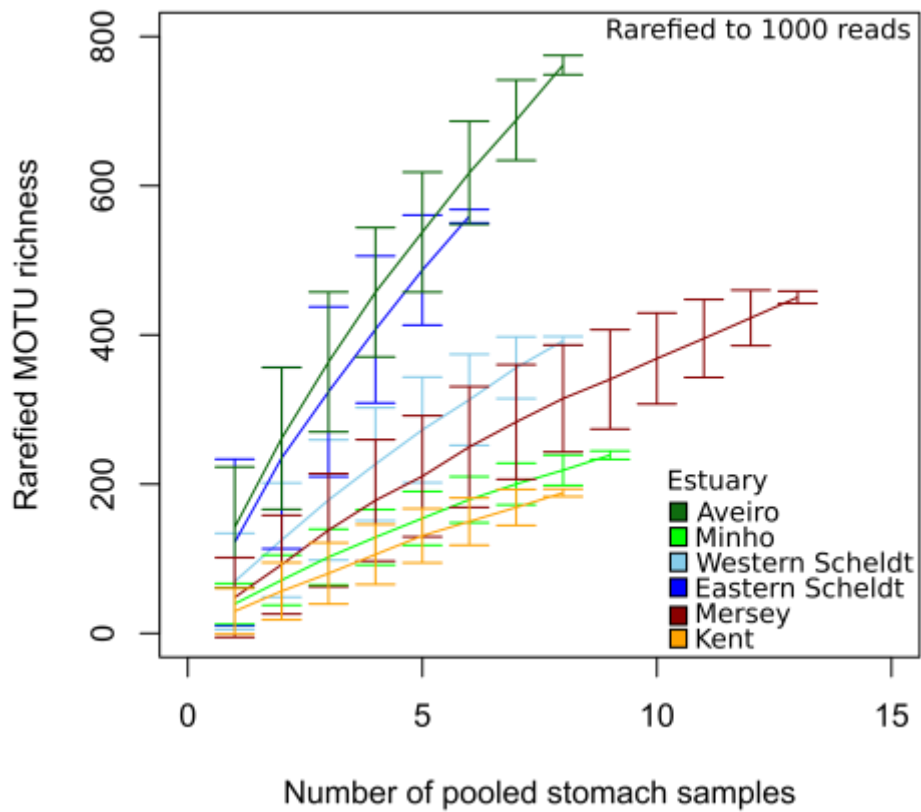
Figure 7. MOTU accumulation curves showing MOTU richness (based on all MOTUs detected) in *Crangon crangon* pooled stomach samples in several European estuaries. Each sample has been rarefied to 1000 reads prior to the construction of the accumulation curves. Stomach samples consisted of a pool of up to 8 stomachs.
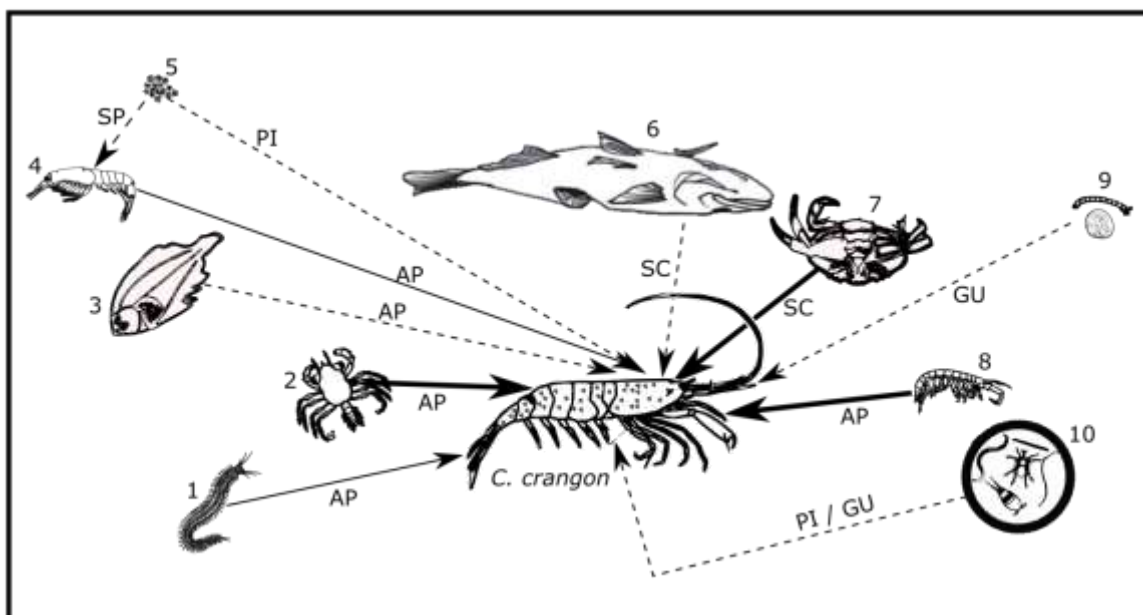
891 Figure 8. Schematic representation of the most important food items of adult *Crangon crangon* and
892 their probable method of capture/ingestion. Line thickness represents trophic significance: high
893 (bold); medium (thin); low (dashed). Numbers identify prey categories: annelids (1); decapod
894 larvae/instars (2); fish 0-year-juveniles (3); 4 mysids (4); (pico) phytoplankton (5); fish carcasses (6);
895 decapod carcasses (7); amphipods (8); chironomid, mollusc and barnacle larvae (9); meiofauna (10).
896 Letters define method of ingestion: Secondary predation (SP); ambush predation (AP); gulping
897 predation (GP); passive ingestion (PI); scavenging (SC). Images not to scale.

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