



## Niche separation between two dominant crustacean predators in European estuarine soft-bottom habitats

Andjin Siegenthaler<sup>a,\*</sup>, Owen S. Wangensteen<sup>b</sup>, Chiara Benvenuto<sup>c</sup>, Riccardo Lollobrigidi<sup>c</sup>, Stefano Mariani<sup>d</sup>

<sup>a</sup> Faculty of Geo-Information Science and Earth Observation (ITC), University of Twente, Hengelosestraat 99, P.O. Box 217, 7500 AE Enschede, The Netherlands

<sup>b</sup> Norwegian College of Fishery Science, UiT the Arctic University of Norway, Tromsø, Norway

<sup>c</sup> Ecosystems and Environment Research Centre, School of Science, Engineering and Environment, University of Salford, Salford, UK

<sup>d</sup> School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

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### ABSTRACT

Epibenthic predators in estuarine shallow soft-bottom environments are generally considered to have broad ecological niches with a wide overlap. This allows them to cope with abundant but highly variable prey communities. The assessment of trophic relationships in shallow soft-bottom habitats is, however, challenging and often complicated by the bias and low resolution of the analytical tools available to study diet in relatively small invertebrates. This study investigates niche overlap between two dominant epibenthic predators in European estuarine soft-bottom environments, the brown shrimp (*Crangon crangon* L.) and the European green crab (*Carcinus maenas* L.), by means of trophic DNA-metabarcoding with universal primers for cytochrome c oxidase I (COI). Results show differences in diet composition between the two predators, despite the fact that both species are opportunistic generalists with a high overlap in prey items (218 overlapping Molecular Taxonomic Units). The European green crab showed a richer, more even, and more geographically consistent diet than the brown shrimp, with fairly balanced consumption of algal, arthropod, annelid and mollusc food items. The brown shrimp instead preferred arthropods and annelids, and showed more spatial variation in diet. The observed niche separation could be linked to behavioural variation between the two predators, with European green crab showing more active movements over shallow soft-bottom environments compared to the brown shrimp, which regularly stays buried in the sediment, ambushing its prey. An accurate understanding of the trophic ecology of these estuarine crustaceans is important to capitalise on their role as sentinel organisms since their diet reflects local diversity and can result in toxin bioaccumulation. This study provides new insights in the predator-prey relationships and food web dynamics in European estuaries and highlights the importance of trophic DNA-metabarcoding to study marine food webs.

### 1. Introduction

Estuaries provide essential ecosystem functions and services. These include nursery and refuge for many ecologically and economically important species, coastal protection, recreational values, and carbon and nutrient recycling (Martinez et al. 2007, Hyndes et al., 2014, Sheaves et al. 2014). Estuaries are very productive but environmentally stressed systems, due to both natural and anthropogenic pressures, to which only a subset of species are adapted (Chapman and Wang 2001, Kennish 2002, Elliott and Quintino 2007, Teuchies et al. 2013). For these reasons, the study of food webs in these dynamic ecosystems is of

great ecological interest and management importance (Vinagre and Costa 2014). The shallow, sandy, soft-bottom habitats within these estuaries are notable for the high predation pressure and large temporal variation in benthic prey communities. In combination with the limited refuge opportunities in these open, dynamic and scarcely vegetated habitats, this resulted in the evolution of highly flexible predator-prey interactions (Evans 1983, Ruiz et al. 1993, Laurel and Brown 2006, Nanjo et al. 2011). Both the main prey and predator species in soft-bottom habitats are considered to have high ecological and economical relevance, either directly, as fisheries targets (Campos and Van Der Veer 2008), or as food source for shorebirds and other vertebrates of

\* Corresponding author.

E-mail address: [a.siegenthaler@utwente.nl](mailto:a.siegenthaler@utwente.nl) (A. Siegenthaler).

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conservation importance (Freitas et al. 2007). Trophic generalism is a predominant feeding strategy in these habitats (Evans 1983, Evans and Tallmark 1985), with multiple consumers competing for the same food sources (Pihl 1985). The brown shrimp, *Crangon crangon* L., and the European green crab *Carcinus maenas* L., are dominant invertebrate predators in European soft-bottom habitats (Jensen and Jensen 1985, Freitas et al. 2007, Chaves et al. 2010, Friese et al. 2021). Within European estuaries and tidal flats, they are ubiquitous, highly abundant and, in contrast to other predators, present during the whole year (Evans and Tallmark 1985, Jensen and Jensen 1985, Bamber and Henderson 1994, Polte et al. 2005). Both species are opportunistic generalists, feeding on a wide range of prey items (Chaves et al. 2010, Siegenthaler et al. 2019a, Cordone et al., 2021). Both predators can have a significant impact on prey communities, including commercially important species such as bivalves and fish (Jensen and Jensen 1985, van der Veer et al. 1998, Oh et al. 2001, Beukema and Dekker 2014), and are themselves an important food source for fish and sea birds (Walter and Becker 1997, Mendonça et al. 2007). Furthermore, *C. crangon* is a commercially important target species for fisheries (Campos and Van Der Veer 2008), and *C. maenas* is considered to be one of the worst invasive species in the world (Lowe et al. 2000), causing considerable economic damage outside its European native range (Kouba et al. 2022).

Epibenthic predators with similar diets are known to coexist in shallow soft-bottom habitats. They are assumed to have broad ecological niches with a wide overlap which allows them to cope with abundant but highly variable prey communities, which is typical for habitats characterised by low shelter availability and high predation pressure (Evans 1983, Evans and Tallmark 1985, Beukema and Dekker 2014). Also *C. crangon* and *C. maenas* are considered to fill the same ecological niche due to their omnivorous and opportunistic feeding patterns (Horn et al., 2020, van der Heijden et al. 2020). The assessment of trophic relationships in shallow soft-bottom habitats is, however, complex due to the presence of intricate predator-prey interactions which can have variable and mixed effects (Posey and Hines 1991, Cordone et al., 2021). Furthermore, most studies on the diet of *C. crangon* and *C. maenas* are performed using traditional methods which can be challenging due to the presence of soft-bodied and highly macerated prey remains (Asahida et al. 1997, Berry et al. 2015). The use of molecular techniques, such as DNA metabarcoding, is considered to be most effective and versatile for the identification of prey remains, showing a higher taxon resolution, precision and speed of analysis compared to traditional morphological methods (Pompanon et al. 2012, Berry et al. 2015). Although some recent studies analysed the diets of *C. crangon* and *C. maenas* using molecular methods (e.g. Albaina et al. 2010, Siegenthaler et al. 2019a, Siegenthaler et al. 2019b, Campos et al. 2020, Cordone et al., 2021), no direct comparison of their diets using trophic metabarcoding has been performed yet. Furthermore, the assessed impact of epibenthic predators on prey communities is not consistent between studies (e.g. Evans 1983, Pihl and Rosenberg 1984, Jensen and Jensen 1985, Feller 2006, Allouche et al. 2021), calling for a more detailed evaluation of the diet of these predators. This study aims to determine the niche overlap between *C. crangon* and *C. maenas*, by means of trophic metabarcoding using nearly universal primers for mitochondrial cytochrome *c* oxidase, at three estuaries in Western Europe. By establishing the accurate trophic role of these two key predators, this study produces improved understanding of predation and competition dynamics, contributing new ideas pertaining to the assessment and monitoring of food web structure in European estuaries.

## 2. Methods

### 2.1. Sample collection and DNA extraction

A total of 163 shrimp (*Crangon crangon*) and 26 crabs (*Carcinus maenas*) were collected, by push net, on sandy substrates in the intertidal zone from 10 sites distributed over three European estuaries. Sampling

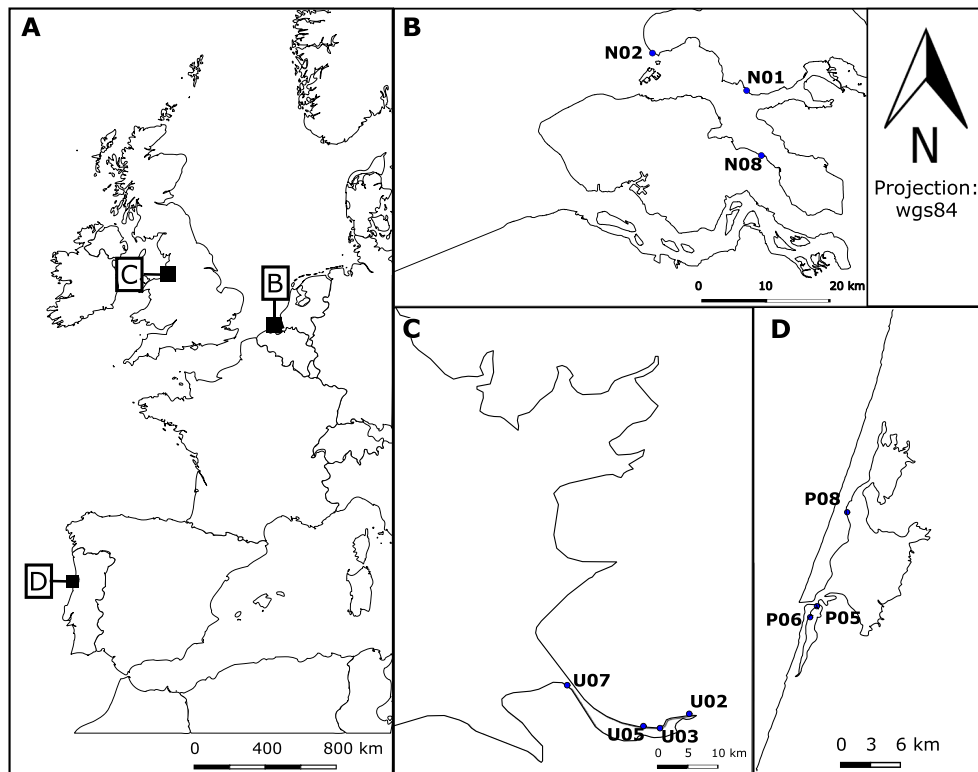
was performed in May-July 2016, during low tide ( $\pm 3$ h) at day time. The following estuaries were sampled: the mesotidal Aveiro estuary in Portugal, the mesotidal Eastern Scheldt in the Netherlands and the macrotidal Mersey estuary in the United Kingdom (Fig. 1). Environmental parameters were measured in triplicate for each site (Supplementary Table S1). Shrimp had a mean ( $\pm$ SD) total length of  $3.5 \pm 0.8$  cm and wet weight of  $0.4 \pm 0.3$  g. Crabs had a mean ( $\pm$ SD) carapace width of  $2.4 \pm 1.0$  cm and wet weight of  $4.4 \pm 5.7$  g (Supplementary Table S2). Sediment samples (one pool of three samples per site) were collected from the upper 2-cm sediment layer using a 3.2 cm  $\varnothing$  corer. Sediment was collected for the assessment of sedimental environmental DNA to characterize the biological community present at each site and to determine selectivity for specific prey items (Jacobs 1974). Shrimp and sediment samples were collected as part of a larger study concerning the trophic ecology of the brown shrimp (Siegenthaler et al. 2019a) and crabs were initially caught as by-catch but retained for this study. Samples were kept cold until transport to the laboratory, where they were stored at  $-20^\circ\text{C}$  until dissection using flame-sterilized tools. Only samples with full stomachs were kept. Sediment samples were stored in 96% ethanol and kept at  $-20^\circ\text{C}$ . DNA of stomach contents was extracted from homogenized stomach contents (0.25 g) using the Qiagen PowerSoil® DNA Isolation Kit whereas environmental DNA from sediment (10 g) was extracted using the Qiagen PowerMax® DNA Soil Kit. Crab stomachs were extracted individually while shrimp stomachs were pooled in batches of eight (up to three batches per site; Supplementary Table S3) following Siegenthaler et al. (2019a). Five pools contained less than eight shrimp due to low catches at some sites (Supplementary Table S3). Individual analysis of crab stomachs while pooling shrimp stomachs may be a cause of variation in results between the two species. Pooling samples allowed for a higher comparability between species in terms of analysed stomach biomass since the predators showed a ten-fold difference in stomach size and mass (0.007 vs. 0.07 g mean stomach weight for shrimp and crabs respectively; Supplementary Tables S2 and S3). Pooling is regularly conducted to reduce cost and to deal with heterogeneous substrates (Taberlet et al. 2012), but could cause a minor bias in alpha diversity estimates (Sato et al. 2017, Mata et al. 2019).

### 2.2. DNA amplification and high-throughput sequencing

DNA concentrations of the purified extracts were determined with a Qubit fluorometer (Thermo-Fisher Scientific) and pre- and post-PCR procedures were conducted in separate laboratories to avoid contaminations. A 313-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) region was amplified using a single set of versatile, highly degenerated primers: mICOIntF-XT (5'-GGWACWRGWT-GRACWITITAYCCYCC-3'; Leray et al. 2013, Wangenstein et al. 2018) and jgHCO2198 (5'-TAIACYTCIGGRTGICCRARAAYCA-3'; Geller et al. 2013). Eight-base oligo-tags were attached to the metabarcoding primers, in order to label different samples in a multiplexed library, and a variable number (2–4) of fully degenerate positions (Ns) were added to the amplicons during a single PCR step. The PCR mix recipe and PCR profile are described in Siegenthaler et al. (2019a). PCR products (including two negative controls) were pooled at equimolar concentration into three multiplexed sample pools and purified using MinElute columns (Qiagen). Library preparation and quantification was performed using the NextFlex PCR-free library preparation kit (BIOO Scientific) and the NEBNext qPCR quantification kit (New England Biolabs). The three libraries (final molarity of 8 pM) were sequenced on an Illumina MiSeq platform using v2 chemistry (2  $\times$  250 bp paired-ends), along with 0.7% PhiX v3 (Illumina).

### 2.3. Bioinformatic and data analyses

Raw sequence data were demultiplexed and filtered using the OBI-Tools metabarcoding software suite (Boyer et al. 2016), chimeric sequences were then removed using the uchime\_denovo algorithm



**Fig. 1.** Overview of sampled estuaries (A) and close up of the collection sites. (B) Eastern Scheldt; (C) the Mersey estuary; (D) the Aveiro estuary. Small dots within estuaries represent individual collection sites for shrimp, crab and sediment samples. Source map: OpenStreetMap.

implemented in VSEARCH (Rognes et al. 2016). Unique sequences were clustered into molecular operational taxonomic units (MOTUs) using the SWARM 2.0 algorithm (Mahé et al., 2014; Mahé et al., 2015) with a distance value of  $d = 13$ . The ecotag algorithm (Boyer et al. 2016) was used for taxonomic assignment using a local reference database (Wangensteen et al. 2018). See Siegenthaler et al. (2019a) for more details on the bioinformatic pipeline used. LULU (Frøslev et al. 2017) was used for post clustering curation, and further refinement of the data was achieved by clustering MOTUs assigned to the same species, and the removal of false positives arising from tag switching following the abundance renormalization algorithm described in Wangensteen and Turon (2017). MOTUs with less than five reads per sample were removed on a sample by sample basis (Alberdi et al., 2017; Siegenthaler et al. 2019a). Reads from bacterial, human or terrestrial origin and one MOTU for which the abundance in the PCR-negative controls was higher than 10% of the total reads of that MOTU were removed as well. Since this study focusses on food-derived reads, reads from the predator hosts,

and of known parasites (*Hypocreales* sp., *Hematodinium* sp., and *Eimeria* sp. [Apicomplexa]) were removed prior to analyses (Table 1; Molnar et al. 2012; Davies et al. 2019; Siegenthaler et al. 2019a). For simplicity, the few *Platyhelminthes* spp. reads detected (78 reads) were considered to be parasitic, even though it was not possible (due to their low taxonomic resolution) to establish whether these reads belonged to free-living or parasitic taxa (Torchin et al. 2001). The remaining food-derived read counts varied between 13 and 67,190 reads per sample (median: 5,046). Samples ( $N = 18$ ) with a low read depth ( $<1,000$ ) were removed (Siegenthaler et al. 2019a). The remaining samples were rarefied to the lowest read number (1,000 food-derived reads) to normalize sequencing depth among samples using the rarefy function of the package vegan (version 2.5–6, Oksanen et al. 2016) in R version 4.0.3 (<https://www.R-project.org/>). Removed samples were not included in the catch numbers reported earlier in this section.

All statistical analyses were performed in R with the packages vegan and metacoder (version 0.3.4; Foster et al. 2017). Alpha diversity was determined per sample type (sediment, crab stomachs, shrimp stomachs) using individual (crabs) or pooled (shrimp and sediment) samples and was represented as MOTU accumulation curves using the specaccum function (100 permutations) in vegan. The effect of sample type and estuary on trophic community structure was tested using PERMANOVA (Jaccard similarities, using 999 permutations). Site ID was included as a random factor in this analysis to account for spatial-pseudoreplication within sites. For all other analyses, samples were combined per site (based on read averaging) to correct for variation in sample numbers (Supplementary Table S3). The effect of sample type and estuary on trophic community structure was further determined using nonmetric multidimensional scaling (nMDS) and Mantel tests (999 permutations), based on Jaccard similarities. Diet overlap between the two predators was studied by plotting the trophic significance – which combines relative read abundance, detection frequency and the Jacobs' selectivity index (Siegenthaler et al. 2019a) of phyla detected in the predator's stomachs – and by plotting heat trees to highlight significant differences

**Table 1**

Mean proportion (%) of predator and parasitic reads in crab (*C. maenas*) and shrimp (*C. crangon*) stomach samples.

Sample type	Taxon	Type of reads	Mean read %	SD
Crab	<i>C. maenas</i>	Predator	79.01	25.92
Crab	<i>Eimeria</i> sp. (Apicomplexa)	Parasite	0	0.01
Crab	<i>Hematodinium</i> sp.	Parasite	1.2	7.24
Crab	<i>Hypocreales</i> sp.	Parasite	0.05	0.08
Crab	<i>Platyhelminthes</i> spp.	Parasite	1.01	4.36
Shrimp	<i>C. crangon</i>	Predator	37.7	31.93
Shrimp	<i>Eimeria</i> sp. (Apicomplexa)	Parasite	0.14	0.7
Shrimp	<i>Hematodinium</i> sp.	Parasite	0.04	0.15
Shrimp	<i>Hypocreales</i> sp.	Parasite	32.99	34.87
Shrimp	<i>Platyhelminthes</i> spp.	Parasite	0	0.01

in relative read abundances of consumed prey taxa between the two predators over multiple taxonomic levels (Foster et al. 2017). Differentially abundant families in the diet of the two predators were identified using linear discriminant analysis effect size (LEfSe) analysis (<https://huttenhower.sph.harvard.edu/galaxy/>) using  $\alpha = 0.05$  (factorial Kruskal-Wallis test) and minimum logarithmic LDA score = 3 (Segata et al. 2011).

### 3. Results

#### 3.1. Data processing

A total of 3,831,640 reads and 8,239 MOTUs were retained after the bioinformatic pipeline and manual curation. A further 1,315 MOTUs were discarded during LULU post clustering curation and 18,618 reads and 495 MOTUs were removed since they contained less than five reads on a sample-by-sample basis. Shrimp samples contained on average 38% predator (*C. crangon*) reads while crab samples contained on average 79% predator (*C. maenas*) reads. Mean percentage of suspected parasite reads was higher in shrimp samples than in crab samples (Table 1). In total, 73.7% of all reads were classified as either predator or parasite reads and were removed prior to further processing. Shrimp and crab samples did not vary significantly in the number of reads per sample (Wilcoxon rank sum test:  $W = 229$ ,  $p = 0.2449$ ). Sediment samples contained a higher number of reads than the shrimp and crab samples (Supplementary Table S4), mainly due to the fact that no predator or parasite reads had to be removed. Rarefaction resulted in the further removal of 18 samples which failed to reach the threshold of 1,000 food-derived reads. The final dataset contained 3,781 MOTUs and 58,000 reads.

MOTU richness depended on sample type with MOTU accumulation curves showing higher richness in sediment compared to predator samples (Fig. 2; Supplementary Table S5), and crab stomach samples were richer in rarefied prey MOTUs (mean  $\pm$  SD =  $207 \pm 81$ ) than shrimp stomach samples ( $89 \pm 79$ ; Wilcoxon rank sum test:  $W = 86$ ,  $p < 0.001$ ). Also after adjustment for total weight of the extracted stomach contents per sample, rarefied prey MOTU richness was significantly higher (ANCOVA:  $F_{1,45} = 29.32$ ,  $p < 0.0001$ ) in individual crab samples compared to pooled shrimp samples. The number of taxa that could not be assigned to any phylum was higher in the crab stomach samples than

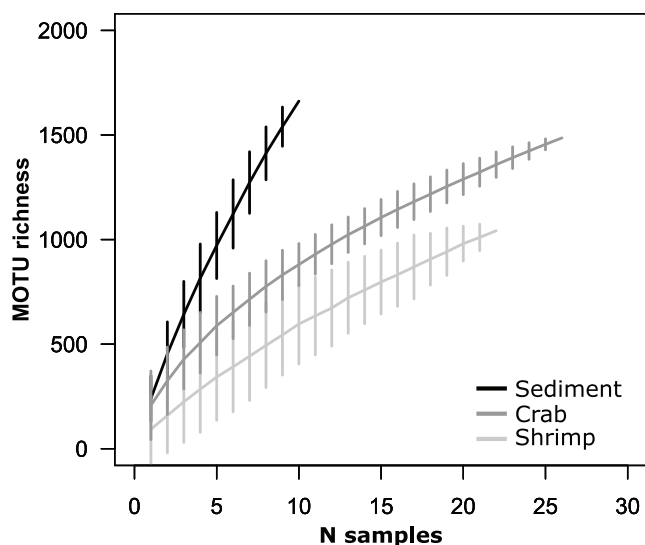


Fig. 2. Sample-based MOTU accumulation curves based on 100 permutations of random sample addition. Shrimp (*C. crangon*) samples are pooled by eight, sediment samples are pooled by three, and crab (*C. maenas*) samples are single individuals. All samples are rarefied to 1,000 reads. Error bars show 95% confidence intervals.

the shrimp stomach samples (Fig. 3B). A total of 218 MOTUs were shared by both predators. Only five of those shared MOTUs were abundant ( $>1\%$ ) in the sediment samples (2 Dinoflagellata, 1 Bacillariophyta, 1 Discosea and 1 unassigned Eukaryota). The most abundant prey items, in either crab or shrimp stomach samples are shown in Table 2.

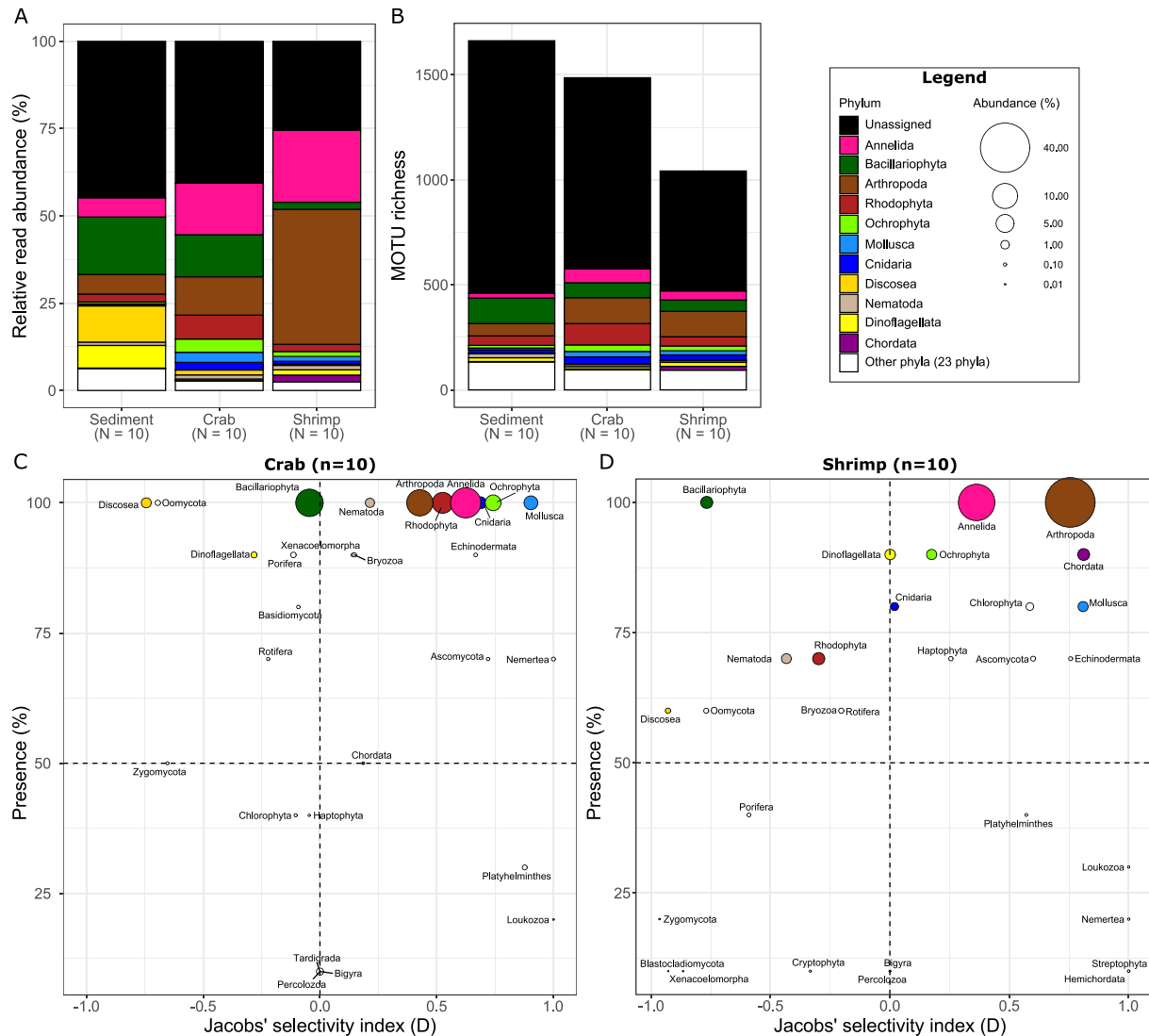
#### 3.2. Crab and shrimp diet overlap

Multivariate analysis on the stomach contents showed clear differences in dietary communities between the two species (Fig. 4). PERMANOVA (Jaccard similarities) analysis showed significant variation in diet community between species (pseudo- $F_{1,42} = 8.76$ ,  $p = 0.001$ ) and estuaries (pseudo- $F_{2,42} = 1.35$ ,  $p = 0.001$ ). There was a potential interaction effect between species and estuaries, but this effect was non-significant (pseudo- $F_{2,42} = 1.31$ ,  $p = 0.079$ ), possibly due to the low number of sites sampled per estuary. Shrimp samples showed a larger dispersion in the nMDS plot (Fig. 4) compared to the crab samples. Mantel test (based on Jaccard similarities) results showed no significant correlation between the prey community structure detected in the pooled shrimp and crab stomach samples ( $r = -0.15$ ,  $p = 0.59$ ). Stacked bargraphs showed considerable variation in the relative contribution of the different prey phyla between estuaries for the brown shrimp, while the diet of the European green crab was more consistent across estuaries (Fig. 5). Also a more detailed analysis of the spatial variation in the most abundant prey taxa (Supplementary figure S1) shows large variation in shrimp prey communities within and between estuaries, compared to a much more geographically consistent diet for *C. maenas*.

Arthropods and annelids were important prey items for both predators, in terms of relative abundance (Fig. 3A) and MOTU richness (Fig. 3B). The relative contribution of arthropod reads was significantly higher (Wilcoxon signed-rank test:  $W = 55$ ,  $P < 0.01$ ) in shrimp stomach samples (mean  $\pm$  SE:  $39 \pm 8\%$ ) compared to crab stomach samples (mean  $\pm$  SE:  $11 \pm 1\%$ ). Shrimp stomach samples also contained a significantly higher mean relative abundance of chordate (teleost) reads ( $2 \pm 2\%$  vs.  $0 \pm 0\%$ ; Wilcoxon signed-rank test:  $W = 44$ ,  $P < 0.05$ ). Crab diet, on the other hand, was more omnivorous with a significantly higher relative contribution of Bacillariophyta ( $12 \pm 2\%$  vs.  $2 \pm 1\%$ ; Wilcoxon signed-rank test:  $W = 0$ ,  $P < 0.01$ ) and Rhodophyta ( $6 \pm 1\%$  vs.  $2 \pm 1\%$ ; Wilcoxon signed-rank test:  $W = 0$ ,  $P < 0.01$ ). Relative read abundance of Ochrophyta was also higher in crab stomach samples compared to shrimp stomach samples, but this difference was not significant (Wilcoxon signed-rank test:  $W = 10$ ,  $P = 0.08$ ). Mollusca ( $3 \pm 0\%$  vs.  $1 \pm 0\%$ ; Wilcoxon signed-rank test:  $W = 5$ ,  $P < 0.05$ ) and Cnidaria ( $2 \pm 0\%$  vs.  $1 \pm 0\%$ ; Wilcoxon signed-rank test:  $W = 1$ ,  $P < 0.01$ ) showed a significant higher relative contribution in crab versus shrimp stomach samples. The crab's main prey phyla (based on relative abundance and Jacobs' selectivity index) were detected in samples from all sites, while for the shrimp only its most abundant prey phyla (arthropods and annelids) were regularly detected (Fig. 3C and 3D). Other phyla, such as molluscs and chordates, showed high selectivity in the shrimp diet, but were present in a lower proportion of the samples. Unicellular organisms (Bacillariophyta, Dinoflagellata and Discosea) were most likely passively consumed by both predators. They showed a low selectivity index and relatively high abundance in the environment (based on sedimentary DNA reads; Fig. 3A).

Analyses using Linear Discriminant Analysis (LDA) and heat trees captured clear differences between crab and shrimp diets (Figs. 6 and 7). Both predators consumed a large variety of arthropod taxa (supplementary figures S2 and S3), but LDA analyses showed clear differences at the family level. Multiple decapod families were distinctive for the crab diet (Diogenidae, Epialtidae, Paguridae and Xanthidae) while, with the exception of Carcinidae, no decapod families were found to be distinctive for the shrimp diet. Several amphipod families (Caprellidae, Dexaminidae and Melitidae) were found to be distinctive in *C. maenas*'s diet while others (Aoridae, Corophiidae and Ischyroceridae) were more





**Fig. 3.** Differences in prey phyla detected in sediment, crab (*C. maenas*) stomach and shrimp (*C. crangon*) stomach samples by COI metabarcoding. (a) Mean relative read abundance of phyla detected in sediment, crab stomach and shrimp stomach samples. (b) Total number of MOTUs detected per phylum in sediment, crab stomach and shrimp stomach samples (after rarefaction to 1,000 reads). (c-d) Phylum trophic significance in crab (c) and shrimp (d) stomach samples based on percentage of sites where the phylum was found (after rarefaction to 1,000 reads), mean relative abundance (%) and Jacobs' selectivity index. Stomach samples were averaged per predator species and per site (N = 10). Predator and parasitic reads were removed from the crab and shrimp samples prior to analysis. The category "other phyla" (represented in white) contains phyla with < 1% COI reads in both the shrimp and crab stomach samples. [Colour figure can be viewed online].

abundant in *C. crangon*'s diet. Annelids also showed a clear division between the shrimp and crab diets, with *C. crangon* specialising in Naididae and *C. maenas* selecting Amphinomidae and Eunicidae. The ragworm *Hediste diversicolor* was abundant in the shrimp's diet (Table 2) but was not distinctive (Fig. 7). Molluscs were mainly associated with the diet of *C. maenas*, with *Patella rustica* being the only taxon distinctive for the shrimp's diet. The cnidarian families Aiptasiidae, Olindiidae were found to be more associated with the crab diet while Campanulariidae were distinctive of the shrimp's diet. Within these families, the sea anemone *Aiptasia pulchella* (Aiptasiidae) was the only annotated species that showed significant differences in abundance between the diets of the two predators (Fig. 7). Multiple Ochrophyta and Rhodophyta families were distinctive for the crab's diet (Fig. 6). The LDA analysis determined one Rhodophyta family (Peyssonneliaceae) to be distinctive for the shrimp diet, but this family did not significantly differ in relative abundance compared to the crab's diet (Wilcoxon test  $p > 0.05$ , Fig. 7). Several distinctive Porifera families were determined by LDA, but were not further taken into consideration due to the low relative abundance of Porifera reads in general (Fig. 3 and supplementary figures S2 and S3).

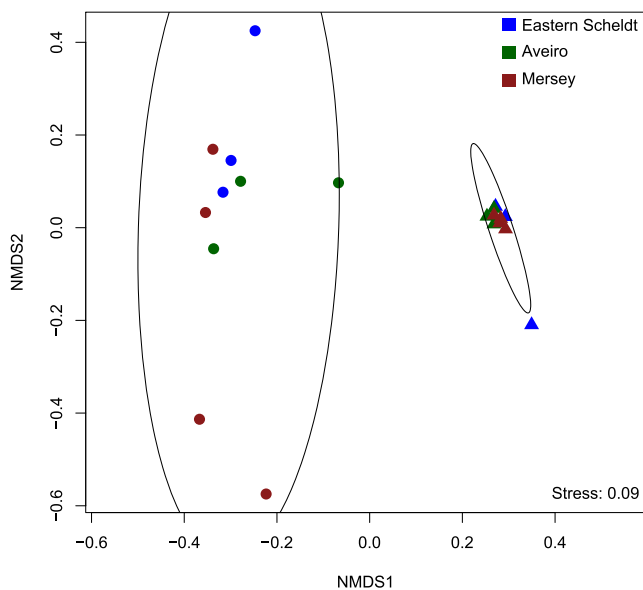
#### 4. Discussion

The European green crab (*C. maenas*) and the brown shrimp (*C. crangon*) both showed a rich diet consuming the majority of phyla present in the studied areas within European estuaries, confirming their generalist feeding strategy in soft-bottom habitats (Evans 1983, Pihl and Rosenberg 1984, Baeta et al. 2006, Cordone et al., 2021). The crab's diet was richer than the shrimp's diet, consisting especially of a notable higher diversity of algal taxa. The algal contribution to the predators' diets was likely underrepresented due to the low affinity of the COI primers for chlorophytes (Wangensteen et al. 2018). In addition to this taxon-specific bias, trophic DNA-metabarcoding has several other limitations which should be considered when interpreting these results. Examples of these biases are: false positive results due to secondary predation (taxa present in the stomach of preyed organisms) or species-specific digestion and degradation rates, primer-bias, amplification and sequencing errors, and the lack of reliable biomass estimates in metabarcoding (Deagle et al. 2010, Pompanon et al. 2012, Berry et al. 2015, Barnes and Turner 2016). Also, cannibalism cannot be detected using metabarcoding

**Table 2**

Most abundant prey MOTUs detected in crab (*C. maenas*) or shrimp (*C. crangon*) stomach samples. Prey items are shown of which the read count was at least 1% of the rarefied total read count of either all crab or shrimp stomach samples. For each prey item, the percentage of samples where the taxa was detected and relative read count (%) per sample type are shown. Shrimp samples are pooled (eight individuals), crab samples are single individuals. A dash indicates MOTUs could only be assigned to the phylum or family level. MOTUs (N = 11) not assigned to a phylum are not included.

Prey item Phylum	Family	Species	Crab stomachs Samples (%)	Reads (%)	Shrimp stomachs Samples (%)	Reads (%)
Annelida	Amphinomidae	<i>Eurythoe</i> sp.	92	4.30	5	0.06
	Eunicidae	<i>Eunice gagzoi</i>	81	1.43	0	0.00
	Nereididae	<i>Hediste diversicolor</i>	8	0.05	36	4.64
	Spionidae	<i>Spio</i> sp.	4	0.02	18	1.50
	Terebellidae	<i>Pista cristata</i>	0	0.00	23	5.58
	–	–	92	2.95	5	0.00
Arthropoda	–	–	88	1.12	0	0.00
	Caprellidae	<i>Caprella</i> sp.	96	1.16	0	0.00
	Carcinidae	<i>Carcinus maenas</i>	–	–	59	7.95
	Centropagidae	<i>Centropages typicus</i>	4	0.05	9	1.38
	Chironomidae	<i>Chironomus</i> sp.	0	0.00	5	4.06
	Corophiidae	<i>Corophium volutator</i>	0	0.00	27	7.73
	Mysidae	<i>Mesopodopsis slabberi</i>	8	0.31	9	1.25
	Mysidae	<i>Neomysis integer</i>	8	0.03	18	8.23
	Pontoporeiidae	<i>Bathyporeia sarsi</i>	0	0.00	5	2.43
	–	–	88	1.03	5	0.00
	Bacillariophyta	Bacillariaceae	–	92	2.57	5
Bacillariaceae		<i>Cylindrotheca closterium</i>	92	1.14	36	0.23
–		–	96	2.16	5	0.06
–		–	92	1.01	5	0.05
Chordata	Atherinidae	<i>Atherina presbyter</i>	0	0.00	5	2.04
Mollusca	Haminoeidae	<i>Haminoea orteai</i>	92	1.50	5	0.01
Nematoda	–	–	8	1.05	9	1.07
Ochrophyta	Dictyotaceae	<i>Canistrocarpus cervicornis</i>	92	1.57	5	0.05
Tardigrada	Echiniscoididae	<i>Echiniscoides sigmundi</i>	4	1.01	0	0.00



**Fig. 4.** nMDS of Jaccard similarities (999 permutations) of MOTUs detected in crab and shrimp stomach samples (rarefied to 1,000 reads). Replicate samples were combined per site. Circles: shrimp (*C. crangon*) stomach samples; Triangles: crab (*C. maenas*) stomach samples. [Colour figure can be viewed online].

(Berry et al. 2015) and both *C. maenas* and *C. crangon* are known to be cannibalistic. We further acknowledge that pooling and combining samples can bias prey diversity estimates, on the one hand, by under-representing rare species (Mata et al. 2019) and, on the other hand, by potentially increasing the number of prey taxa by combining individuals with different feeding preferences. Pooling does allow, nevertheless, for a more comparable biomass to be analysed per site between the two predator species (i.e. to compensate for the 10-fold difference in

stomach mass) and its effect on diversity estimates is generally considered low, especially in case of site-by-site comparisons (Sato et al. 2017, Van den Bulcke et al. 2021). Averaging multiple samples per site may also have inflated MOTU richness depending on the number of samples combined. Despite these limitations, we believe that this study provides a sound in-depth analysis of the diet of these two dominant estuarine predators and that trophic metabarcoding can be considered to be more sensitive and reliable than traditional morphological examination of stomach contents, especially in the case of small, cryptic and decomposed species which often contribute greatly to the diet of crustaceans (Pompanon et al. 2012, Berry et al. 2015, Cordone et al., 2021).

The results of this study show that the European green crab and the brown shrimp have distinct diets, even though both species are generalists and their diets show a high overlap in prey taxa. When combining relative read abundance and absence-presence data with Jacob's selectivity index calculations, *C. maenas* shows a less selective and more omnivorous diet compared to *C. crangon*. Selectivity values may, however, be overestimated since the sediment samples may not have captured a total picture of the available prey community (but see: Turner et al. 2015, Siegenthaler et al. 2019b). Crabs consume arthropod, annelid, rhodophyte, ochrophyte, mollusc and cnidarian prey items with equal preference, while the brown shrimp shows a clear preference for arthropod and annelid prey. The prey items detected in the stomachs of these predators are in line with the results of multiple studies using traditional and molecular methods (e.g. Elner 1981, Jensen and Jensen 1985, Oh et al. 2001, Baeta et al. 2006, Mendonça et al. 2007, Chaves et al. 2010, Siegenthaler et al. 2019a, Campos et al. 2020, Cordone et al., 2021). Due to their opportunistic nature, the diet of both predators can vary spatially and temporally depending on their prey availability (Chaves et al. 2010, Siegenthaler et al. 2019a, Young and Elliott 2019). European green crab diet showed a more consistent pattern over estuaries and sites compared to brown shrimp diet. The relative abundance of the shrimp's main prey taxa showed large variation between estuaries and sites, probably reflecting local variation in prey communities and environmental variables (Supplementary Table S1; Siegenthaler et al. 2019a). It should be noted that this study was conducted during one

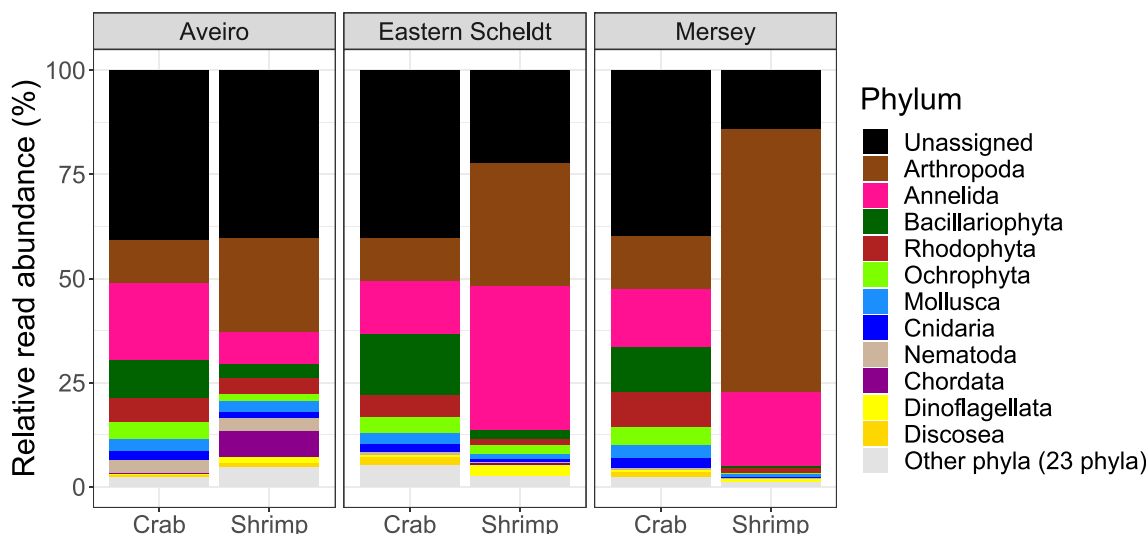


Fig. 5. Mean relative read abundance per estuary of phyla detected in individual crab (*C. maenas*) stomachs and pooled shrimp (*C. crangon*) stomach samples (eight shrimp per pool). Relative read abundance is based on 1,000 rarefied reads and averaged per estuary. [Colour figure can be viewed online].

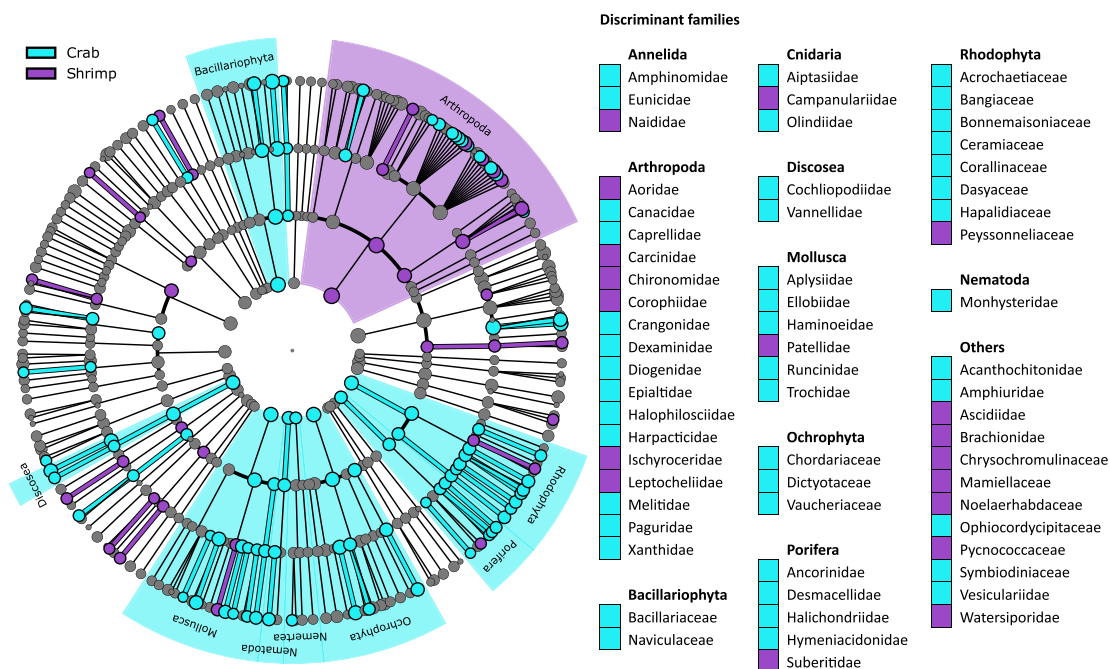
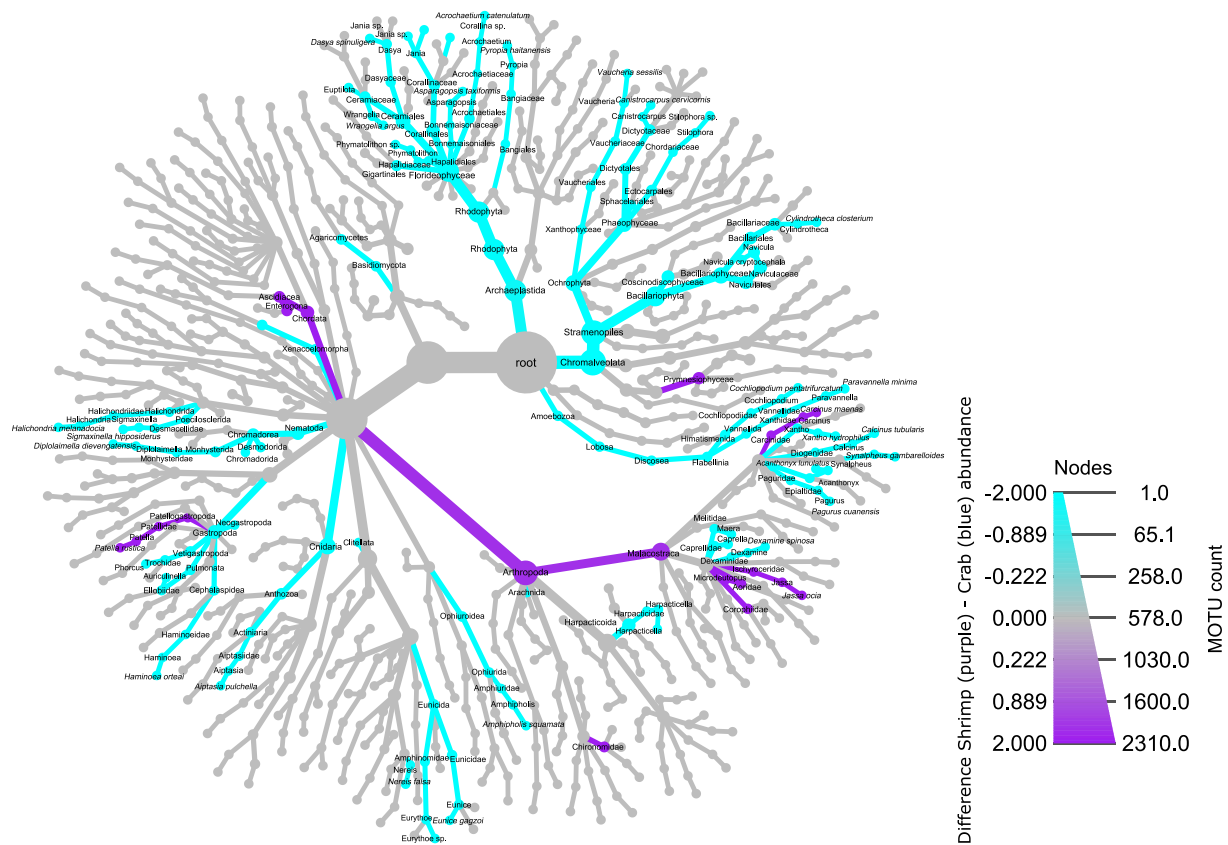


Fig. 6. LEfSe cladogram demonstrating taxonomic differences in stomach contents between crab (*C. maenas*) and shrimp (*C. crangon*) samples. Relative abundances of individual crab samples and pooled shrimp samples (pools of eight) were averaged per site prior to LEfSe analysis. The taxonomic levels detected in crab or shrimp stomach samples is represented by rings with phyla in the innermost ring and family in the outermost ring. Families and nodes highlighted in blue and purple were discriminant for crab and shrimp diets, respectively ( $P < 0.05$ ; LDA score  $> 3$ ). Grey nodes represent taxa that were not significantly differentially represented. The diameter of each node is proportional to the relative abundance of the taxon. [Colour figure can be viewed online].

season only (spring-summer 2016) while crustacean predators are known to show seasonal variation in their diet (Campos and Van Der Veer 2008), which may influence the extent of the observed niche separation. A detailed study of geographical or temporal variation was, however, not a goal of this study. Samples were collected at multiple estuaries to capture as much variation in diet as possible but did not aim to capture inter-estuary or inter-seasonal variation.

The brown shrimp and the European green crab show distinct feeding behaviour which could explain the observed differences in diet. The brown shrimp utilizes a wide range of feeding strategies, including ambush predation, foraging in open water, and scavenging (Siegenthaler et al. 2019a), resulting in wide variation in the composition of

its stomach contents (Supplementary fig. S1). Feeding strategies and food selection are also known to vary with food availability, season and shrimp size (Pihl and Rosenberg 1984, Oh et al. 2001). During daytime, *C. crangon* mainly obtains its food as an ambush predator (Gibson et al. 1995), capturing prey while hiding camouflaged and buried just below the sediment surface (Pinn and Ansell 1993, Siegenthaler et al. 2018). The annelid and small crustacean prey items in shrimp’s diet, e.g. *Hediste diversicolor* and Corophiidae, are species typically found on intertidal soft-bottom substrates (Meadows 1964, Scaps 2002) and are likely susceptible to ambush predation. Larger prey species such as fish and decapods are likely caught as larvae/juveniles or consumed from carcasses found during tidal migrations or night time movements



**Fig. 7.** Heat tree summarising differences in relative read abundance of taxa detected in crab (*C. maenas*) and shrimp (*C. crangon*) stomach samples over different taxonomic levels. Nodes highlighted in blue and purple showed a significant higher relative abundance ( $P < 0.05$ ; Wilcoxon rank-sum test followed by Benjamini-Hochberg correction) in crab and shrimp stomach samples, respectively. Grey nodes represent prey taxa that do not differ significantly in relative abundance between the two predators. Relative abundances of individual crab samples and pooled shrimp samples (pools of eight) were averaged per site prior to analysis. Node size varies with MOTU richness. [Colour figure can be viewed online].

(Siegenthaler et al. 2019a). The European green crab, on the other hand, actively moves around and can visit multiple different habitats (e.g. sandy, vegetated, rocky) within a brief timeframe (Edwards 1958, Powell 1962). In intertidal habitats, *C. maenas*' food consumption increases with rising water level when some of its prey taxa moves with the tidal cycle while the *C. crangon*'s gut fullness is highest during high tide when the environmental conditions are most stable for burying (Feller 2006). Due to its fast active movements and strong claws, *C. maenas* is capable of catching large motile decapod prey items such as crabs and hermit crabs. Its claws also allow it to predate on hard-shelled organisms such as molluscs (Wilcox and Rochette 2015). Our data clearly show that they are also largely used to manipulate vegetation and other sessile organisms. Due to its active movements, *C. maenas* can encounter a wider diversity of prey items, including sessile food items such as macroalgae, sea anemones and sponges (Mascardo and Seed 2001). Many of the smaller prey species detected in the green crab's stomachs, e.g. caprellids, bryozoans and the tardigrade *Echiniscoides sigismundi*, are associated with macroalgae and were most likely consumed while foraging in vegetated habitats (Green 1950, Manríquez and Cancino 1996, Woods 2009). The European green crab's active predation strategy and access to food sources in adjacent habitats results in a rich and remarkably consistent diet between sample sites (Figs. 4 and 5 and S1). The brown shrimp is restricted to soft bottom substrates and can show a high site fidelity (Pinn and Ansell 1993, Friese et al. 2021). As a result, its diet varies more according to local variation in the prey community (Figs. 4 and 5 and S1; Siegenthaler et al. 2019a). The brown shrimp's diet can hence be considered as a good indicator for local biodiversity assessments (Siegenthaler et al. 2019b).

This study shows that epibenthic crustacean predators with a wide

diet overlap can show clear niche separation related to variation in feeding behaviour. An accurate understanding of trophic interactions is essential for the management and conservation of estuarine ecosystems since these interactions form the basis of ecological networks and are essential for ecosystem functioning (Raffaelli 2006, Clare 2014, van der Heijden et al. 2020). Estuaries are important nursery and feeding grounds for many animals, and are thus intrinsically linked with the adjacent marine ecosystems (Mendonça et al. 2007, Chaves et al. 2010, Sheaves et al. 2014). In addition to their ecosystem services, estuaries are some of the most anthropogenic impacted areas in the world and bioaccumulation of contaminants such as heavy metals are a well known problem in estuarine predators (Dauvin 2008). Crustaceans are considered to be potential sentinel organisms of environmental quality in estuarine environments. They are generally abundant, easy to collect, readily bioaccumulate toxins and their opportunistic diet provides a good reflection of local biodiversity (Stentford and Feist 2005, Siegenthaler et al. 2019b). Assessing the trophic interactions in these estuaries is thus essential for modelling anthropogenic impacts (Loizeau et al. 2001). Trophic metabarcoding can complement traditional and environmental DNA based assessments of estuarine biodiversity, especially regarding the identification of finely macerated prey remains (Asahida et al. 1997, Siegenthaler et al. 2019b). Cordone et al. (2021) showed, for example, the power of combining complementary techniques (metabarcoding, traditional visual identification, and stable isotope analysis) when assessing the diet and environmental impact of invasive green crabs. Metabarcoding provided quantitative information about diet richness while stable isotope analysis was essential for determining species trophic levels and trophic pathways. Visual assessments of prey remains complemented metabarcoding information



by ruling out cannibalism and highlighting limitations of the metabarcoding data. Metabarcoding data can also be effectively combined with stable isotope data in order to build food webs; the metabarcoding data provides information on food web size and complexity, and the stable isotope data is used for trophic niche determination (Compton et al. 2019). A complementary approach of trophic metabarcoding and traditional techniques can also have implications for conservation management. The power of diet metabarcoding could be harnessed in these ubiquitous organisms to detect and track the occurrence of rare or invasive species, which may escape other more traditional survey methods. Furthermore, given the considerable interest in *C. maenas*' impact as an invasive alien species (Grosholz and Ruiz 1996), the broad diet shown by this species in its native habitat may help to explain its success as invasive species. Overall, our study provides a framework for the coexistence of these two competitive predators (Lagardère 1982, Young and Elliott 2019) in estuarine soft-bottom habitats, and allows for the re-evaluation of estuarine predatory interactions, which is essential to properly assess and eventually predict ecosystem changes in estuaries and adjacent ecosystems.

#### CRedit authorship contribution statement

**Andjin Siegenthaler:** Conceptualization, Methodology, Investigation, Writing – original draft. **Owen S. Wangenstein:** Conceptualization, Software, Methodology. **Chiara Benvenuto:** Conceptualization, Funding acquisition, Writing – review & editing. **Riccardo Lollobrigidi:** Investigation. **Stefano Mariani:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2022.108839>.

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