Using molecular and stable isotope markers to identify the main predators of *Nephrops norvegicus* in Mediterranean deep-water ecosystems

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ABSTRACT: To obtain a better understanding of the functioning of ecosystems and how they respond to disturbance, it is necessary to identify the relevant biotic interactions and specific trophic roles. Predation is one of the most important biotic interactions that can also define the spatial patterns of other species. Many predators are considered key species for the functioning and maintenance of ecosystems, as they play an important ecological role that can influence the dynamics at lower trophic levels. The Norway lobster *Nephrops norvegicus* is one of the most valued European fishing stocks. However, its value and capture have declined over the last decade. In the Atlantic Ocean, Atlantic cod *Gadus morhua* is the main predator of *N. norvegicus*. However, this species is not present in the Mediterranean Sea, and little is known about which species might prey on *N. norvegicus* in this area. Here, we combine 2 methodologies — genetic identification of stomach contents and stable isotope analyses — to identify, for the first time, the main predators of *N. norvegicus* in the Mediterranean Sea. Moreover, we have created the Predation Index, which determines the most influential predator affecting *N. norvegicus* population dynamics. Our results reveal that the major predators are the cephalopods *Sepietta* spp. and *Abralia veranyi*, which probably affect the early stages of *N. norvegicus*, followed by the elasmobranch *Scyliorhinus canicula* and the bony fishes *Merluccius merluccius*, *Trigla lyra*, and *Conger conger*. To evaluate possible fluctuations in the *N. norvegicus* population, we consider the assessment of these predator populations crucial.

KEY WORDS: Ecological role · Feeding ecology · Fisheries · Food web · Norway lobster · Predation Index · Prey DNA · Resource management · Trophic ecology

1. INTRODUCTION

Determining the trophic role of a particular organism is pivotal to understanding its ecological function in the ecosystem and to designing effective management actions. This involves identifying the environmental factors and biotic interactions that could influence or play an essential role in population dynamics and species distribution (D'Amen et al. 2018). Recognizing these factors could also be useful for predicting how marine ecosystems will respond to changing environmental conditions (Wisz et al. 2013). Among biotic interactions, predator–prey interactions are key in affecting the spatial patterns of species (Ritchie et al. 2012). Predators play an important role in all ecosystems, influencing the dynamics of species at lower trophic levels (Estes 1996, Ritchie & Johnson 2009).

Ecosystem modelling, which also considers consumer–prey interactions, is being widely used for

ecosystem-based fishery management (Buchary et al. 2002). This holistic approach includes the type and magnitude of the species interactions involved, analyzing biomass and abundance at several trophic levels from plankton to apex predators (Fogarty 2014). This entails a prior understanding of which prey–predator interactions exist in the environment to be managed. Similarly, in the assessment of the effectiveness of marine protected areas (MPAs), evaluating predator densities is crucial, even in small MPAs, as an increase in predators could prevent the restoration of overexploited species (Clements & Hay 2017) or a lack of them could lead to habitat regime shifts (Daskalov et al. 2007). Nevertheless, the trophic role that individual species play within marine ecosystems is still often unclear, precluding the prediction of the consequences of their removal or recovery. In the case of ecosystem-based fisheries management, a lack of knowledge of biotic interactions could lead to a misinterpretation of the consequences of overfishing, resulting in inconsistent trophic patterns constraining the viability of stocks and their predators (Shackell et al. 2010).

Despite the importance of this ecological information, there is still a huge knowledge gap regarding the main predators of some well-studied marine species. This is the case for Norway lobster *Nephrops norvegicus* in the Mediterranean Sea. This demersal decapod is one of the most important crustacean species for the European fishing industry, being considered an important target species across European waters. It is a common decapod crustacean distributed in the northeast Atlantic Ocean and the Mediterranean Sea, inhabiting the muddy bottoms of the continental shelves and slopes, at depths from 10 to 800 m (Johnson et al. 2013, Ungfors et al. 2013). The management of this stock involves a mixture of EU regulations (Common Fisheries Policy; CFP) and national legislation, though *N. norvegicus* stocks seem to have been fished above scientific advised levels (Sardà 1998, Letschert et al. 2021). Over the last decade the Mediterranean stocks of *N. norvegicus* from the northwestern Mediterranean Sea (Spanish waters) have declined in abundance (from 400 t in 2009 to 200 t in 2019). In the Mediterranean Sea, most management policies are not successfully enforced, as the current state of the stocks is not reviewed (Sardà 1998). To better understand the temporal changes in species abundance, distribution and biology, this approach to fisheries management needs to be changed. Moreover, to prevent possible trade-off consequences due to fluctuations of other species populations that may be associated with *N. norvegicus*, it is essential to understand their ecological role and importance as a trophic resource for predators. Despite the large amount of research conducted on *N. norvegicus*, knowledge about its main predators in the Mediterranean Sea is scarce. In the Atlantic waters, its main predator is the Atlantic cod *Gadus morhua* (Chapman 1980, Brander & Bennet 1986, Dombaxe 2002), which is distributed within a similar depth range (Johnson et al. 2013). In contrast, information about its main predators in the Mediterranean Sea, where *G. morhua* is not present, is limited to only a few studies that suggest that cephalopods, some demersal elasmobranchs, and the bony fish *Conger conger* could be important (Coll et al. 2006, El-Amine Abi-Ayad et al. 2011, Šantić et al. 2012, Navarro et al. 2014). However, describing the diet of cephalopods and elasmobranchs, and identifying specific prey using traditional stomach content analyses, is complicated because they usually have empty stomachs and, in the case of cephalopods, it is difficult to identify stomach contents due to the high efficiency of their beaks in crushing food and their rapid enzymatic stomach activity (Guerra 1978, Ibáñez et al. 2021). For this reason, the actual trophic importance of *N. norvegicus* for these predators may be underestimated.

As an alternative to stomach content analysis, the use of trophic indicators such as molecular or stable isotope analyses (SIA) could help to determine the diet composition of marine predators (Guerreiro et al. 2015, Olmos-Pérez et al. 2017). In the case of molecular techniques, it is possible to determine the presence of a specific prey by detecting its DNA in the stomach contents of a potential predator (Dunshea 2009). Also, stable isotopes of nitrogen (denoted as $\delta^{15}N$) and carbon (denoted as $\delta^{13}C$) have been used extensively to study the trophic ecology of marine predators (Shiffman et al. 2012, Navarro et al. 2013, Giménez et al. 2021). This approach is based on the fact that $\delta^{15}N$ and $\delta^{13}C$ values are transformed from dietary sources to consumers at predictable ratios (Kelly 2000): the incorporation of the carbon isotope ratio ($^{13}C/^{12}C$) of primary producers into consumer tissues and the enrichment of the nitrogen isotope ratio ($^{15}N/^{14}N$) due to the incorporation of the heavy isotope of nitrogen (^{15}N) by the consumer from its diet. Stable isotopes alone cannot completely resolve the consumption of *N. norvegicus* due to the limitation of the technique (i.e. simulated predators based on a predator that only feeds on *N. norvegicus*). Combination with other techniques, such as DNA analysis, is beneficial and enhances the interpretation of the results. A more precise interpretation

would be possible if stable isotope information on all putative prey were available to infer diet through stable isotope mixing models. The combination of different methodologies could solve complex ecological questions by providing more accurate information about the structure and functioning of marine food webs and be a useful tool to validate results. Moreover, due to the different integration time of each of these 2 methodologies, their combination could provide information about the trophic importance of a particular prey at the short-term scale (DNA integrates in days due to rapid digestion times; Aguilar et al. 2017) and the long-term scale (SIA in muscle tissues integrates ~2 to 8 mo of the food consumed by a predator, depending on the species; Vander Zanden et al. 2015). For example, in SIA of muscle tissues, the isotopic half-life ($ln(2)/\lambda$, days), defined as the time required to reach 50% equilibration with the diet, averages 47 d in the cod *G. morhua* (Ankjaerø et al. 2012), 147 d in the red rock lobster *Jasus edwardii* (Suring & Wing 2009) and 105.3 d in the leopard shark *Triakis semifasciata* (Kim et. al 2012, Malpica-Cruz et al. 2012).

In the present study, our principal objective was to identify the main predators of *N. norvegicus* in the deep-sea ecosystems (315 to 475 m depth) of the western Mediterranean Sea using both molecular

and stable isotope techniques. We also considered possible predators of the early stages of *N. norvegicus*, such as the cephalopods *Sepietta* sp. and *Abralia veranyi*, which are smaller than adult *N. norvegicus*. Moreover, we estimated the predation impact on *N. norvegicus* to portray the significance of the predator–prey interactions taking into account the predator density, which could adversely affect prey consumption (Arditi et al. 2001). For this purpose, a Predation Index (PI) combining the results of this study and the density of each predator identified in the study area was calculated to elucidate the real importance of each predator in the *N. norvegicus* fishing grounds.

2. MATERIALS AND METHODS

2.1. Fieldwork procedures

The study was conducted in the northwestern Mediterranean Sea, in a deep-sea *Nephrops norvegicus* fishing ground (300 to 500 m depth; Fig. 1). This area is composed of muddy habitats of the upper slope of the continental shelf margin, which is crossed by several submarine canyons. These submarine canyons are major geomorphological struc-
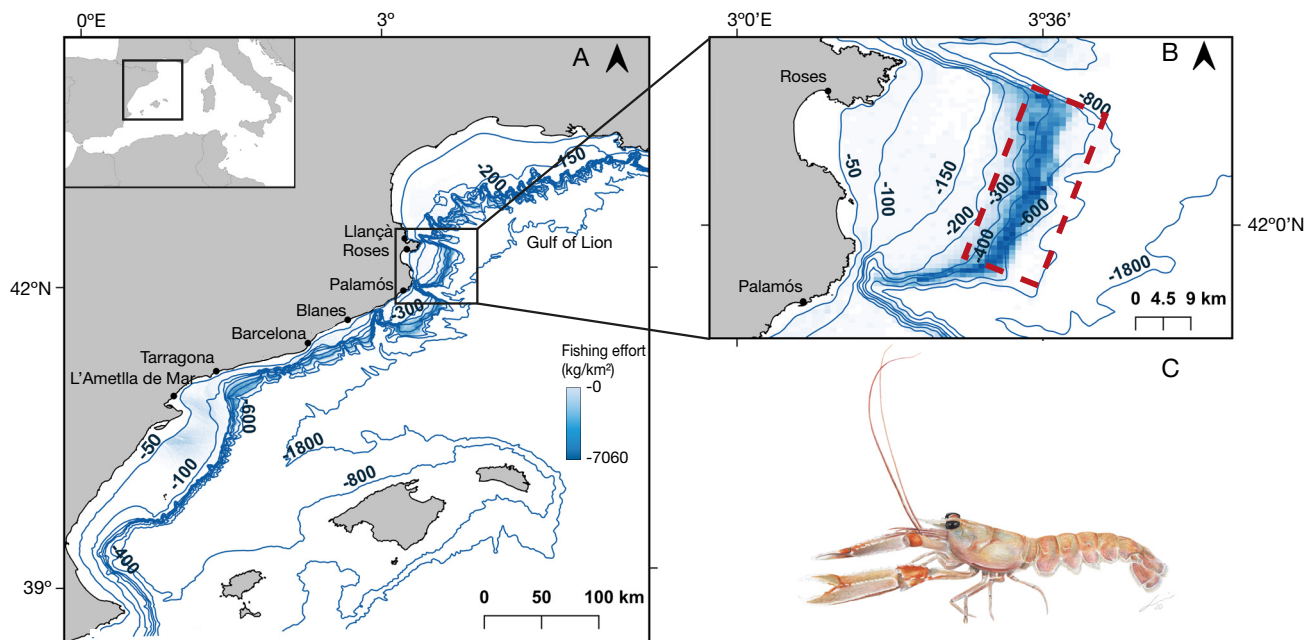

Fig. 1. (A) Study area of Norway lobsters *Nephrops norvegicus* in the northwestern Mediterranean Sea, Spain. (B) Spatial distribution of *N. norvegicus* along the Catalan coast, obtained by combining Vessel Monitoring System (VMS) information and official landing data from the time period 2005–2018 (European Commission 2002–2018, Garcia et al. 2016), and the study area (red rectangle). (C) *N. norvegicus* (illustration by Joan Mir-Arguimbau)

tures of the western Mediterranean Sea, hosting a fishing ground of great economic importance for the local and regional fisheries (Fernández-Arcaya et al. 2017).

To collect potential predators and estimate their abundance, a total of 28 experimental hauls (18 hauls in September 2017 and 10 in September 2019) were performed in this area using commercial trawling gears (square mesh size of 40 mm) covered with a net with a diamond mesh size of 12 mm to collect the maximum number of species and sizes. The headline height of the trawl was around 1.3 m, and the horizontal trawl opening was about 22 m. The total wire of the fishing trawl was between 850 and 950 m. Towing was maintained at a constant speed (2.4 to 2.5 knots) and the duration of each haul ranged between 1 h 31 min and 1 h 44 min. All hauls were conducted in local daylight, coinciding with the diurnal emergence of *N. norvegicus* on the continental slope of these deep-water habitats (Aguzzi et al. 2003, Vigo et al. 2021).

2.2. Abundance of potential predators of *N. norvegicus*

The total catch was classified into *N. norvegicus* and the different species of its potential predators. The abundances of *N. norvegicus* and the different predators were estimated considering the swept area, which is the area that the trawl net has towed, in km^2 following the equation:

$$A = V \times BT \times H \times 1852 / 10^6 \qquad (1)$$

where A is the total number of individuals per km^2, V is the average speed of the trawls (knots), BT is the towing time (h), H is the horizontal opening of the net (m) and the constant 1852 is the equivalent in nautical miles to make the conversion to km^2 (Sparre & Venema 1998).

2.3. Muscle and stomach content sampling

A total of 20 *N. norvegicus* individuals and potential predators (cephalopods [n = 133], bony fish [n = 78], and elasmobranchs [n = 38]) were collected from the fishing hauls. They were identified at the species level and frozen on board until their dissection at the Institut de Ciències del Mar (ICM - CSIC). In the laboratory, all collected individuals were weighed (in g) and measured (standard body measures in mm: mantel length [ML] for cephalopods, total length [TL],

standard length [SL], or anal length [AL] for fish species). We obtained 269 muscle samples, 20 from *N. norvegicus* and 249 from the potential predators of *N. norvegicus*, which were later frozen and preserved for stable isotopic determination. We also extracted the stomach contents of these potential predators of *N. norvegicus* and kept the contents in ethanol-sterilized sample pots.

2.4. Extraction and amplification of DNA from the stomach contents of predators

All collected stomachs were dissected using ethanol-sterilized tools and DNA extraction from the stomach contents was performed using the NZY Tissue gDNA Isolation Kit (NZYTech) following the manufacturer's instructions. DNA quality was analyzed with the Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific).

We designed primers with sequences corresponding to a mitochondrial gene from the 16S subunit of the ribosomal ribonucleic acid (following Palero et al. 2009) to identify predation only on Norway lobster. We used the program PRIMER3Plus (Untergasser et al. 2007) to design the primers: R 5′-ACG CTG TTA TCC CTA ARG TAA CTT-3′ and F 5′-GGT GTA GAT TAA GGA ATT CG-3′.

We followed the protocol of the NZYTaq 2x Colourless Master Mix (NZYTech) using a thermocycler with pre-programmed temperatures and times. The optimal PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 19 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; 20 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 30 s; and a final step at 72°C for 5 min to make sure that all DNA extracts were amplified. Finally, the sample was stored at 12°C to preserve the reaction for the short term. PCR products were visualized on 2% agarose gels prior to cloning and sequencing to verify amplicon presence and size. We analyzed the results as binary variables: the absence of bands was classified as no detection (value 0) and the presence of a band was considered detection of Norway lobster (value 1).

2.5. Stable isotopic analysis

All muscle samples (*N. norvegicus* and its potential predators) were freeze-dried and powdered, and 0.28 to 0.33 mg of each sample was packed into tin capsules. Stable isotopic analyses were performed at the Laboratory of Stable Isotopes of the Estación Bio-

lógica de Doñana CSIC (www.ebd.csic.es/lie/index. html). Samples were combusted at 1020°C using a continuous flow isotope ratio mass spectrometry system (Thermo Electron) by means of a Flash HT Plus elemental analyzer interfaced with a Delta V Advantage mass spectrometer. This applies international standards run every 9 samples: LIE-CV and LIE-PA, previously normalized with the international standards IAEA-CH-3, IAEACH-6, IAEA-N-1, and IAEA-N-2. Stable isotope ratios were expressed in the standard δ-notation (‰) relative to Vienna Pee Dee Belemnite (δ^{13}C) and atmospheric N_2 (δ^{15}N). Based on laboratory standards, the measurement errors (SD) were ±0.1 and ±0.2 for δ^{13}C and δ^{15}N, respectively. The C:N ratio of all tissues was always lower than 3.5‰, and hence, no correction of the δ^{13}C values was required to account for the presence of lipids in muscle samples (Logan et al. 2008).

To compare the differences in the δ^{13}C and δ^{15}N values between potential predators we used Kruskal-Wallis and Wilcoxon post-hoc tests, using the statistical software R version 4.0.3 (R Core Team 2020).

2.6. Identification of the potential predators of *N. norvegicus* using isotopic values

Based on the stable isotope values, we estimated the qualitative importance of *N. norvegicus* for each sampled predator. This approach consisted of determining whether the isotopic niche of a potential predator overlapped with that of a hypothetical predator (bony fish, elasmobranch, or cephalopod) that only feeds on *N. norvegicus*. If so, then we could assume that this predator includes *N. norvegicus* in its diet (see a similar approach in Popa-Lisseanu et al. 2007). We also checked whether *N. norvegicus* was segregated from the other crustaceans present in the study area. For this, we revised all the published data on the stable isotope values of the cohabiting crustaceans present in the study area, confirming that *N. norvegicus* was segregated in its stable isotope values from the other cohabiting crustaceans (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m695p095_supp.pdf).

As isotopic niche, we calculated 3 different kernel utilization density (KUD) estimators that contained 50%, 75%, and 95% of the isotopic niche of each species. KUD is estimated across a regular network of equally spaced points, with the extent of the grid larger than that of the observations (Venables & Ripley 2002, Eckrich et al. 2020). The contour lines used are defined in relation to the Euclidean distance of each observation to the centroid in bivariate space (Robinson 2021). For these predictions, different diet-to-tissue discrimination factor values based on published studies were used for cephalopods ($\Delta\delta^{13}$C = −0.20 ± 0.5‰, $\Delta\delta^{15}$N = 3.37 ± 0.95‰; Golikov et al. 2020), bony fish ($\Delta\delta^{13}$C = 1.3 ± 0.1‰, $\Delta\delta^{15}$N = 3.35 ± 0.2‰; Caut et al. 2009), and sharks/rays ($\Delta\delta^{13}$C = 0.49 ± 0.32‰, $\Delta\delta^{15}$N= 1.95 ± 0.26‰; Hussey et al. 2010). We used the rKIN package (rKIN; https://github.com/salbeke/rKIN) in R version 4.0.3 (R Core Team 2020) to compute the KUDs and calculate their overlap.

2.7. Combining molecular and trophic markers

We combined and plotted the results for the percentage of stomachs containing *N. norvegicus* DNA (% DNA) for each of the different potential predators and the 95% contour of the KUD overlap (K 95%) of the isotopic niches estimated from the isotopic values for each predator. Species with low values for both metrics should not be considered as predators of *N. norvegicus*, while species with high values could be major predators of *N. norvegicus*. Species that appear in the lower-right section of the plot (only high values of % DNA; see Fig. 5) could be casual predators of *N. norvegicus* with short-term predation importance. On the contrary, if species appear in the upper-left section of the plot (high values of K 95%, stable isotopes), they present a long-term marker, indicating that they had preyed on *N. norvegicus* in the past months (~2 to 8 mo).

2.8. Estimating the impact of each predator on the population of *N. norvegicus*

By using % DNA or K 95%, we estimated the relative importance in number (*I*) of each potential predator of *N. norvegicus* by calculating the percentage of abundance of each predator in relation to the abundance of all predators together. The abundance of predators was obtained from the fishing surveys conducted in the study area. The importance index (I_x) was obtained with the following equation:

$$I_x\,(\%) = [(PA_x/100) \times (E/100)] \times 100 \qquad (2)$$

where *PA* is the percentage of abundance of a specific predator *x* in relation to the abundance of all potential predators and *E* is the % DNA or the K 95%. For each predator, we estimated a Predation Index (PI) based on the average *I* values obtained with % DNA and K 95%.

3. RESULTS

3.1. DNA results

From the 249 stomachs of 20 potential predator species (see Table 1), *Nephrops norvegicus* DNA was detected mainly in the cephalopods *Todaropsis eblanae* (% DNA = 73.33%), *Abralia veranyi* (72.73%), *Sepietta* sp. (66.67%), *Pteroctopus tetracirrhus* (50%), *Todarodes sagittatus* (42.86%), and *Illex coindetii* (38.46%). In bony fish, *N. norvegicus* DNA was detected mainly in *Lophius budegassa* (75%), *Merluccius merluccius* (35.71%) and *Conger conger* (33.33%). In the other 13 potential predators analyzed, less than 25% of individuals had *N. norvegicus* DNA in their stomachs, and it was absent in 7 of the species (Table 2).

3.2. Stable isotope results

When comparing the stable isotopic values of *N. norvegicus* and its potential predators, we found that *N.*

norvegicus was completely segregated from them all in δ^{15}N and δ^{13}C values (Fig. 2). Among predator species, we found significant differences (Kruskal Wallis tests, $p < 0.05$) in both δ^{15}N and δ^{13}C values (Table 1, Fig. 2). The species that presented the lowest values of δ^{13}C and were the most significantly different from *N. norvegicus* (-19.91 ± 0.46‰) (based on Wilcoxon post-hoc tests, $p < 0.05$) were the bony fishes *C. conger* (-18.63 ± 0.27‰) and *Trigla lyra* (-18.86 ± 0.24‰), and the elasmobranchs *Scyliorhinus canicula* (-17.80 ± 0.48‰), *Raja* sp. (-17.80 ± 0.48‰), and *Galeus melastomus* (-18.65 ± 0.13‰). For δ^{15}N values, the predators most segregated from *N. norvegicus* (5.86 ± 0.36‰) and with the highest values of δ^{15}N (based on Wilcoxon post-hoc tests, $p < 0.05$) were the cephalopod *A. veranyi* (10.34 ± 0.56‰), the bony fish *L. budegassa* (10.51 ± 0.28‰), and the elasmobranch *S. canicula* (9.13 ± 0.23‰) (see Table 1).

Regarding the 95% contour of KUD overlap between the isotopic area of a potential predator that consumes only *N. norvegicus* and each predator species (Figs. 3 & 4), we found higher K 95% values for the elasmobranch *G. melastomus* (64.3%) and the

Table 1. Results obtained from the stable isotope analyses of each species considered as a potential predator of Norway lobster. n: number of individuals or samples; values are mean ± SD

Species	n	Body mass (g)	δ^{13}C (‰)	δ^{15}N (‰)
Crustacea				
Nephrops norvegicus	20	26.39 ± 7.52	−19.91 ± 0.46	5.86 ± 0.36
Cephalopods				
Abralia veranyi	9	4.89 ± 1.15	−19.76 ± 0.55	10.34 ± 0.56
Eledone cirrhosa	16	223.10 ± 93.71	−18.97 ± 0.28	8.27 ± 0.46
Illex coindetii	12	139.71 ± 58.03	−19.20 ± 0.30	9.74 ± 0.77
Rossia macrosoma	20	76.91 ± 31.94	−18.49 ± 0.41	8.20 ± 0.22
Octopus saluti	2	282.50 ± 307.59	−18.87 ± 0.21	7.31 ± 0.65
Pteroctopus tetracirrhus	4	412.50 ± 251.58	−18.44 ± 0.19	7.09 ± 1.12
Scaeurgus unicirrhus	2	56.36 ± 0.00	−19.15 ± 0.08	8.04 ± 0.41
Sepietta oweniana	20	9.89 ± 14.30	−19.33 ± 0.17	9.55 ± 0.24
Sepietta spp.	18	5.20 ± 2.20	−19.32 ± 0.22	9.57 ± 0.27
Todarodes sagittatus	9	205.40 ± 306.10	−19.24 ± 0.30	9.85 ± 0.85
Todaropsis eblanae	15	121.37 ± 35.19	−19.38 ± 0.23	9.46 ± 0.37
Bony fish				
Conger conger	29	197.29 ± 229.51	−18.63 ± 0.27	9.30 ± 0.44
Lepidopus caudatus	2	46.00 ± 0.00	−20.16 ± 0.00	9.35 ± 0.00
Lophius budegassa	4	435.00 ± 189.47	−18.62 ± 0.32	10.51 ± 0.28
Merluccius merluccius	14	353.14 ± 124.38	−19.01 ± 0.19	9.79 ± 0.33
Molva macrophtalma	10	29.90 ± 7.47	−19.24 ± 0.19	10.12 ± 0.41
Trigla lyra	18	79.61 ± 12.44	−18.86 ± 0.24	9.46 ± 0.37
Elasmobranchs				
Galeus melastomus	10	15.44 ± 4.21	−18.65 ± 0.13	8.43 ± 0.21
Scyliorhinus canicula	30	207.83 ± 102.86	−17.80 ± 0.48	9.13 ± 0.23
Raja clavata	1	260.00	−17.76	9.08
Raja polystigma	3	77.50 ± 10.61	−18.05 ± 0.64	7.85 ± 1.19
Raja spp.	2	46.00 ± 0.00	−17.67 ± 0.29	8.71 ± 0.32

Table 2. Results obtained from the DNA determination and overlap between the isotopic niches of the potential predators and the simulated predator generated from the *Nephrops norvegicus* isotopic values. K 95 %: 95 % contour of the isotopic area overlap between the simulated predator and the species analyzed in kernel utilization densities; % DNA: percentage of individuals that contained Norway lobster DNA in their stomach; *A*: abundance (ind. km^{-2}) in the study area; PI: Predation Index value based on % DNA and K 95 % values of each species (see Section 2.8.)

Species	K 95 %	% DNA	*A*	PI (%)
Cephalopods				
Abralia veranyi	30.3	72.7	274	4.94
Eledone cirrhosa	34.5	0	11	0.04
Illex coindetii	23.9	38.4	59	0.76
Rossia macrosoma	8	19.5	35	0.16
Octopus saluti	0	0	2	0
Pteroctopus tetracirrhus	22.8	50	0	0
Scaeurgus unicirrhus	9.9	0	2	0
Sepietta oweniana	55.9	15	848	6.42
Sepietta spp.	58.1	66.7	233	4.45
Todarodes sagittatus	25.9	42.9	5	0.08
Todaropsis eblanae	22.1	73.3	15	0.30
Bony fish				
Conger conger	25	33.3	21	0.19
Lepidopus caudatus	14	0	0	0
Lophius budegassa	0	75	13	0.21
Merluccius merluccius	0	35.7	161	1.21
Molva macrophtalma	0	0	77	0
Trigla lyra	15.8	0	393	0.49
Elasmobranchs				
Galeus melastomus	64.3	10	91	0.75
Scyliorhinus canicula	6.4	9.1	762	1.8
Raja spp.	21.8	0	1	0

cephalopods *Sepietta* spp. (58.1 %) and *S. oweniana* (55.9 %), representing the species most likely to be specialized predators of *N. norvegicus*. Other predators that presented relevant overlap values were the cephalopod species *Eledone cirrhosa* (34.5 %), *A. veranyi* (30.3 %), and *Todarodes sagittatus* (25.9 %) (Table 2). The K 95 % of the other 14 sampled predators, including all bony fishes and the rest of cephalopods and elasmobranchs, was lower than 25 % (Table 2, see Figs. 3 & 44).

3.3. Short- and long-term markers of *N. norvegicus* in the diet of its predators

The potential predators *Sepietta* spp. and *A. veranyi* presented high values for both % DNA and K 95 % (Fig. 5A). For other predators, such as *G. melastomus*, the trophic marker of *N. norvegicus* was high for K 95 % and low for % DNA (Fig. 5A), while some, such as *L. budegassa*, presented high % DNA values and low K 95 % values (Fig. 5A).

3.4. Abundance of predators in the study area and their estimated predation impact on *N. norvegicus*

The most abundant predators in the *N. norvegicus* grounds were the cephalopods *Sepietta oweniana* (848 individuals [ind.] km^{-2}), *A. veranyi* (274 ind. km^{-2}), *Sepietta* spp. (233 ind. km^{-2}), the bony fish *Trigla lyra* (393 ind. km^{-2}), and the elasmobranch *Scyliorhinus canicula* (762 ind. km^{-2}) (see Table 2).

The PI, which incorporates the importance of % DNA and K 95 % in relation to the relative abundance of each potential predator in the environment, was higher for the cephalopods *Sepietta oweniana* (6.42 %), *A. veranyi* (4.94 %), and *Sepietta* spp. (4.45 %), followed by the shark *Scyliorhinus canicula* (1.81 %) and the bony fish *M. merluccius* (1.21 %) (see Table 2, Fig. 5B).

4. DISCUSSION

In the present study, we identified the main predators of *Nephrops norvegicus* in the deep-sea Mediterranean waters combining 2 methodologies: genetic determination of stomach contents and stable isotope analysis. Our results demonstrate that the major predators of *N. norvegicus* are the cephalopods *Sepietta* spp., as they presented high values using both methodologies. Considering only the short-term marker (% DNA), which consists of the identification of *N. norvegicus* DNA in the stomach contents (i.e. recent consumption), we also identified the bony fish *Lophius budegassa* as a major predator. Considering only the long-term marker (K 95 %), which reflects the assimilation in the muscle tissues of *N. norvegicus* consumed a few months ago, we identified the elasmobranch *Galeus melastomus* and the cephalopod *Sepietta oweniana*. Moreover, according to the estimated PI, which also considers the abundance of the predators in the *N. norvegicus* grounds, the main predators are still *Sepietta* spp. and *S. oweniana*, followed by *Abralia veranyi* (see Fig. 5C). These results are useful for understanding prey–predator interactions and their ecological role in *N. norvegicus* grounds, and could also provide pivotal information for ecosystem-based fisheries management, as well as for evaluating the
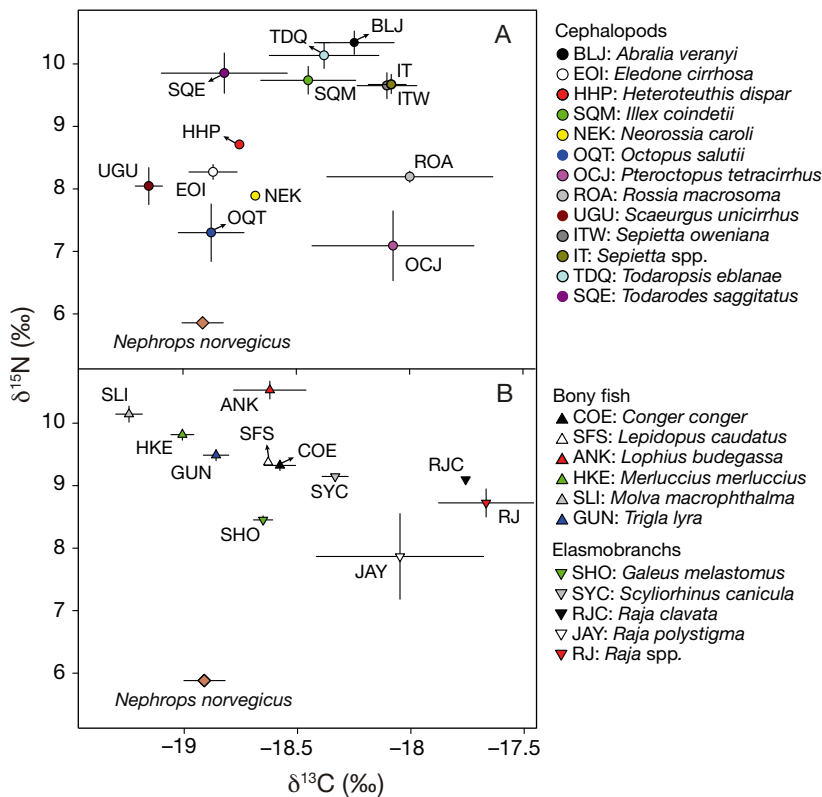
Fig. 2. Mean and standard error of δ15N and δ13C values of *Nephrops norvegicus* and potential predators sampled in the northwestern Mediterranean Sea. (A) Cephalopods; (B) bony fish

effectiveness of marine protected areas for the conservation of demersal species.

Using genetic analyses of stomach contents, we identified species that had recently preyed on *N. norvegicus*, while stable isotope analyses from muscle tissue showed the assimilation of all the prey consumed by predators in recent months (Boecklen et al. 2011). As expected, all potential predators showed higher δ15N values than *N. norvegicus,* reflecting its lower position in the food web. There was isotopic variability among predators, reflecting different trophic habits. For example, within the cephalopods, we found species that occupy higher trophic levels, such as *A. veranyi* that can prey on decapods, larger fish, and other cephalopods (Guerra-Marrero et al. 2020), whereas species such as the cephalopod *Pteroctopus tetracirrhus*, which preys mainly on crustaceans such as *Alpheus glaber* and some fishes (Quetglas et al. 2009), occupy lower trophic positions (Navarro et al. 2013). The range of values found suggests that some predators can exploit resources across entire food webs. *L. budegassa* and *A. veranyi* both showed the highest values, indicating that they

may prey on other potential predators, such as *Merluccius merluccius*, and even other cephalopods (Negzaoui-Garali et al. 2008, López et al. 2016, Guerra-Marrero et al. 2020, Lloret-Lloret et al. 2020). The overlap in the different isotopic niches helped us determine if the diet composition of each predator was similar to that of a predator that only consumes *N. norvegicus*. The most similar predators were the demersal shark *G. melastomus*, which has a diet consisting mainly of decapod crustaceans (Fanelli et al. 2009, Barría et al. 2018), and the cephalopods *Sepietta* spp. and *S. oweniana*, which tend to forage from dusk till dawn as adults, preying on demersal and benthic crustaceans (Bergström 1985).

Even though the combined results of the genetics and isotopic niche overlap indicate some specific species as the major predators, it is crucial to keep in mind the density effects of predators, such as predation rate, on *N. norvegicus* (Kunert & Weisser 2003). Predator density could indicate that some species, despite showing less importance based on genetics and isotopic analyses, could have a stronger influence on *N. norvegicus* population dynamics and distribution due to a higher abundance within the habitat (Mchich et al. 2007). Considering the estimated PI along with the % DNA and K 95% results indicates that the major predators were the cephalopods *S. oweniana*, *Sepietta* spp., and *A. veranyi*. Though some bony fishes and elasmobranchs prey occasionally on *N. norvegicus*, the main predators in their grounds are cephalopods, in contrast to the Atlantic Ocean, where the main predator is the bony fish *Gadus morhua* (Björnsson & Dombaxe 2004). The bony fish most closely related to cod in the Mediterranean is *M. merluccius*, which showed a low consumption of *N. norvegicus* based on the % DNA value. In the western Mediterranean, *M. merluccius* is mainly distributed between 100 and 200 m depth (Recasens et al. 1998), segregated from the habitat occupied by *N. norvegicus* (300 to 600 m) (Sardà 1998), which could explain why the crustacean is only occasionally predated by this species. The trophic marker of *N. norvegicus* for *Conger conger*, based on stable isotopes and % DNA values, was
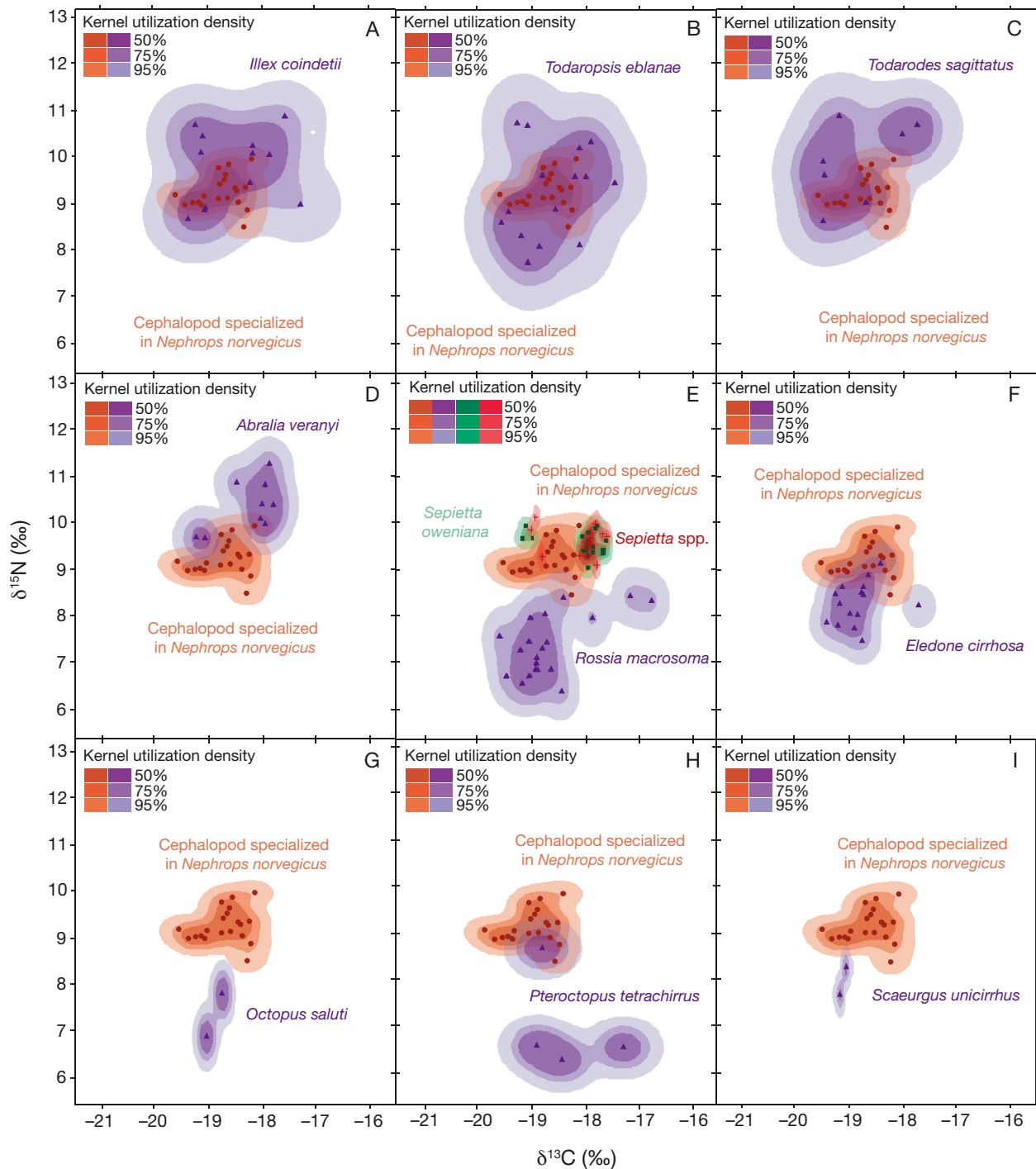
Fig. 3. (A−I) Isotopic niche and overlap of a potential cephalopod that only feeds on *Nephrops norvegicus* and 11 cephalopods inhabiting deep-water ecosystems of the northwestern Mediterranean Sea. Niche size and overlap estimates were generated for the 50%, 75%, and 95% contour levels of the kernel utilization densities. The potential predators that only feed on *N. norvegicus* represent the isotopic niche of the expected stable isotope values of 20 *N. norvegicus* individuals corrected for specific-group isotopic fractionation factors (see Section 2.6.)

similar to that determined for *M. merluccius*, and this species has been directly observed preying on *N. norvegicus* (Fig. S2; O'Sullivan et al. 2004, Matić-Skoko et al. 2012). However, the PI was not high enough for this fish to be defined as a major predator, probably due to its low abundance in the habitat.
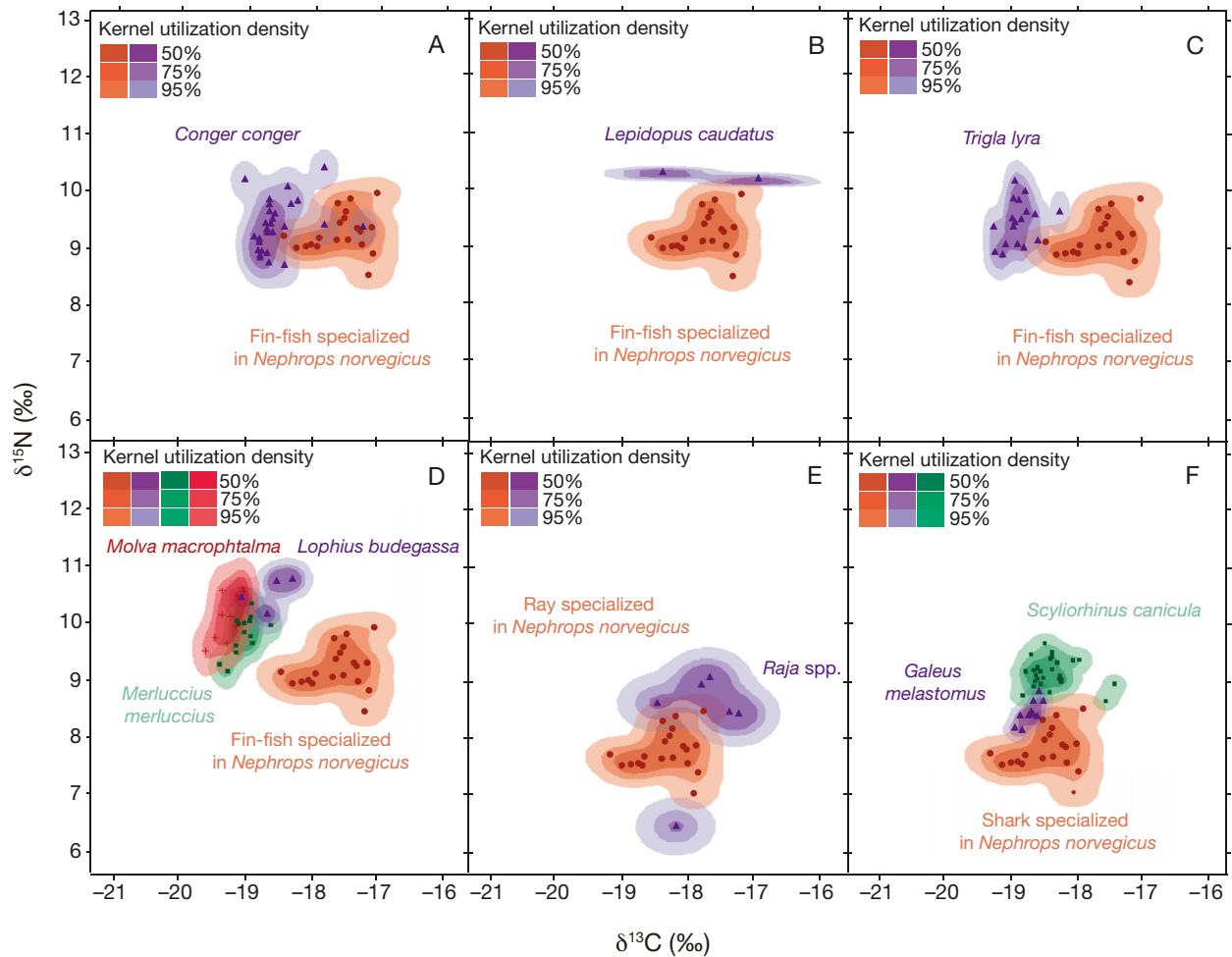
Fig. 4. Isotopic niche and overlap of a potential bony fish, ray and shark that only feed on *Nephrops norvegicus* and (A–D) 6 bony fishes, (E) rays (*Raja* spp.), and (F) 2 shark species inhabiting deep-water ecosystems of the northwestern Mediterranean Sea. See Fig. 3 for an explanation of the niche size and overlap estimates and the potential predators

Some studies have indicated that the combination of unavailability of *N. norvegicus* due to its burrowing behavior and the potential invulnerability of larger individuals could explain the absence of specialized predators (Smith & Herrkind 1992, Wahle 1992, Serrano et al. 2003). In addition, *N. norvegicus* is not an easy prey to catch and digest because of its exoskeleton and its low fat content (Björnsson & Dombaxe 2004); thus, its predation might be more a matter of prey availability than a preference (Chapman 1980).

The identification of predators offers key information for interpreting biological linkages among species and is critical for identifying nature-based solutions for ecosystem-based management used both in fisheries and marine spatial planning. Ecosystem-based modelling can generate predictions of the eco-

logical consequences of activities such as overfishing (Jacobsen et al. 2016). Most predators are considered keystone species in the environments they inhabit (Power et al. 1996), playing an essential ecological role in restoring ecosystems and conferring resilience against future negative impacts, such as biological invasions or climate change (Ritchie et al. 2012). The information provided in the present study could be useful for adapting and creating effective fisheries management and restoring the population of *N. norvegicus* in the Mediterranean Sea while preventing negative future scenarios. An improvement for fisheries management could be the parallel assessment of the major predators identified, to compare the fluctuations between populations. In the management of no-take marine reserves, predators should also be evaluated in terms of abundance and bio-
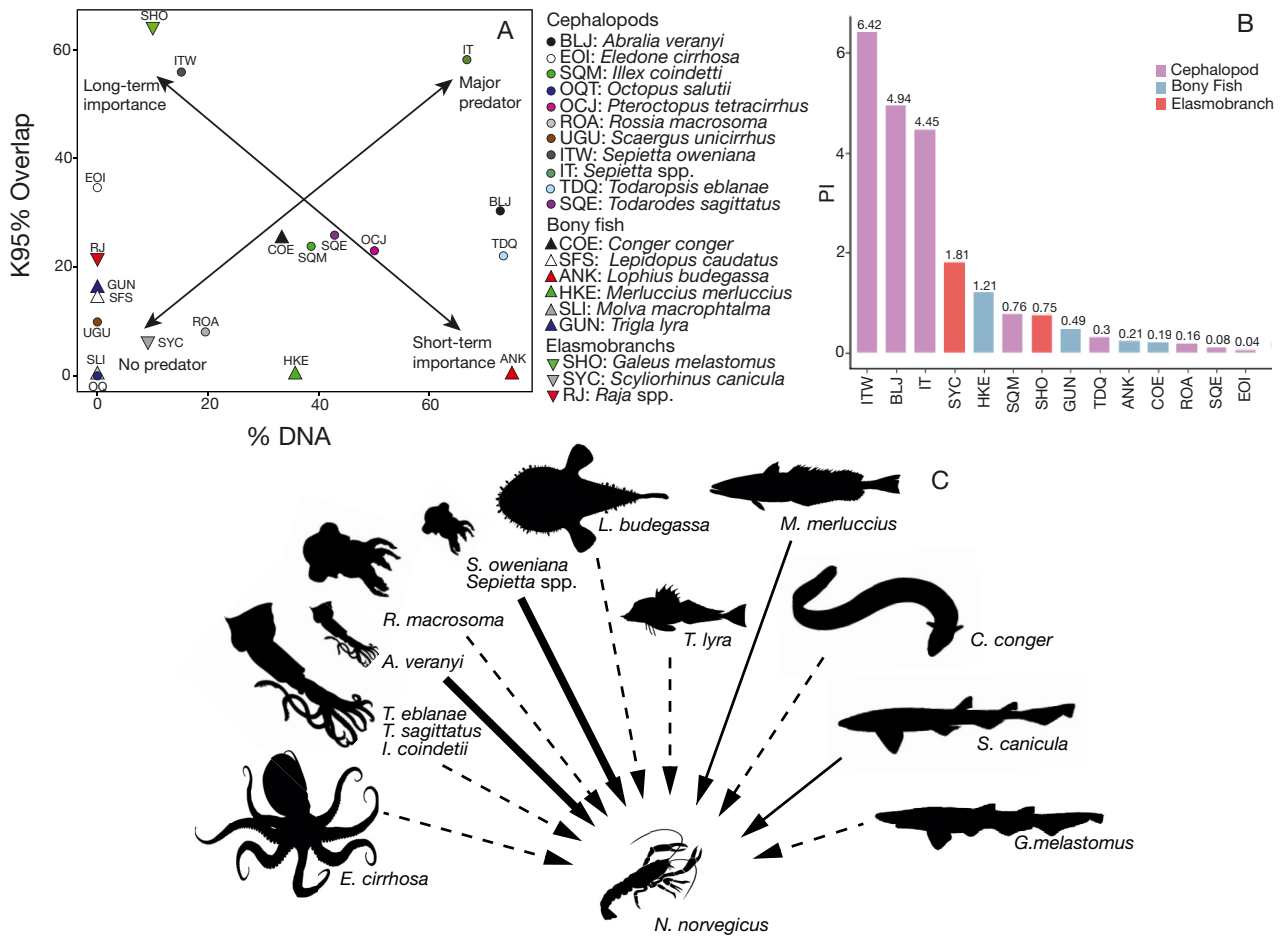
Fig. 5. (A) Classification of the potential predators of Norway lobsters according to their % DNA (percentage of individuals containing *Nephrops norvegicus* DNA in their stomach) and K 95 % (95 % contour overlap between the isotopic area of the simulated predator and the potential predator) values. Long-term importance: high K 95 % value; Short-term importance: high % DNA value; Major predator: % DNA and K 95 % values both high; No predator: % DNA and K 95 % values both low, meaning that this species does not prey on Norway lobster. (B) Predation Index for each species, from the highest value to the lowest. (C) Trophic network relating the species (silhouettes obtained from https://pngtree.com/ and http://clipart-library.com/) that prey on *N. norvegicus*. Solid and wider arrows: major predators; solid and thin arrows: species of moderate importance; dashed arrows: predators with minor importance

mass, to properly assess the effectiveness of the protected area. Predators such as *Sepietta* spp. and *A. veranyi* might also directly affect the dynamics of *N. norvegicus* populations by reducing recruitment and/or survival.

In this study, we used indirect methods and, therefore, we did not consider whether *Sepietta* spp. and other predators prey only on small-sized *N. norvegicus* individuals or larvae. Consequently, next steps could involve assessing and testing the predation of *Sepietta* spp. and *S. oweniana* on *N. norvegicus* of different sizes (including larvae) and evaluating the presence of predator avoidance of larger *N. norvegicus* across all the predator species analyzed.

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