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A multimethod approach to analyse the dietary composition of the Baltic grey seal (*Halichoerus grypus*) in the southern Baltic

Master thesis in the study program: Integrated Zoology

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Content

Declaration of Originality

I confirm that the submitted thesis is original work and was written by me without further assistance. Appropriate credit has been given where reference has been made to the work of others. The thesis was not examined before, nor has it been published. The submitted electronic version of the thesis matches the printed version.

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Abstract

Baltic grey seals (*Halichoerus grypus*) returned to the Southern Baltic Sea in the early 2000s, and in 2021 more than 40,000 were counted in the Baltic Sea, with about 10 % of the population found in the southern Baltic. As top predators, grey seals compete to some extent with coastal fisheries for the same resource. Estimation of total consumption of key fish species by grey seals is hampered by the low natural abundance of some species such as northern pike (*Esox lucius*) as well as high mobility of seals, which move among inner and outer coastal waters where the fish communities differ. Moreover, grey seals are known to feed on soft tissue of large-bodied species such as pike or marine mammals. Therefore, hard parts of those species may not be detectable with morphological analyses. We anticipated a better understanding of the grey seal diet composition by including genetic analyses. In this study, morphological and genetic methods are combined to compare the approach of these two methods in connection to the estimation of the diet composition of Baltic grey seals. Samples were collected during necropsies within the marine mammal stranding network of the German Oceanographic Museum in Mecklenburg-Western Pomerania (Germany). In total, 104 intestine- and 23 stomach-content samples were taken and following DNA was extracted. The mitochondrial 16S rRNA gene was targeted for DNA metabarcoding, using a combination of existing primers and newly designed primers that cover the expected local diet of grey seals, for 26 grey seals. For direct comparison, 7 whole intestines and stomachs were analysed morphologically and genetically. Additionally, samples of 32 seals of stomach and intestines were analysed morphologically separately to investigate the results of this method. With morphological analysis, herring, cod and round goby were found to important prey species of grey seals. Prey-fish size and species was related to seal size. Genetic analysis detected 40% more species than morphological analysis, but was subject to contamination in some negative controls, thus conclusions to be drawn are limited. Nonetheless, herring, sculpin, and roach were found to be important prey species. DNA concentration decreased with increasing decomposition state, which may influence the detection of prey species with this method. There were no significant differences in prey abundance among different areas along the coast of Mecklenburg-Western Pomerania, or in different years and seasons (quarters). Gamma diversity revealed a high diversity in prey-fish community, with no single species overly dominating the diet.

It suggests an opportunistic feeding behaviour of grey seals. Taken together, more species could be identified using genetic methods, compared to morphological, but there were still differences in the prey taxa detected. To cover a wider spectrum and try to reveal a more detailed insight, both methods could be performed or combined (for future work with a larger sample size).

Zusammenfassung

Ostsee-Kegelrobben (*Halichoerus grypus*) kehren seit Anfang der 2000er Jahre in die südliche Ostsee zurück. In 2020 wurden erstmals mehr als 40.000 Tiere in der Ostsee gezählt, wobei etwa 10 % der Population in der südlichen Ostsee zu finden sind. Als Spitzenpredatoren konkurrieren Kegelrobben in gewissem Maße mit der Küstenfischerei um dieselbe Ressource. Die Schätzung des Gesamtverbrauchs der wichtigsten Fischarten durch Kegelrobben wird durch die geringe natürliche Häufigkeit einiger Arten wie des Hechts (*Esox lucius*) sowie durch die hohe Mobilität der Robben, die sich zwischen inneren und äußeren Küstengewässern bewegen, wo die Fischgemeinschaften unterschiedlich sind, erschwert. Darüber hinaus ernähren sich Kegelrobben bekanntermaßen von Weichgewebe großvolumiger Arten wie Hechten oder Meeressäugetieren. Daher sind Skelettstrukturen dieser Arten mit morphologischen Analysen möglicherweise nicht nachweisbar. Mittels genetischen Analysen wird ein besseres Verständnis der Nahrungszusammensetzung der Kegelrobben erwartet. In dieser Studie werden morphologische und genetische Methoden kombiniert, um die Nahrungszusammensetzung baltischer Kegelrobben abzuschätzen. Die Proben wurden im Rahmen von Obduktionen innerhalb des Meeressäugetier-Strandungsnetzes des Deutschen Meeresmuseums in Mecklenburg-Vorpommern (Deutschland) gesammelt. Insgesamt wurden 104 Proben aus dem Darm und 23 aus dem Magen entnommen und anschließend DNA extrahiert. Das mitochondriale 16S rRNA-Gen wurde für die DNA-Metakodierung von 26 Kegelrobben mit einer Kombination aus bestehenden Primern und neu entwickelten Primern, die das Beutespektrum der Kegelrobben abdecken, gezielt untersucht. Zum direkten Vergleich wurden 7 ganze Därme und Mägen morphologisch analysiert. Zusätzlich wurden Proben von 32 Kegelrobben aus Magen und Darm unabhängig von zu morphologischen Proben analysiert, um die Ergebnisse dieser Methode zu untersuchen. Die Ergebnisse der morphologischen Analyse deuten darauf hin, dass die Verwertung von Hartteilen von Fischteilen im Verdauungstrakt im Hinblick auf die Schätzung der Biomasse der Beutetierarten schwach zu sein scheint. Generell zeigt die morphologische Analyse, dass Hering, Dorsch und Schwarzmundgrundel wichtige Beutetiere für Kegelrobben sind. Die Größe der Beutetiere scheint mit der Größe der Robben zusammenzuhängen. Bei der genetischen Analyse wurden 40 % mehr Arten nachgewiesen als bei der morphologischen Analyse, doch waren einige Negativkontrollen verunreinigt, so dass nur begrenzte Schlussfolgerungen gezogen werden können. Bei der genetischen Analyse wurden große Mengen an Hering, Seeskorpion und Plötze analysiert. Außerdem wurde festgestellt, dass die DNA-Konzentration mit zunehmendem Zersetzungszustand abnimmt. Dies könnte sich auf den Nachweis von Beutetierarten mit dieser Methode auswirken, auch wenn die DNA-Konzentration von Probe zu Probe variiert. Bei der Untersuchung des Einflusses ökologischer Variablen konnten keine signifikanten Unterschiede in der Beutetierhäufigkeit zwischen verschiedenen Gebieten entlang der Küste Mecklenburg-Vorpommerns, verschiedenen Jahren und Jahreszeiten (Quartalen) festgestellt werden, was das opportunistische Fressverhalten der Kegelrobben widerspiegelt. Die Gamma-Diversität zeigte eine große Vielfalt in der Beutefisch-Gemeinschaft, wobei keine Art die Ernährung übermäßig dominierte. Mit genetischen Methoden konnten mehr Arten identifiziert werden als mit morphologischen, aber es gab immer noch Unterschiede bei den Taxa der Beutetierarten. Um jedoch ein breiteres Spektrum abzudecken und einen detaillierteren Einblick zu erhalten, müssen beide Methoden kombiniert werden (für zukünftige Arbeiten mit einer größeren Stichprobe).

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1. Introduction

Pinnipeds inhabit most marine aquatic environments, including estuaries and continental shelves, tropical seas, the deep ocean, Arctic and Antarctic polar seas, mostly in regions with high productivity (Bowen, 1997; Kovacs et al., 2012; Berta, 2018). Like most marine mammals, pinnipeds are apex predators and feed near or at the top of marine food webs (Pauly et al., 1998). As a result, pinnipeds are prominently reported in interactions with commercial fisheries, largely due to marine protection and population recoveries (Jorgensen et al., 2007; Kuparinen & Merilä, 2007; Magera et al., 2013; Roman et al., 2015; Jackson et al., 2022).

Seal-fishery interactions serve as a prime illustration of a persistent and challenging conflict between human activities and wildlife. These interactions take place worldwide wherever seal habitats intersect with fishing grounds (Cronin, 2011; Jackman et al., 2018; Olsen et al., 2018; Sepúlveda et al., 2018), making conflicts between the fishing industry and marine predators such as pinnipeds a global issue (Jackson et al., 2022). Within South American waters, the frequency of interactions generally exceeded 50% of the observed hauls (de Oliveira et al., 2006; Sepulveda et al., 2007; De La Torriente et al., 2010; Sepúlveda et al., 2018b; Szteren et al., 2018). Although estimated losses caused during the fisheries interactions have ranged from less than 1% to 3% of the total catch from gillnets (Machado et al., 2016; Oliveira et al., 2020). Hückstädt and Antezana (2003) estimated the amount of fish consumed by the South American Sea Lions to be as little as 0.4% of the catch. These numbers were comparable to sea lion consumption in South Africa (Wickens et al., 1992). Aquaculture worldwide, including in Scotland, the USA, Canada, Australia, New Zealand, Norway, and Chile, is negatively affected by pinnipeds (Heredia-Azuaje et al., 2022). Overall, the global proportion of loss to small-scale/recreational fisheries is five times greater (21.8 %) than to large-scale fisheries (4.86 %) (Jackson et al., 2022).

Grey seals are the largest predators in German waters. After being absent for many decades, the Baltic grey seal has regained its native presence along the German coastline (Galatius et al., 2020; Westphal & Liebschner, 2021). Effective international conservation management is reflected by the increasing abundance of grey seal subpopulations (Maschner et al., 2014). Coordinated surveys conducted since 2003, which count individuals during the moulting season, have revealed a consistent increase in the Baltic grey seal population. After a stagnation period of 30,000 individuals from 2014 to 2017, approximately 38,000 grey seals were counted in 2019. In 2020 and 2021 numbers exceeding 40,000 were counted (HELCOM, 2021; ICES, 2020), although in 2022 fewer than 37,000 seals were counted (HELCOM 2023). The southern Baltic includes ca. 8 % of the Baltic population (HELCOM, 2021). Along the coast of Mecklenburg-Western Pomerania (MWP) in Germany, the seal numbers fluctuate seasonally (Figure 1). A peak (>300 individuals) occurs between March and April at the main haul-out site “Greifswalder Oie” (Buschhaus & von Rönn, 2022). This increase is also represented by the rise of carcass discoveries, from 10 per year until 2007 to over 50 in 2020 and even 125 carcasses in 2021, along the MWP coasts (Herrmann et al., 2016; Maschner et al., 2014; Reif et al., 2021; Reif et al., 2023). Since 2004, grey seals can again be found in the waters of the Greifswald Bay on a regular basis (Nestmann & Harder, 2014). Grey seals have also been reported in Grabow, Barther lagoon, Kubitzer lagoon and the Strelasund on a regular basis in recent years (Figure 3) (Sichtungskarte DMM). The preferred sites within the eastern German Baltic waters include the Stubber bank, the islands Ruden and Oie, and Cape Arkona on Rügen. An additional haul-out site in western Mecklenburg is the sandbank Lieps, where both grey seals and harbour seals are found together (Nestmann & Harder, 2014; Schwarz, 2003b) (Figure 3). For grey seals, the population is continuously increasing by about 5 % per year in the Baltic Sea, with the strongest rate of increase currently taking place in the southern areas (HELCOM, 2018).

Whereas the return of the Baltic grey seal is an enormous success for marine nature conservation, the growing numbers of seals have led to more interactions with fisheries in the Baltic Sea, as grey seals and costal fisheries compete to some extent for the same resource (Harding & Härkönen, 1999; Lunneryd, 2001; Lundström et al., 2010; Olsen et al., 2018).

The Greifswald Bodden is part of the NATURA 2000 network and belongs to a protected area (1747-301) under the Flora-Fauna-Habitat directive (StaLu 2011). The grey seal is listed as protected species in this area (annex two FFH). Moreover, the shallow lagoons of the German Baltic coastline are the main spawning area of the Western Baltic herring (Kanstinger et al., 2018) and, therefore, this area is an important herring fisheries area (ICES, 2022). Gill nets and large fish traps are the fishing gear primarily used in these shallow and tide-independent waters.

Several fish species occurring in estuaries and lagoons are of high economic value. The role of fish as a coastal fishery resource is site-dependent. For example, within the Greifswalder Bodden, catches, especially of spring-spawning population, contribute importantly to the local economy, including recreational fishing tourism (Winkler & Schröder, 2003; Arlinghaus et al., 2023; Koemle et al., 2023). Whereas in the Darß-Zingst Bodden chain fisheries depend on pike-perch (*Sander lucioperca*), Wismar Bay fisheries targeting flounder and eel and the Baltic fisheries herring, sprat, plaice and flounder (LALLF 2022). Throughout herring displays an important target species of the coastal fisheries within Mecklenburg-Western Pomerania (Winkler, 2001; Winkler & Debus, 2006; LALLF 2022). Due to increased anthropogenic impact as the historical overfishing in the Baltic Sea and its inner waters along with different other abiotic stressors such as warming, eutrophication, oxygen depletion and acidification in the Baltic lagoons (Asp & Lassen, 2012; Möllmann et al., 2021; Arlinghaus et al., 2023), have led to reduced fish stocks and decreased productivity, particularly affecting commercially important species like Baltic cod (*Gadus morhua*) (Sguotti et al., 2018; Möllmann et al., 2021) and Western-Baltic spring-spawning herring (*Clupea harengus*) (ICES, 2022). These changes have a high economic as well as social importance (Henking, 1923; Rechlin & Fadschild, 1991; Döring, 2001). The number on reports of decreasing freshwater fishes that inhabit brackish coastal ecosystems in the Baltic Sea also has increased, including declines in Eurasian perch (*Perca fluviatilis*), pike-perch (*Sander lucioperca*) and pike (*Esox lucius*) (Olsson, 2019; Olsson et al., 2023). The role of seals in this decline has been emphasized in Sweden (Bergström et al. 2022). Over time, this shift of fish communities was registered in the diet of top predators, such as grey seals. On the one hand a decline of 18 % of Atlantic cod within 30 years in the diet composition of seals from the Baltic Proper using morphological analysis, was reported (Söderberg, 1972; Lundström et al., 2007). Whereas the importance of fatty herring increased to 78 % of the seals diet in the Gulf of Bothnia and the Baltic proper, identified by morphological analysis (Lundström et al. 2007; 2010).

Pike within the Baltic Sea inshore areas is particularly vital to local commercial fisheries and has a major importance in the high socio-economic value of the recreational fishing industry (Crane et al., 2015; Arlinghaus et al., 2018; Kuparien & Lethonen, 2018). Angling tourism is a growing economic sector in the lagoon areas and various professional guided fishing companies are based in this area (Koemle et al., 2021, 2022). Due to unique foraging opportunities in the brackish water system, the pike in the Baltic Sea often reaches, so-called, trophy sizes over one meter and is a praised target for recreational anglers (Arlinghaus et al., 2021; Koemle et al., 2021, 2022). The Baltic Sea's southern lagoons stand out as an area where the high number of large fish, measuring between 100-120 cm, are caught (Arlinghaus et al., 2023). As pike is occurring in lagoon systems, as well as these are main haul-out sites of grey seals and reports of predation is increasing, this could affect population size and economical importance of prey fish. Most likely grey seals follow herring and garfish to their spawning grounds in the Greifswald lagoon (Nestmann & Harder, 2014; Larsson et al., 2015; Reckendorf et al., 2019).

Coastal fisheries have declined in Mecklenburg-Western Pomerania from around 1,000 businesses in 1990 to roughly 330 (LALLF 2022). It is an extremely difficult situation, as commercial fish stocks being overexploited, the return of grey seals is a major challenge, as the fishing gear that has become established in their absence is very vulnerable to predation damage (Schwarz et al. 2003).

High seal abundance may also result in a change of fish behaviour (Varjopuro, 2011). Since 2020 the ministry of Mecklenburg-Western Pomerania compensates for up to 80 % of the verified seal-induced damages (catch loss/ fishing gear damage) of coastal herring and eel fisheries by paying of the repairment costs (Ministerium).

Additionally, within the lagoon areas, conflicts between commercial and recreational fisheries are heated, due to different regulations in quotas and of pike and other freshwater species (Arlinghaus et al., 2022).

As mentioned, conflicts among fish eating mammals and fisheries are rising, therefore to understand the habitat use of marine mammals is of greater importance. Within the southern Baltic, there have been studies in Swedish waters, but little research has been carried out on grey seals along the German Baltic coast. This thesis provides a detailed insight into the dietary composition of the Baltic grey seal in in different coastal areas of the southern Baltic Sea. To analyse if large fish species, such as pike has been underestimated in the diet of Baltic grey seals so far, the morphological analysis of hard-parts is compared to the metabarcoding approach. As the estimation of the predation of grey seals (*Haliocherus grypus*) upon important fish species, is hampered by the low abundances (e.g. northern pike) of certain species and furthermore the high mobility of the seals within inner bays and the open Baltic Sea.

This thesis studied the diet of grey seals by analysing the prey-fish found in stomachs and intestines using morphological and genetic methods. The overall aim was to characterize the prey spectrum, the impact on particular prey species of interest, as well as predator-prey relationships and the food web of the southern Baltic.

The specific objectives were:

1) To estimate and compare the species composition in the diets of grey seals assessed with morphological and metabarcoding methods, evaluating the efficiency and accuracy of each approach.
2) To assess the diet composition of grey seals in five different areas of the coastline of Mecklenburg-Western Pomerania: North-Western Mecklenburg (NWM), the west coast of Rügen (WCR), the surrounding coast eastern Rügen (SC), Greifswald Bay (GB), and the surrounding coast of Vorpommern (SCV).
3) To assess the influence of the differences in the diet of grey seals based on different years (2014-2022), as well as the season (winter, spring, summer, autumn) and body length.
4) To compare DNA extraction success among different decomposition states of analysed seals.
5) To calculate prey-fish biomass in order to estimate seal consumption.

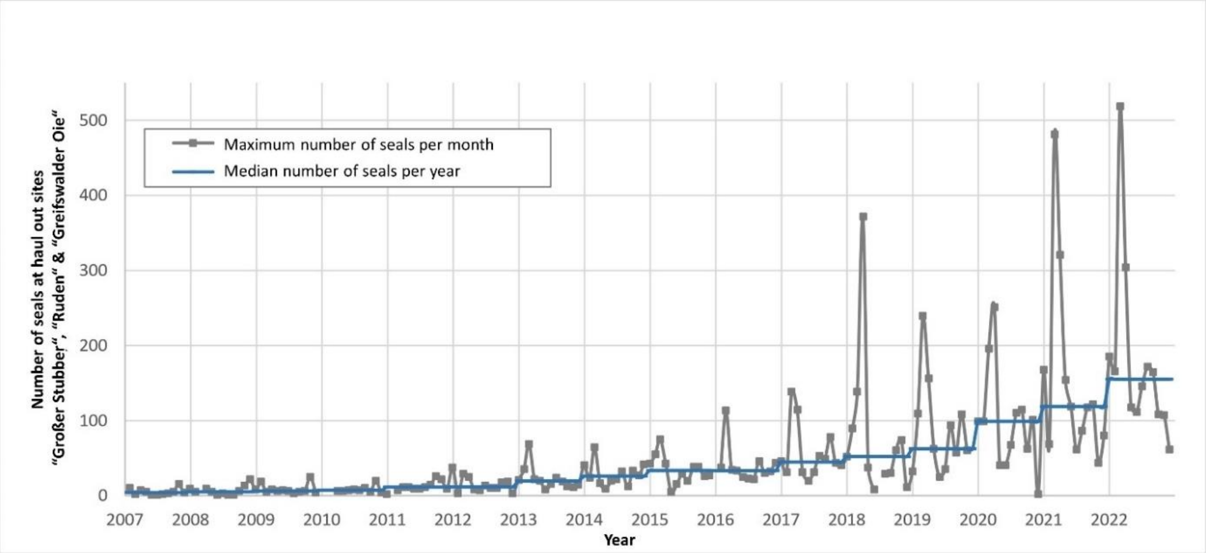

Figure 1 Development of the Baltic grey seal (*Halichoerus grypus*) population 2007-2022. Median yearly (blue line) and maximum monthly (grey line) number of seals present at the haul-out sites, Großer Stubber in Greifswalder Bodden and "Ruden" and "Greifswalder Oie" east of Greifswalder Bodden (data sources: Verein Jordsand, Weisse Flotte, Bundesamt für Naturschutz, Biosphärenreservatsamt Südost-Rügen and Deutsches Meeresmuseum) (© *Arlinghaus et al., 2023*).

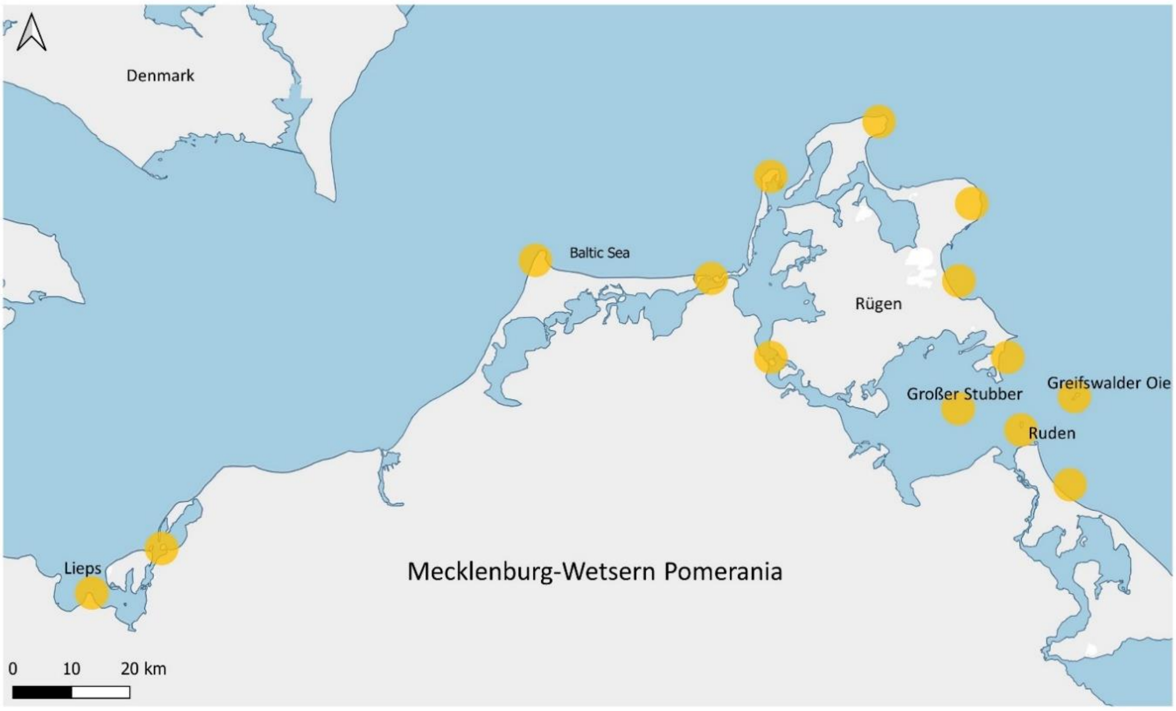

Figure 2 Common haul out sites (filled yellow circles) for grey seals (*Halichoerus grypus*) along the coast of Mecklenburg-Western Pomeraniain the southern Baltic Sea (Sichtungskarte DMM).

Figure 3 Typical haul out site along the southern Baltic area. Male Baltic grey seals (*Halichoerus grypus*) resting on stone sticking out of the water at the Greifswalder Oie (March 2022, © Linda Westphal)

2. Literature review

2.1. Baltic grey seal

Three phocid seal species are present in the Baltic Sea, the Baltic ringed seal (*Phoca hispida baltica*), the harbour seal (*Phoca vitulina vitulina*) and the Baltic grey seal (*Halichoerus grypus grypus*) (Olsen et al., 2018). The most frequent species in the southern Baltic Sea and the waters of Mecklenburg-Western Pomerania is the Baltic grey seal (Nestmann & Harder, 2014; HELCOM, 2018; Galatius et al., 2020) (Figure 3). The recent population rebound, after a long period of absence, is a big win for conservation following many years of hunting, culling, and contamination events (Figure 1).

The decline of the Baltic grey seal population in the mid-20th century was precipitated by multiple factors. Hunting and extensive culling in the 1940s, and the introduction of ecotoxins such as organochlorines, polychlorinated biphenyls (PCBs), and dichlorodiphenyltrichloroethanes (DDT) in the mid-1970s led to a reduction of the grey seal population down to 3,000 – 3,600 animals with additional high infertility rates of 70 – 80 % (Helle et al., 1976; Helle, 1980; Harding & Härkönen, 1999; Schwarz, 2003; Harding et al., 2007; Maschner et al., 2014). At that point only 1-2% of the native stock were still present in the Baltic Sea, and the population was thought to be extinct along the coast of Mecklenburg-Western Pomerania (Almkvist, 1982).

After the introduction of protective measures, decided by the Helsinki Commission (HELCOM) including all the Baltic Sea states in 1992, a consequent ban of the use of these contaminants and an additional general culling and hunting ban, the population had been growing exponentially (Harding & Härkönen, 1999; Härkönen et al., 2007; HELCOM, 2018). Furthermore, the Baltic grey seal is a specially protected species according to the Federal Act for the Protection of Nature (BNatSchG §7 Abs. 2 Nr. 13) and additionally, listed to be protected by the EU directive 92/43/ECC on habitats, flora and fauna in the Appendix II and V in 1980s. Since the beginning of the 2000s, seal population development across the Baltic Sea and current protection and management measures have been observed and evaluated by an international body within the framework of the Helsinki Convention (HELCOM), the so-called Marine Mammal Expert Group (EG MAMA). The seal expert group conducts monitoring and gives recommendations upon conservation measures (HELCOM RECOMMENDATION 27-28/2). According to the assessment "unfavourable/inadequate" (meaning the species is not in a satisfactory state to ensure its long-term survival or well-being) of the FFH directive in 2013, implementations of appropriate measures are required by member state authorities (in this case Mecklenburg-Western Pomerania) in order to subsequently improve the conservation status of this species (von Nordheim et al., 2019). For semi-aquatic pinnipeds hauling out, a behaviour described as crawling outside the water is essential in order to rest, give birth, and molt (Hall & Thompson, 2009). Historically important haul-out places, in the southern Baltic were destroyed by actively removing stones for industrial purposes, particularly noticeable in the Greifswald Bay. However, conservation efforts successfully established two national parks and a biosphere reserve in 1990, creating possible resting sites (Harder & Spielmann, 2003).

Despite year-round use of haul out sites, the animals hardly reproduce on the southern coasts. In 2020, the birth rate was only 0.5 %, even lower than at the beginning of the return in 2003 with 2 % (Galatius et al., 2020). Rødsand (Denmark) is the only site in the southern Baltic Sea with regular annual pupping events since the recolonization of the Baltic grey seal, where annually 5 - 6 pups (2018–2020) were reported (Galatius et al., 2020). Only sporadic breeding events in Danish Kattegat, southern Sweden, Poland and Germany occur. In 2018 on Rügen and 2019 on Poel evidence of grey seal births in Mecklenburg-Western Pomerania could be proven (von Nordheim et al., 2019; Westphal & Liebschner, 2021).

2.2 Seals and fisheries

Whereas the return of the Baltic grey seal is an enormous success for marine nature conservation, the growing numbers of seals have led to more interactions with fisheries (Harding & Härkönen, 1999; Lunneryd, 2001; Lundström et al., 2010; Olsen et al., 2018). There can be direct interactions, such as stealing fish out of the fishing gear, but also indirect effects such as damaging gear and changing the behaviour of prey and reducing their abundance can affect the fishing industry (Varjopuro, 2011; Hansson et al., 2018; Bergström et al., 2022). Due to the technological development, seals were considered to be the main competitor to fisheries starting at the end of the 19th century (Schwarz et al., 2003), rising numbers have re-emerged todays conflicts with human fishing activities in an increasing number of areas (Mehtonen, 2019). Estimated damaging impacts on the fishing industry increased over the past years, but overall fishery catch is considerably larger than the predation by birds and seals combined (Hansson et al., 2018).

In Swedish waters 2004 damage to fishing gear and catches was estimated to exceed 5 million euro, 15–20 % of the annual catch value for the total coastal fisheries (Westerberg et al., 2006). In the northern Baltic, a similar situation happens within trap-net fisheries, depending on the area, seal damage up to 37 % on the salmon catch occurs (Kauppinen et al., 2005). Within the coastal fisheries of Mecklenburg-Western Pomerania, the damages increased from 12.000€ in 2019 to 27,000€ in 2020 and up to 300,000 € in 2022 (Ministerium MV; FIUM, 2020; LALLF 2022). Due to exploitation and fisheries management 2018 - 2020 proceeds of fisheries declined by 30 % due to quotas upon herring and cod, which cannot be compensated by other target species (LALLF 2022).

2.3 Management

Management conflicts are often arise regarding how pinnipeds affect mortality upon fish stocks. Density-dependent mortality determined by predation, especially of juvenile fish, and competition of a stock is a key compensatory mechanism damping down inter-annual variability of recruitment (Myers & Cadigan, 1993). Size-dependent effects within adult fish are mostly additive. Total mortality (Z) is determined by natural mortality (M) and fishing mortality (F), increasing M will result therefore in higher Z and overall stocks mortal (Connell, 1998; Pauly, 1980; Lorenzen, 2000; Powers, 2014; Stige et al., 2019). Relatively fewer old fish in the population, being important for the recruitment. Hence, natural predators and fisheries become direct competitors (Lee et al., 2011; Maunder et al., 2023). It should be mentioned that on the other side increasing interactions with fishing industries can also affect the seal population negatively, as they get entangled and drown in fishing gear (McIntosh et al., 2015). Between 4 - 10 % of the Baltic grey seal population die as cause of bycatch every year (Vanhatalo et al., 2014). Unwanted bycatch depicts one of the greatest threads to seals and is highest in stationary fishing gears such as gillnets and large traps (Vanhatalo et al., 2014; Westphal & Liebschner, 2021).

The increase in damages over the years resulted in a renewed interest in managing the seal populations, as grey seals are only one of them (Harding & Härkönen, 1999). Several methods are applied by different countries. Regulated hunting, with the intention of reducing their predation on valued resources, is prohibited in Germany (ICES, 2020; BNatSchG §7 Abs. 2 Nr. 13). As well as acoustic seal deterrent devices to keep the predators away from the nets are prohibited in Germany (Brandt et al., 2012; Schaffeld et al., 2019, Commission Delegated Regulation (EU) 2022/303) have developed over the past years (Varjopuro, 2011). Studies form the Swedish west coast present that if pots where equipped with seal exclusion devices (SEDs) the bycatch of seals was reduced to zero without negatively affecting the pot's catchability, or even resulted in retention of heavier salmons (Königson et al., 2015; Calamnius et al., 2018). Furthermore, Königson et al. (2013) presented that generally adult male grey seals developed a characteristic behaviour and specialized in raiding fishing gear.

2.3.1 Seal plan for Mecklenburg - Pomerania

Due to the increasing numbers of grey seals, the local gillnet fishery is increasingly complaining about seal-related damage to catch and gear (Figure 4) (Arlinghaus et al, 2023b). At the same time, more dead grey seals showing clear signs of drowning in fishing gear are found on the coast (Reif et al., 2023). To contribute to a sustainable coexistence of coastal fisheries and grey seals and mitigate this conflict, an advisory board consisting of representatives of the State Ministry for Agriculture and Environment of the State of MV, nature conservation authorities, fisheries and nature conservation associations and research institutes was formed. The “Konfliktmanagement – Fischerei - Kegelrobben” (KFK) (Conflict Management - Fisheries - Grey Seals) includes in the working packages such as compensation payments for fisheries, development of alternative fishing gear to minimise damage and bycatch and monitoring and biology of grey seals (Ferretti, 2021).

An important first step is to elucidate ecosystem functions, such as illustrating predator–prey relationships with empirical evidence (Granquist et al., 2018). As there is not much known upon the diet of grey seals in German Baltic waters, this is a crucial information to elucidate the dietary composition of grey seals in German Baltic areas.

2.3.2 Baltic lagoon systems

World-wide, the Baltic Sea is one of the largest brackish ecosystems, covering a surface of 415,000 km^2 (Bonsdorff, 2006). The semi-enclosed nature of the Baltic Sea is characterized by a significant inflow of freshwater from rivers, resulting in a notably variable salinity gradient (Schubert & Telesh, 2017). The western region (Kattegat), connected to the North Sea, has marine conditions (20 - 30 Practical Salinity Units, PSU), while the northeast and certain coastal areas maintain nearly freshwater conditions (2 - 4 PSU) (Håkanson & Bryhn, 2008). Additionally, a vertical salinity gradient occurs, as the denser saltwater sinks into the deep central Baltic basin induced by inflow from the North Sea. This induces in a barrier for vertical transport, which leads to low oxygen levels (Schubert et al., 2010). Along the coast of the southern Baltic Sea, especially in the German state of Mecklenburg-Western

Pomerania, lagoons (so called “Bodden”) and Haffs (non-tidal estuaries and inlets) are dominant water bodies (Schiewer, 2002). Functioning as transitional zones between marine and terrestrial ecosystems, these areas play a crucial role in the ecosystem, serving as spawning and nursery grounds for migratory organisms of ecological and economical importance, including stenohaline freshwater species e.g. northern pike (*Esox lucius*) (Thiel 1990, Schubert & Thelesh, 2017). Lagoons are known for their significant primary productivity, often being brackish ecosystems with soft sediments and a moderate presence of macrophytes (Schubert & Telesh, 2017). Salinity levels in coastal zones are crucial in determining species diversity, reproduction, and growth (Remane, 1934; Nissling et al., 2006). Bodden lagoons, distinguished by their limited connectivity to the Baltic Sea, typically exhibit lower salinity than the nearby coastlines due to freshwater inputs from surrounding rivers (Schiewer, 2008). Depending on their ties to the Baltic Sea, lagoons fall into two categories. Firstly, those like the western Darss-Zingst Bodden Chain (DZBC), dominated by pike-perch in oligo- to mesohaline conditions and secondly, regions around Rügen, such as West-Rügen-Bodden and Greifswalder Bodden, marked by freshwater predators and diminished pike-perch populations (Winkler, 1990). Salinity varies across these lagoons, with Rügen's surroundings averaging below 9 PSU annually.

The southern Baltic ecosystems, diverse in prey species, have Bodden lagoons characterized by minimal tidal shifts and relatively stable salinity, often within 2 – 4 PSU (Oertl and Birr, 1995; Sagert et al., 2008; Schubert et al., 2017; Schiewer, 2008). However, some lagoons, especially those closer to the Baltic Sea, experience more pronounced salinity fluctuations due to wind-induced water exchange.

2.3.3 Fish biodiversity in the southern Baltic region

The existing salinity gradient within the Baltic Sea has contributed to the development of a unique species assemblage comprising both marine and freshwater species within the Baltic Sea (Ojaveer et al., 2010; Olsson, 2019; Reusch et al., 2018; Wennerström et al., 2013). As salinity changes across gradient, marine species get gradually replaced by freshwater species (Ojaveer et al., 2010).

Within the Baltic Sea, the biomass in the open-sea fish communities are dominated by herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) as pelagic species and cod (*Gadus morhua*), a demersal species with up to 80 %. Furthermore, flounders (*Platichthys flesus*) play a high ecological role as benthic species in the central and southern Baltic Sea, particularly in coastal areas (ICES, 2022).

Lagoon habitats serve as essential feeding areas for species adapted to brackish conditions, notably roach, bream, and perch, which thrive in these settings (Adjers et al., 2006; Ojaveer et al., 2010). The southern Baltic lagoons boast a rich species diversity with up to 87 species in the Strelasund and 61 in the Greifswald Bay (Winkler, 1989a; Thiel et al., 2005). Yet, only a handful of species account for most of the biomass (Rittweg et al., 2023). Cyprinid abundance, particularly species like bream and roach, tends to rise with eutrophication, especially in oligohaline waters. Conversely, mesotrophic species like perch and pike flourish in mesohaline waters. This trend is accentuated from mesohaline to oligohaline regions, with locales like Saaler Bodden and Peenestrom showcasing more cyprinids. These species often traverse between these salinity zones, exploiting the nutrient-rich lagoons for sustenance (Henking, 1923). Populations of roach, bream, perch, and pike-perch, in particular, thrive under such conditions (Henking, 1923, 1929). In Bodden lagoons, freshwater and migratory marine species often eclipse marine species in biomass terms. Previous research, provides insights, but a gap remains for consistent fishery-independent longitudinal data (Fredrich, 1975; Löser, 2004). Consequently, commercial landing records have become the go-to for discerning fish community shifts, especially during the 1970s and 1980s marked by rampant eutrophication (Winkler, 1990, 1991). This era witnessed a notable shift from pike to pike-perch dominance in the western DZBC, spurred by nutrient surges and consequent environmental upheavals (Winkler & Debus, 2006). Relying exclusively on commercial data, however, might overlook pivotal food web components. Elaborating on younger fish populations, various studies have probed the age-0 fish community across lagoons (Fredrich, 1975; Pribbernow et al., 1985; Thiel, 1990). Löser (2004) revealed a spectrum of 17 species in the Strelasund, affected markedly by factors like wind and turbidity. Of these, the three-spined stickleback reigned supreme in shallower regions, while herring dominated deeper waters. Notably, younger pikes were predominantly found in wind-sheltered areas (Löser, 2004). These lagoons' shallow littorals emerge as vital reproductive zones for diverse species, including freshwater, estuarine, and some euryhaline marine types (Thiel, 1990), though successful reproduction for most freshwater species is primarily confined to oligohaline settings (Klinkhardt and Winkler, 1989). In sum, lagoon fish communities are shaped by a multiple factors, ranging from salinity to vegetation cover (Pribbernow et al., 1985; Thiel, 1990; Löser, 2004; Winkler et al., 1995). Recent studies of fish communities in the lagoons around Rügen, using different sampling methods, showed gobies (*Neogobius* spp., *Pomatochistus* spp.) and percids (perch, ruff, pike perch) were found in highest numbers in the Greifswald lagoon, Strelasund and western Rügen. High numbers of three-spined sickleback (*Gasterosteus aculeatus*) in the Greifswald lagoon as well as cyprinids and marine sprat and herring were revealed. The biomass in the lagoons is dominated by lower trophic levels (Rittweg et al., 2023). Strong localized variations in species composition occur due to factors such as oxygen levels, temperature, eutrophication, and wave exposure (Schubert & Telesh, 2017; Rittweg et al., 2023).

Grey seals have been reported to consume a range of species, taking advantage of locally and seasonally abundant prey, geographic variations in the diet within the Baltic Sea were identified, due to the distinct salinity gradient (Lundström, 2010). Seals might follow resources pulses (herring) within the Greifswald lagoon and therefore diet will differ by lagoons as species community shifts (e.g. from oligohaline to mesohaline).

2.4 Foraging behaviour

Foraging grounds of grey seals are shallow waters both near and far from the coast, as well as submarine slopes and reefs (Schwarz, 2003a). Since most dives of the animals reach the seafloor, it is suggested that they use predominantly a benthic hunting method (Sjöberg et al., 1992, 1999).

Furthermore, telemetric studies on grey seals in Great Britain and Sweden revealed that grey seals are capable to migrate several 100 km, and hunting grounds can be more than 50km away from haul out sites, therefore, these trips can take several days (Sjöberg et al., 1992, 1999). Oksanen et al. (2014) investigated a high site fidelity of grey seals in the Bothnian Sea and the Gulf of Finland, with a majority of tagged seals showing a 'resident' behaviour. Only two showed a 'transient' foraging, exceeding 400 km, transitioning to the wintering areas in the Baltic Proper or the Gulf of Riga, which include drift-ice breeding areas (recording 136 days per mean). The resident seals showed active core areas near river estuaries or shallow water areas, indicating a site-fidelity to those areas, overlapping with trap-net fisheries.

2.4.1 Dietary analysis of Baltic grey seals

Grey seal diet is known to vary with occurring prey availability and assemblages, as well as within different sex (Bowen et al., 1993; McConnell et al., 1999; Beck et al., 2005; Beck, Iverson, et al., 2007). For opportunistic feeders like grey seals the most important species are the ones occurring in high biomasses, as it is known that grey seals take advantage of locally and seasonally abundant prey (Ojaveer et al., 1981; 2010; de Jong et al., 2002; Suuronen & Lehtonen, 2012). On average grey seals eat 5 - 7 kg fish per day with a variance throughout the year. During moulting, mating and weaning they hunt less (Bowen et al., 1993).

Lundström et al. (2010) showed, by using hard part analysis, the geographic region significantly influenced the largest variation in the diet within the northern and central Baltic area, likely reflecting variation in local fish assemblages. The dietary patterns of seals in the Baltic Proper in western Sweden differed from those from both areas in the Gulf of Bothnia. Among the seals collected from the Baltic Proper, European sprat and sandeel were relatively more abundant than among the seals from the Gulf of Bothnia, where Atlantic herring and, to a lesser degree, common whitefish were relatively more abundant (Lundström, 2010). Pups consumed relatively small non-commercial species than older seals, such as sandeels (*Ammodytidae*), European eelpout (*Zoarces viviparus*), and shorthorn sculpin (*Myoxocephalus scorpius*) (Lundström et al., 2010). Controversy within studies on prey size exists grey where seals appeared where no size-selective predation by grey seals was suggested and even consumed smaller prey fish (Bowen & Harrison, 1994; Fowler, 2005). Previous studies undertaken on seals have inferred prey preference as they use data from seal diet combined with fish abundance surveys, although these abundance surveys do not necessarily reflect the prey available to individuals (Bowen & Harrison, 1994; Thompson et al., 1997). In contrast, individual size preference of prey consumption by grey seals could be reflected in captive experiments, where seals generally selected the larger number of prey items and showed consistent preferences for particular species, which could be explained due to size selection (Gallon et al., 2017). Within harbour seals in the Atlantic Ocean they will select for forage fish that are larger than the average size of available prey (D. J. Tollit et al., 1997).

Based on morphological analysis, area-dependent differences are evident, as evidenced by the proportion of pike in the diet of grey seals foraging in Swedish lagoons. Pike consumption was four times higher in the inner and central archipelago (around Stockholm archipelago) compared to the outer archipelago (Baltic Proper) (Lundström et al., 2007; Hansson et al., 2018; Strömberg et al., 2012; Svensson, 2021). Prey size estimations showed that relatively large pike (28 -73 cm with a mean length of 44 cm) are part of their diet, along with perch (46%) and herring (24%) as important prey species in terms of biomass, as the estimation was based on morphological analysis of scats (Svensson, 2021). A potential correlation between the increasing grey seal population and the declining pike population in the western Baltic Sea archipelagos has been indicated (Bergström et al., 2022; Bergström et al., 2022; Svensson, 2021).

Worth mentioning is that the abundance of grey seals per km in Swedish waters is similar to the abundance of grey seals in the Greifswald lagoon(HELCOM, 2021; Bergström et al., 2022) and up to date there is little published data upon the diet of grey seals from the German Baltic area. Therefore, a detected dependence of seal predation on fish stock size could yet not be proven within the lagoons of the German southern Baltic area. Nevertheless, evidence of damage to fish catches, occasionally on pikes, has been increasingly documented by many coastal and lagoon fishermen in Mecklenburg-Western Pomerania over the past years (Figure 4) (FIUM, 2020; Arlinghaus et al., 2023). The most recent study comprised a hard part analysis of 32 stomachs along the coast of Western-Mecklenburg Pomerania. In accordance to other studies, a significant amount of herring was detected in the diet with 45.7 - 62.8 %. Surprisingly the second most abundant fish species in the diet displayed roach with up to 35.7 % Another significant species of the grey seal diet was cod with 8.1- 14.6 %, whereas sprat accounted for only 1 % of the total biomass (Hoffmann, 2019a). Up to date, no pike could be detected with dietary analysis in the estuaries and lagoons of the German southern Baltic coast (Westphal, unpubl.; Arlinghaus et al., 2023).

2.4.2 Dietary analysis methods

Dietary analyses are important to understand die ecological role of species and their trophic interactions within food web structures, as well as formulating appropriate management approaches in terms of their interactions with fisheries (Lundström et al., 2010; Tollit et al., 2010).

Different approaches have been established to estimate the prey consumption. A simple direct observation of feeding occurs to be limited within free-ranging marine mammals, most diet estimations rely on indirect methods (Pierce & Boyle, 1991; Bowen & Siniff, 1999; Bowen & Iverson, 2013). A common technique to study pinniped diets is the analysis of hard parts of prey remains found in the stomach or faeces (Bowen & Siniff, 1999; Walker et al., 1998; Santos et al., 2004; Pierce & Santos, 2003; Ridoux et al., 2007). Well known biases limit the method of hard part analysis, such as the requirement that hard parts are present and identifiable (Pierce & Boyle, 1991; Bowen, 2000; Tollit et al., 2004; Hauser et al., 2008; Phillips & Harvey, 2009). It is not uncommon for seals to consume the body of a large prey fish but not ingest the heads (Pitcher, 1980; Phillips & Harvey, 2009; Benoїt et al., 2011; O'Boyle & Sinclair, 2012; Benoit-Bird et al., 2013; Scheffer & Slipp, 2016). Bergström et al. (2022) presented that there might be similar size-selectivity indicated by frequent observations of large pike carcasses eaten by seals, with the head left intact. Hard part identification is dependent on the consumption of prey species, but enables the estimation of prey size and mass percentage and is a useful method as it provides quantified descriptions of diet (e.g. mass) due to specific hard part dimensions (such as otoliths or operculum) (Tollit et al., 2010).

Over the last decade, genetic methods for species identification have undergone extensive development and became of greater importance. Recently, next-generation sequencing techniques were established, where the DNA metabarcoding approach uses high-throughput DNA sequencing to rapidly produce vast quantities of taxonomic data from scats (Thomas, 2015). Analysing prey using DNA barcoding to investigate diet compositions found application in a wide range of species (Casper et al., 2007; Dunshea, 2009; Jarman et al., 2004) and furthermore has previously been successfully used in several pinniped studies (Casper et al., 2007; Méheust et al., 2014). As being a sensitive technique metabarcoding analysis overcomes biases of the conventional hard part method of diet analysis (Massey et al., 2021), but it should be mentioned that converting sequence reads into accurate biomass estimates is still a challenge as taxa-specific biases in the recovery of sequences (Bowles et al., 2011; Boyi et al., 2022; Deagle & Tollit, 2006; Pompanon et al., 2012; Austen et al., 2016).

Other methods reflecting a long-term diet analysis over weeks or months include Quantitative Fatty Acid Signature Analysis (QFASA) or stable isotope analysis (Ampela, 2009; Beck et al., 2005, 2007; Tollit et al., 2010; Lerner et al., 2018). The great advantage of these two methods is that they explain assimilated diet of a predator over an integrated period of time, compared to stomach content analysis that depicts ingested prey during a snap shot in time, still it is difficult to obtain taxonomic information on prey species consumed (Bowen & Iverson, 2013; Cherel et al., 2007; Granquist et al., 2018; York et al., 2008).

Each method has its own strength and weaknesses, morphological analysis allow for biomass estimates but is dependent on digestion errors and genetic analysis can detect small prey species not relying on retention of hard parts, it cannot give estimates upon digested biomasses and fatty acid and stable isotopes can elucidate long term patterns and shifts within the diet, but are not specific on taxonomic level (Leopold et al., 2001; Tollit et al., 2003; Bowen & Iverson, 2013; Bergström, et al., 2022). Up to date none can be universally recommended, advised is a combination of complementary methods to cover the whole diet spectrum (Tollit et al., 2010; Bowen & Iverson, 2013).

2.5 Research Hypothesis

Derived from previous work outlines above, this thesis is based on several hypothesis:

1. Diet composition varies among seals and geographical areas, most likely as a result of differences in local fish communities due to differences in the salinity along the coast of Mecklenburg-Western Pomerania.
2. Larger seals target larger prey species.
3. With rising decomposition of the sample, the detectability of prey species will decrease.
4. Taxonomic resolution of prey species will be higher using DNA metabarcoding compared to the morphological analysis

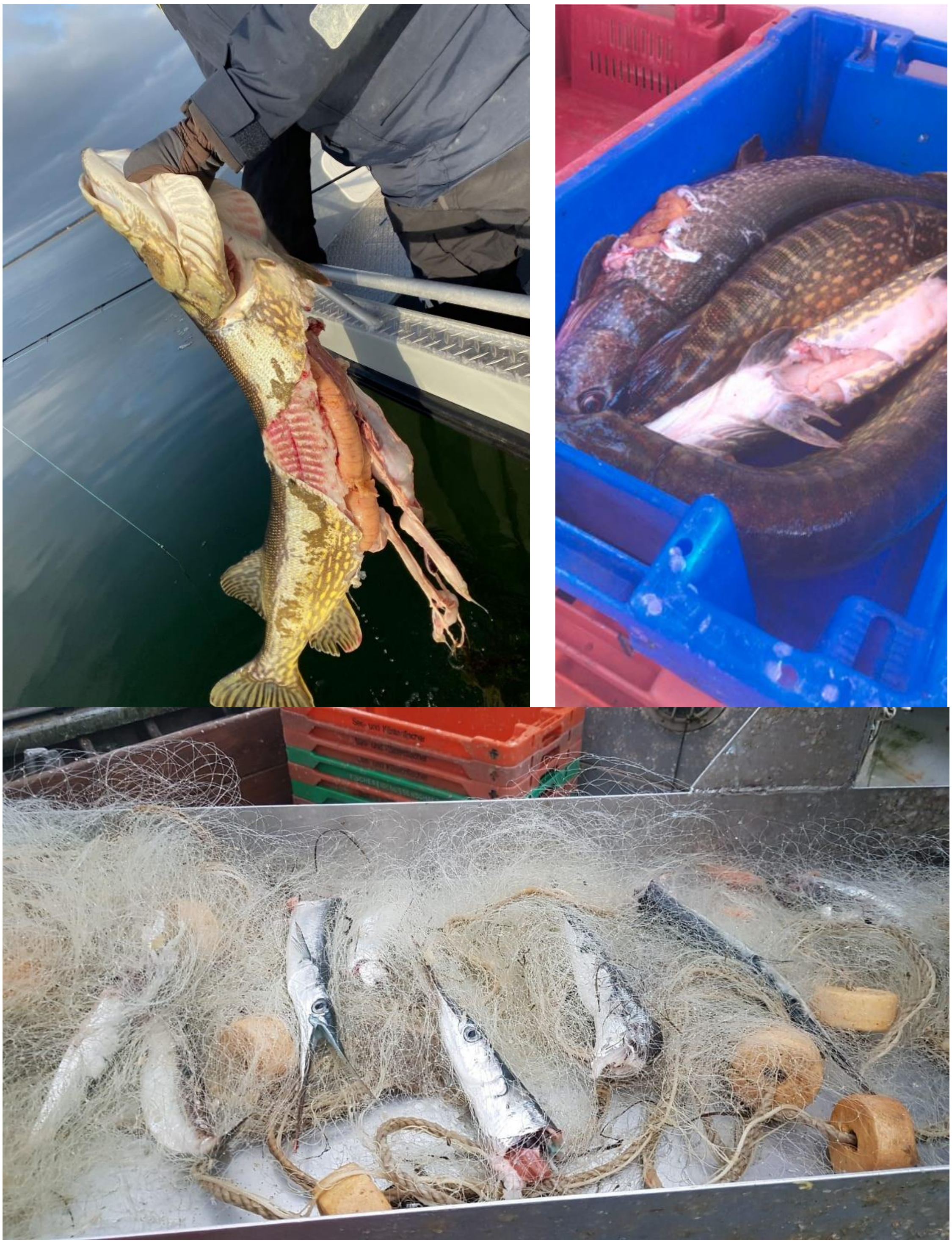

Figure 4 Gnawed fish – top: pike (*Esox lucius*), bottom: garfish (*Belone belone*) found in gear of commercial fisheries, presenting typical lesions caused by grey seals. © Moritz Gabrowski (top left), Steffen Schnorrenberg (top right), FIUM (bottom)

3. Materials and methods

3.1. Sampling

The contents of 23 stomachs and 107 intestines of Baltic grey seals (n = 107) were collected with the help of the monitoring network of the German Oceanographic Museum Stralsund. Necropsies of stranded carcasses or by-caught marine mammals are conducting as part of the marine mammal stranding program (Reif et al., 2023). Samples for this study were available from 2007 and from 2014-2022, and came from carcasses that had been washed up on the shore along the coast of Mecklenburg-Western Pomerania in 5 different areas: the north coast of North-West Mecklenburg and Vorpommern-Rügen (NWM), the west (WCR) and east (SC) coasts of the isle of Rügen, Greifswald Bay (GB), and the surrounding coast of Vorpommern (SCV) (Figure 5). Samples for both morphological analysis and DNA metabarcoding analysis (see below) were taken during standardized necropsies. During each necropsy, a decomposition status of the seals between 1 (very fresh), 2 (fresh), 3 (good), 4 (moderate) to 5 (decomposed)) was assigned to each individual (Lehnert et al., 2021).

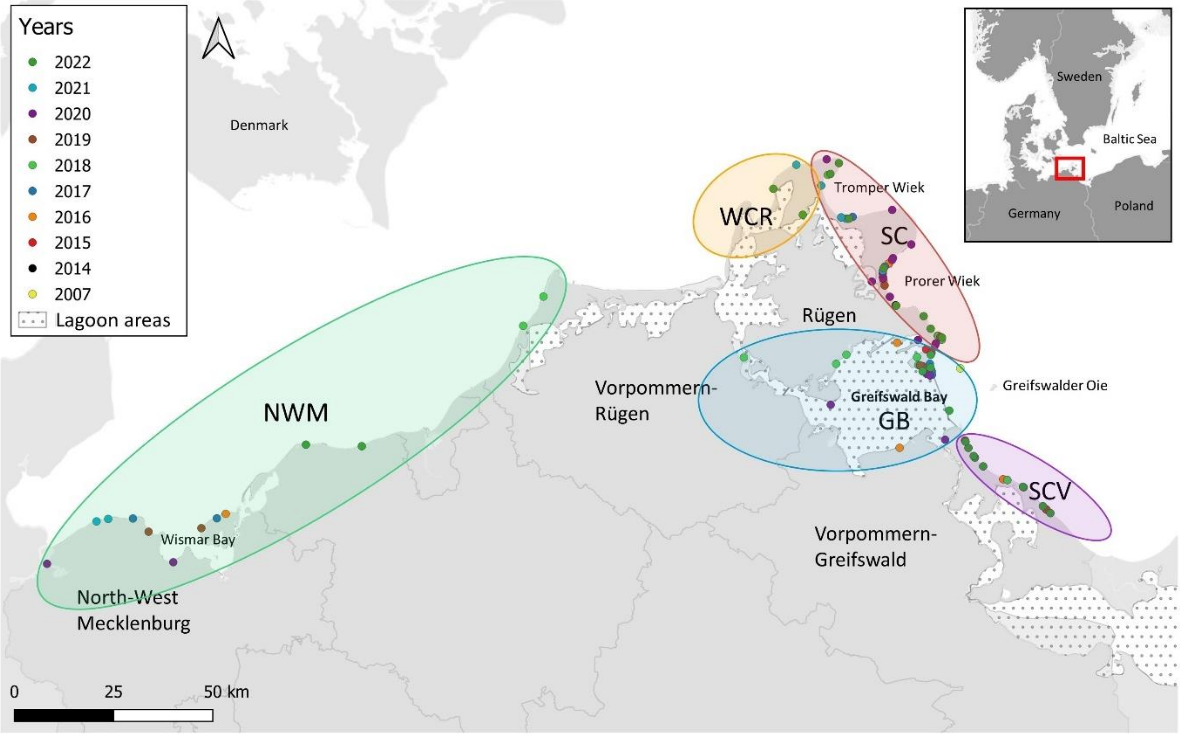

Figure 5 Locations at which 107 individual grey seals (resulting in 23 stomach and 107 intestine samples) were collected in the years 2007 and 2014 - 2022 from the Baltic Sea along the coast of Mecklenburg-Western Pomerania, northern Germany. Samples were divided into five different areas: NWM = North-Western Mecklenburg, WCR = West coast Rügen, SC = Surrounding coast eastern Rügen, GB = Greifswald Bay, SCV = Surrounding coast Vorpommern

Table 1 Number of seals and tissue types (stomach, intestine) collected according to year and area (n = 127). NWM = North-Western Mecklenburg, WCR = West coast Rügen, SC = Surrounding coast eastern Rügen, GB = Greifswald lagoon, SCV = Surrounding coast Vorpommern.

Year	*Total Seal*	*Stomach*	*Intestine*	*GB*	*NWM*	*SC*	*WCR*	*SCV*
2007	1	-	1	-	-	1	-	-
2014	1	-	1	-	-	1	-	-
2015	4	-	4	3	-	1	-	2
2016	6	6	1	2	1	2	-	1
2017	19	19	2	15	2	-	-	2
2018	16	16	-	10	-	3	2	1
2019	9	9	1	3	2	2	-	2
2020	19	19	7	5	2	12	-	-
2021	8	8	2	-	-	5	1	-
2022	21	21	15	6	2	9	1	7

3.2. Morphological analysis

Morphological analysis of prey-fish bones and other undigested hard parts was carried out on 13 whole intestines and 25 stomachs, from a total of 34 seals (Table 2). Samples were selected according to availability and matching genetic samples. The contents of the whole intestines and stomachs were weighed and the content was washed over a sieve system to collect hard parts. To that end, standardized sieve towers were stacked together with mesh sizes of 2 mm, 1 mm to 0.5 mm. The mass of stomach contents was determined by weighing stomachs again after the washing out. This was not possible for whole intestines due to the intestines being soaked with water. All bones and relevant hart parts emerging from the washing process in all three sieves were collected (Figure 6). Hard part structures (bones, chewing pads) and otoliths were used to identify prey fish species using the keys of Härkönen (1986), März (1987), and Leopold (2001). Otolith size was measured to calculate the biomass of prey fish using the regression equations from Leopold (2001). Otolith width was used because it is less prone to digestion (Figure 7). Left and right otoliths were paired, taking into account their size, and right otoliths were measured. If only the left otolith was recovered, this was used for measurement. Estimates for pikeperch, round gobies, and cyprinids rely on pharyngeal bone measures, and regression equations from Härkönen (1986), Azour et al. (2015) and Leopold (2001) were used for each species, respectively. Analyses of the diet were done to describe the frequency of occurrence for each prey species, the possible weight proportion for common species, and the size structure of the species (Lundström et al., 2007). The number of individuals per species in each stomach or intestine was counted based on the number of right- and left-side otoliths, right- and left-side spines (three-spined stickleback), chewing pads or pharyngeal teeth (cyprinids).

For the calculation of prey biomass for seal consumption, if no otoliths were found in the sample but other hard part structures were identifiable (such as scales, spines and pharyngeal teeth), all of them were assigned to one individual, independent of there were originally from different prey individuals (Svensson, 2021). In order to account for the possibility of the total digestion of otoliths, the numerical correction factors (NCFs) from Lundström et al. (2007) were applied on each determined species (Table 3).

Table 2 Samples used for morphological analysis of prey fish (n = 32 seals) including sampling area and year and quarter (Jan – Mar, Apr – Jun, Jul – Sep, Oct – Dec).

Seal_ID	*Year*	*Area*	*Tissue*	*Quarter*
M 13_16	2016	GB	Stomach	2
M 22_17	2017	SC	Intestine	3
M 25_17	2017	S	Intestine & Stomach	1
M 56_17	2017	GB	Intestine	4
M 55_17	2017	GB	Intestine	4
M 61_17	2017	GB	Stomach	4
M 63_17	2017	GB	Intestine	4
M 64_17	2017	SC	Intestine	4
M 25_19	2019	GB	Stomach	2
M 05_20	2020	NWM	Intestine & Stomach	1
M 07_20	2020	GB	Intestine	1
M 10_20	2020	SC	Stomach	1
M 16_20	2020	SC	Stomach	2
M 37_20	2020	SC	Stomach	2
M 56_20	2020	GB	Stomach	2
M 118_20	2020	GB	Stomach	4
M 177_21	2021	WCR	Stomach	4
M 167_21	2021	SC	Stomach	4
M 22_22	2022	SC	Stomach	4
M 23_22	2022	NWM	Intestine	2
M 25_22	2022	GB	Intestine & Stomach	2
M 27_22	2022	GB	Stomach	2
M 28_22	2022	WCR	Stomach	2
M 38_22	2022	SCV	Stomach	2
M 42_22	2022	SC	Intestine	2

M 43_22	2022	SCV	Stomach	2
M 44_22	2022	SCR	Stomach	2
M 45_22	2022	GB	Stomach	2
M 57_22	2022	GB	Intestine & Stomach	2
M 58_22	2022	SCV	Stomach	2
M 70_22	2022	MWP	Stomach	3
M 84_22	2022	GB	Stomach	3
M 88_22	2022	SCV	Stomach	3

Table 3 Numerical correction factors (NCFs) calculated by Lundström et al. (2007) applied to correct for biases caused by total digestion of otoliths.

Common name	**Scientific name**	**NCF**
Gobies	Gobidae	6.3
Sandeels	Ammodytidae	4.5
Hering	*Clupea harengus*	3.7
Pike perch	*Sander lucioperca*	2.4
Perch	*Perca fluviatilis*	1.8
Flounder	*Platychthis flesus*	1.7
Cyprinids	Cyprinidae	1.3
Cod	*Gadus morhua*	1.2

Results of number of non-empty stomachs and intestines (n=38) were used in all calculations. A Vacuity index (VI, Equation (eq.)1) was used to calculate the percentage of empty stomachs. To study the diet composition, an index of relative importance of each prey item (IRI, eq. 2), and a percentage of each prey species (%IRI, eq. 3) was calculated (Hyslop, 1980; Cortés, 1997). The IRI is calculated (eq. 1) as the sum of the wet weight % (W) or volumes of a prey item and the percent number (N), or count of species in the gut/intestine, and the percent frequency of occurrence (FO) (eq. 4), of species in the sample.

[1]

$$VI\ (\%) = A*100/B$$

[2]

$$IRI = \%F \times (\%W + \%N)$$

[3]

$$\%IRI = (IRI/\sum IRI) \times 100$$

where A is the total number of empty stomachs and B is the total number of stomachs examined (Hyslop, 1980).

Figure 6 All hard-parts recovered from the stomach of a subadult male Baltic grey seal (M171_22) on the outer coast of Rügen (SC). Fish bones recovered from stomach sample were used for the identification (*Gadus morhua*, *Clupea harengus* scull bones) and count of prey species. (Foto: K. Mehrwald).

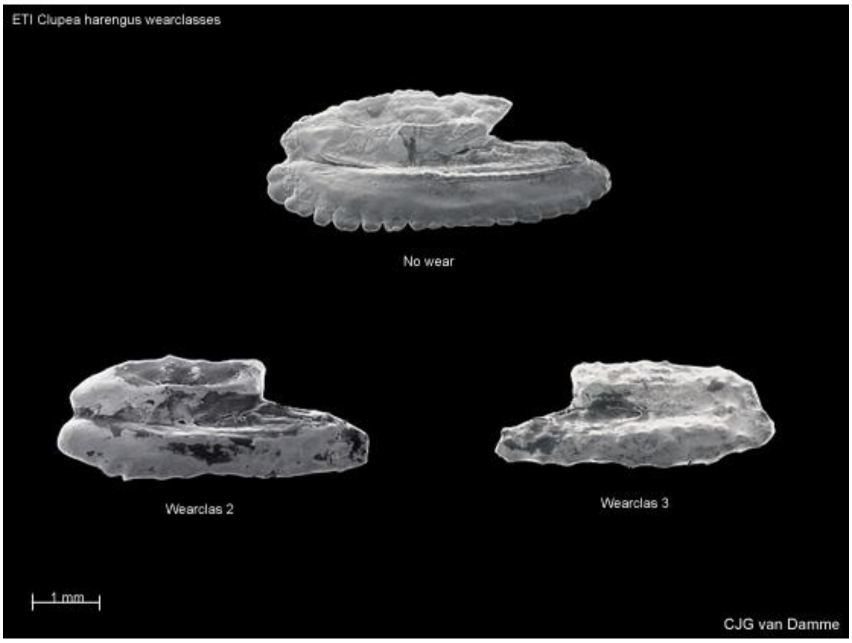

Figure 7 Otoliths from herring (*Clupea harengus*) showing the different erosion classes 1-3. Otoliths were used to estimate the original size of prey species and biomass. Figure: (Leopold et al. 2001)

3.3. Molecular analysis

For the molecular metabarcoding method, subsamples of the intestinal content and stomach content were taken during necropsies from 4 different locations of each intestine and 4 locations within each stomach (

The concentration of isolated DNA was measured by using a fluorometer. If necessary, the DNA concentration was diluted so that all 14 samples had the same DNA concentration. DNA was then stored at −20°C.

Table 4). Samples were placed in 50-ml falcon tunes and either stored at -20°C or placed in 99.9 % ethanol for transport.

DNA was extracted using 200 - 250 mg of homogenized intestinal or gut contents with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). A modification of the protocol based on Boyi et al. (2022) was to incubate the samples at 55°C in the InhibitEX buffer overnight to increase the amount of DNA.

DNA from potential prey fish and from grey seals were used as positive controls. For these, DNA was extracted from muscle tissue using DNeasy Blood & Tissue Kits (Qiagen). Fish DNA, collected during different sampling trips of fishing survey boats, included 14 species: mackerel (*Scomber scombrus*), roach (*Rutilus rutilus*), round goby (*Neogobius melanostomus*), northern pike (*Esox lucius*), sole (*Solea solea*), herring (*Clupea harengus*), flounder (*Platichthys flesus*), turbot (*Scophthalamus maximus*), plaice (*Pleuronectes platessa*), horse mackerel (*Trachurus trachurus*), sprat (*Sprattus sprattus*), whiting (*Merlangius merlangus*), cod (*Gadus morhua*), dab (*Limanda limanda*). Mock communities of four or more different potential prey species were created by equimolar pooling individual fish DNA, based on extracted fish species DNA.

The concentration of isolated DNA was measured by using a fluorometer. If necessary, the DNA concentration was diluted so that all 14 samples had the same DNA concentration. DNA was then stored at −20°C.

Table 4 Samples used for PCR and Illumina sequencing.

Seal_ID	Year	Loction	Tissue	Quater
M 13_16	2016	GB	Intestine	2
M 14_16	2016	GB	Intestine	2
M 38_16	2016	NWM	Intestine	3
M 64_16	2016	SC	Intestine	4
M 68_17	2017	GB	Intestine	3
M 47_18	2017	GB	Intestine	3
M 55_17	2017	GB	Intestine	4

M 78_17	2017	GB	Intestine	3
M 65_17	2017	GB	Intestine	3
M 63_17	2017	GB	Intestine	4
M 64_17	2017	GB	Intestine	4
M 75_17	2017	GB	Intestine	4
M 62_17	2017	GB	Intestine	4
M 25_19	2019	GB	Stomach	2
M 10_20	2020	SC	Stomach	1
M 16_20	2020	SC	Stomach	2
M 56_20	2020	GB	Stomach	2
M 118_20	2020	GB	Intestine	4
M 07_21	2021	NWM	Stomach	1
M 03_22	2021	SC	Intestine	4
M 56_22	2022	GB	Intestine	2
M 58_22	2022	SCV	Intestine	2
M 20_22	2022	GB	Intestine	2
M 22_22	2022	SC	Intestine	2
M 25_22	2022	GB	Intestine	2
M 28_22	2022	WCR	Stomach	2

In order to target species specific mtDNA loci, existing primers were reproduced covering 137 of possible prey species (Winkler & Schröder, 2003; Rittweg et al., 2023) based on Boyi et al. (2022). Furthermore, an additional primer was designed in order to cover every possible species of the southern Baltic region (Table 5). For designing species sequences were extracted from the NCBI database and Unipro UGENE (version 47.0, 2023) was used to align sequences and visually identify suitable primer regions.

Target polymerase chain reactions (PCRs) were performed in 25 µl volumes, containing 5 µl of 5x Reaction buffer, 1 µl of each of the primers, 0.5 µL of dNTP (25 mM), 0.4 µL Herculase II Fusion DNA Polymerase, 0.25 µl $MgSO_4$ (100 mM), 10.1 µl of DEPC- H2O and 5 µl of DNA template in a multicycler pro (Eppendorf). Cycling conditions were: denaturation at 95°C for 2 min, followed by 37 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 30 s. PCR products were visualized on a 1.4 % agarose gel using GelRed DNA gel stain.

PCR purification, indexing PCR, and next-generation sequencing were performed at the Berlin Center for Genomics in Biodiversity Research. PCR products were purified by first washing with ethanol and magnetic beads. After this washing process, a second indexing PCR followed, where a combination of individual indices was attached to each sample for the Illumina sequencing. The adapters consist of 3 components: the sequence complementary to solid support (oligonucleotides on flow cell), the barcode sequence (indices for multiplexing) and the binding site for the sequencing primer (Illumina, 2010). Two further purification steps were performed in order discard any unwanted side products. A quantification of the indexing PCR products was performed to analyze the DNA concentration. A quantification of the indexing PCR products followed and samples were pooled in an equimolar ratio.

Samples were equimolar pooled and sequenced with an Illumina MiSeq, where the added indices serve as reference points during the amplification, sequencing and analysis (Illumina, 2010). The modified DNA was loaded onto a non-patterned flow cell for amplification and sequencing (Bronner & Quial, 2019).

Table 5 Primers used for this study. *Indicates phosphorothioate (PTO) bonds (Boyi et al., 2022).

Primer name	Primer sequence (5‘-3‘)	Gene	Length of amplicon (bp)
Fish_16S_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGAGAAGACCCTRTGRAGC*T**	16S rRNA	190 bp
Fish_16S_Fnew	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGAGAAGACCCTDTGRAGC*T**		
Fish_16S_R1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCRCGGTCGCCCCAACCAA*A**		
Fish_16S_R2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCATGGTCGCCCCAACHGA*A**		
Fish_16S_R3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCGTGGTTGCCCCAACCTA*A**		
Seal_blocker	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGAGAAGACCCTATGGAGCTTTAATTAA*C**		

3.3.1. Bioinformatic process

Primary data analysis was conducted on the sequencing instrument including base calls and quality scores from the imaging during sequencing. Raw sequence data (fastq.gz files) were analysed using the Dada2 package (Callahan et al., 2016) for R.

A quality check was performed upon the dada2 output in which only ASVs at or near the length of the target region (i.e., 73-90 bp) were kept. In the final step all sequences were submitted to NCBI GenBank as a blast file to make taxonomic assignments. Species level identifications based on similarities ≤ 98% were removed from the dataset. Multiple ASVs that were identified to the same species were collapsed and reads counts were summed to produce a total number of reads per species. Seal sequences were removed from the data set.

3.3.2. Data analysis

Metabarcoding data were analyzed as percentage frequency of occurrence depending on presence/absence data for each species (%FO; eq. [4]) and sequence relative reads abundance depending on the read number (RRA; eq. [5]) according to Deagle et al. (2019) calculated as follows:

[4]

$$\%FO_i = \frac{1}{S}\sum_{k=1}^{S} l_{i,k}\ x\ 100$$

where S is the number of samples, and i is an indicator function such that Ii, k = 1 if food item i is present in sample k, and 0 if not (Deagle et al., 2019).

[5]

$$RRA_i = \frac{1}{S}\sum_{k=1}^{S}\frac{n_{i,k}}{\sum_{i=1}^{T} n_{i,k}}\ x\ 100\%$$

where ni, k is the number of sequences of food item i in sample k (Deagle et al., 2019).

To evaluate species richness and within different sampling areas alpha diversity was determined as well as beta diversity for morphological samples. Shannon Index (SHDI see eq. 6) (Shannon & Weaver, 1948) and (SIDI, see eq. 7) (Simpson, 1949) were calculated.

[6]

$$SHDI = 1 - \sum i = 1\ x\ Npi\ x\ lnpi$$

where *N* is the number of area types and p_i the proportional abundance of the *i*th type. This index, ranging in theory from 0 to infinity, estimates the average uncertainty in predicting which area type a randomly selected sub-unit of the landscape will belong to.

[7]

$$SIDI = 1 - \sum_{k=0}^{n} i = 1\ Npi\ x\ pi$$

Producing values from 0 to 1, Simpson's index defines the probability that two equal-sized sub-units of the area, selected at random, belonging to different cover types.

Overall, gamma diversity was calculated for metabarcoding samples and morphological samples in order to allow comparison between different approaches.

3.3.3. Statistical analysis

The software RStudio (2023.06.1, +524, www.r-project.org) was used for all analyses.

Results were analyzed according to year (2014 - 2022), season (quarter of the year 1-4; Jan -Mar, Apr – Jun, Jul – Sep, Oct - Dec), region (GB, WCR, SC, SCV and NWM), and seal length (100cm – 230cm).

For the morphological analysis non-parametric Kruskal-Wallis tests were used on proportional data and post-hoc to determine exact influences of areas. In order to analyze dependency of prey length and seal length cross-validation was performed, generalized linear model (GLM) and generalized additive model (GAM) was applied on log transformed data (Witten et al., 2013).

To calculate frequency of occurrence and IRI, as well as for RDA analysis, *Ammodytes tobianus* was grouped within Ammotidae, as only one sandeel could be identified to species level. Percidae included *Perca fluviatilis* and *Sander lucioperca*, as it could not be identified which of the two species was more commonly consumed based on the available data. Clupeidae were included in *Clupea harengus* since only one individual could be identified and the majority of clupeids consumed could be identified as herring. Furthermore, cyprinids were placed into *Rutilus rutilus*, as being the main species consumed. Trying to avoid strong weighing of several species in the diet and avoiding bias.

PERMANOVA analysis was conducted on abundance data to analyze significant differences. Bray-Curtis dissimilarities were estimated depicting beta diversity on Hellinger transformed abundance data for each species. Morphological samples were visualized by using non-metric multidimensional scaling out of the 'vegan' package (version 2.6-4) and further SIMPER analysis was conducted to see which species accounted for most variation

(Oksanen, 2022). Redundancy analysis was performed to gain insight into relationship of variables (location, year, quarter, seal length, prey length and prey weight) (Legendre & Legendre, 2012).

For the genetic analysis the DNA-concentrations were log transformed in order to match the assumptions of normality in a better way. Non-parametric tests were applied to analyze differences and further post-hoc analysis using Dunn testing to reveal contribution of differences. Comparison of tissue concentration was performed applying a two-way ANOVA, as concentrations matched assumptions on normal distribution. For genetic data, reads amplicon sequence variant (ASV) were produced due to inferred single DNA sequences recovered from a high-throughput analysis (Callahan et al., 2016).

For testing the relationship of decomposition and the relative read abundance, log transformed data was used and cross validation models applied. Due to analysis, multivariate regression was applied for log transformed data. Due to a low sample size, log transformed data was bootstrapped and afterwards the generalized linear model (GLM) was applied to test for differences of decomposition states, identified as most suitable model fit. Analyzing patterns between variables (location, year and quarter) based on species abundance a principal component analysis (PCA) was conducted. Permutation test was run, to analyze significant influence of the tested variables.

To test for similarities between morphological and genetic analysis, PERMANOVA was used on relative abundances of species abundance data to estimate dissimilarities of metabarcoding and morphological approach. Furthermore, SIMPER analysis was performed to identify if prey species causing most of the dissimilarities. Additionally, Hellinger transformed relative abundances were compared within NMDS.

Visualization of the data was conducted using ‘ggplot2’-package v3.3.2 (Wickham 2016).

4. Results

4.1. Morphological analysis

4.1.1 Numerical analysis

A total of 185 individual prey items were successfully identified within 37 intestinal and stomach samples obtained from a total of 32 grey seals. One stomach was found empty, while two stomachs from the surrounding coast and the Greifswald Bay contained solely nematodes. This is making up a Vacuity Index (VI) of 9.4 %. Empty or only nematode particular samples were excluded from further analysis and therefore 30 unique seal samples. Among the remaining samples, 12 different species could be identified to species level. Due to digestion status of the remaining bones/bone fragments, it was possible to classify 5 families as the hard parts did not allow an identification to species level. Figure 8 (a) shows the abundance of individual prey species by the numerical abundance of counted individuals within the diet of all analysed samples. The two most abundant species, in terms of numerical abundance, within the seal diet were *Clupea harengus* (35.2%, n = 64), *Neogobius melanostomus* (30.8%, n = 57) (Figure 8). In 66.6 % of the samples more than one prey species was identified, whereas in the remaining 33.3 % only one prey species could be distinguished. Overall, round goby had the highest mean abundance of prey species averaged across all samples (9 individuals ± 19), followed by herring (4.3 ± 4.5). A long-tailed Duck (*Clangula hyemalis*) was identified in one sample as the only non-fish prey species.

When applying the numerical correlation factors (NCFs) of each species to the analysed data, the most abundant species were *Neogobius melanostomus* (50.3%, n = 352.8), *Clupea harengus* (33.8%, n = 236.8) and *Gadus morhua* (2.6 %, n = 18). Whereas the proportion of herring stayed relatively stable, the importance of all other species is declined, e.g. Gadidae and freshwater species (Figure 8).

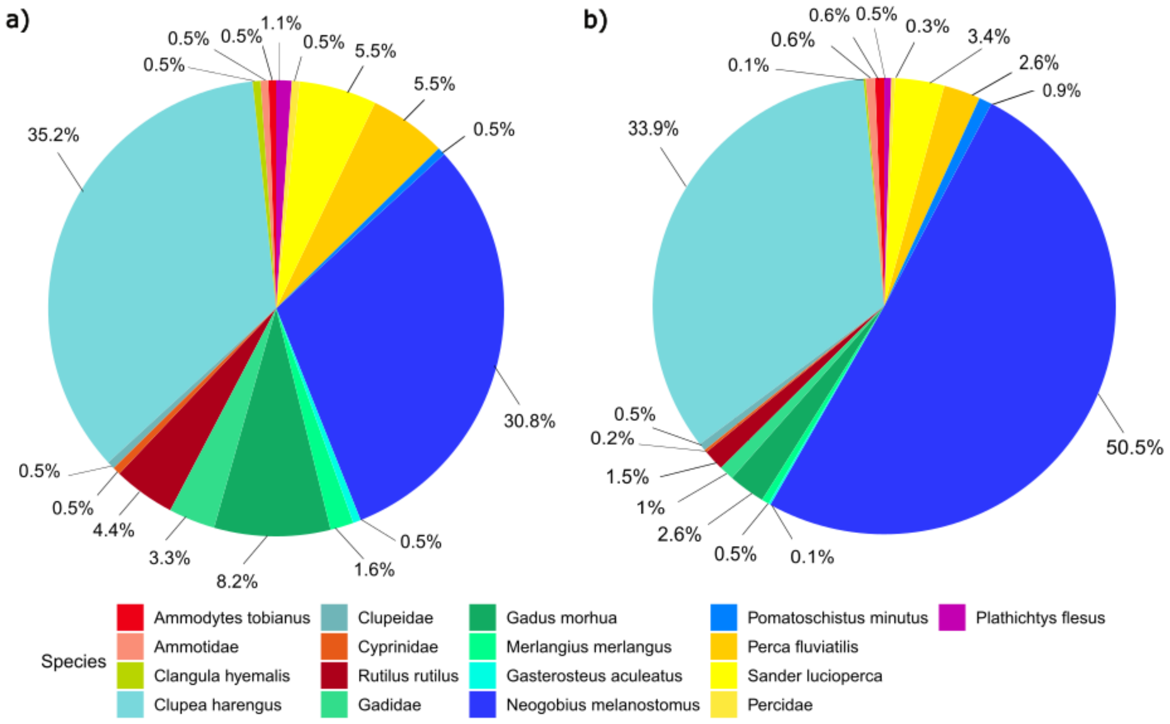

Figure 8 Percentage distribution of the numerical abundance of species identified in 30 samples of grey seal intestines and stomachs collected across various years (2016 - 2022) and locations in Mecklenburg-Western Pomerania. (a) Percentages of the numerical abundance of species analysed/detected in the samples; (b) numerical correction factor (NCF) (Lundström et al., 2007) for specific species included presenting corrected percentage distribution of species.

Table 6 Frequency of occurrence (i.e., proportion of the seals in which the prey species was found) and mean abundances (number of individuals of each prey species in a given seal gut) of identified prey species derived from analysed stomach and intestine samples of grey seals (n = 32).

Species	Common name	FO (%)	Frequency of occurence (n)	Mean abundance (+ sd)
Ammotidae		3.1	1	1
Ammodytes tobianus	small sandeel	3.1	1	1
Clangula hyemalis	long-tailed duck	3.1	1	1
Clupeidae		3.1	15	1
Clupea harengus	herring	46.9	1	4.27 ± 4.53
Cyprinidae		3.1	1	1
Rutilus rutilus	roach	21.9	5	1.14 ± 0.38
Gadidae		15.6	7	1.2 ± 0.45
Gadus morhua	cod	21.9	1	2.14 ± 2.19
Merlangius merlangus	whiting	6.3	2	1.5 ± 0.71
Gasterosteus aculeatus	three-spined sickleback	3.1	6	1
Neogobius melanostomus	round goby	18.8	5	9.33 ± 19.0
Pomatoschistus minutus	sand goby	3.1	1	1
Percidae		3.1	2	1
Perca fluviatilis	perch	15.6	1	2 ± 2.24
Sander lucioperca	pike-perch	12.5	7	2.5 ± 1.29
Plathichthys flesus	flounder	6.3	4	1

Analysing the prey species abundance according to the different areas, the presence/abundance of herring was evident in all areas except the surrounding coast of Vorpommern (Figure 10). Overall, the distribution of samples in each region was as follows: North-West Mecklenburg contained three seals, the west coast of Rügen two seals, the surrounding coast of Rügen nine, Greifswald lagoon twelve and surrounding coast of Vorpommern four. Herring appeared in the analysis with 30.9 % (95 % CI: 22.3 -39.5) within Greifswald lagoon, while round goby had 45.5 (95 % CI: 36.1 – 54.6). Furthermore, herring made up 50 % (95 % CI: 23.8 – 76.2) within NWM and along the surrounding coast of Rügen it appeared with 63.3 % (95 % CI: 47.2 – 80). No herring was found within the samples from the surrounding coast of Vorpommern, where pike-perch was most abundant in the samples (53.8 %, 95% CI: 26.7 – 80.9), followed by round goby (23.1%, 95% CI: 0.2 - 46) and perch (15.4 %, 95% CI: 0 - 35). In NWM a higher proportion of pike-perch could be estimated/observed, accounting for 14.3 % (95% CI: 0 – 32.6). Within two analysed seals of the west coast Rügen cod emerged as the primary species at 58.3 % (95% CI: 30.4 – 86.2), followed by round goby (25 %, 95% CI: 0.5 – 49.5) and herring (16.7 %, 95% CI: 0 -37.8). Most species were identified from animals in 2017 and 2022 (Figure 9). Herring was present in all years except 2019, when only one sample was analysed (39 % in 2017 to 19.5 % in 2022). The amount of roach increased from 10.7 % in 2017 to 24.6 % in 2022. Separated into different quarters of the year, most species were found in quarters 2 and 4. Roach were most present in quarter 3 (32. 4 %) and percids in quarter 4 (23.4 %) (Figure 9).

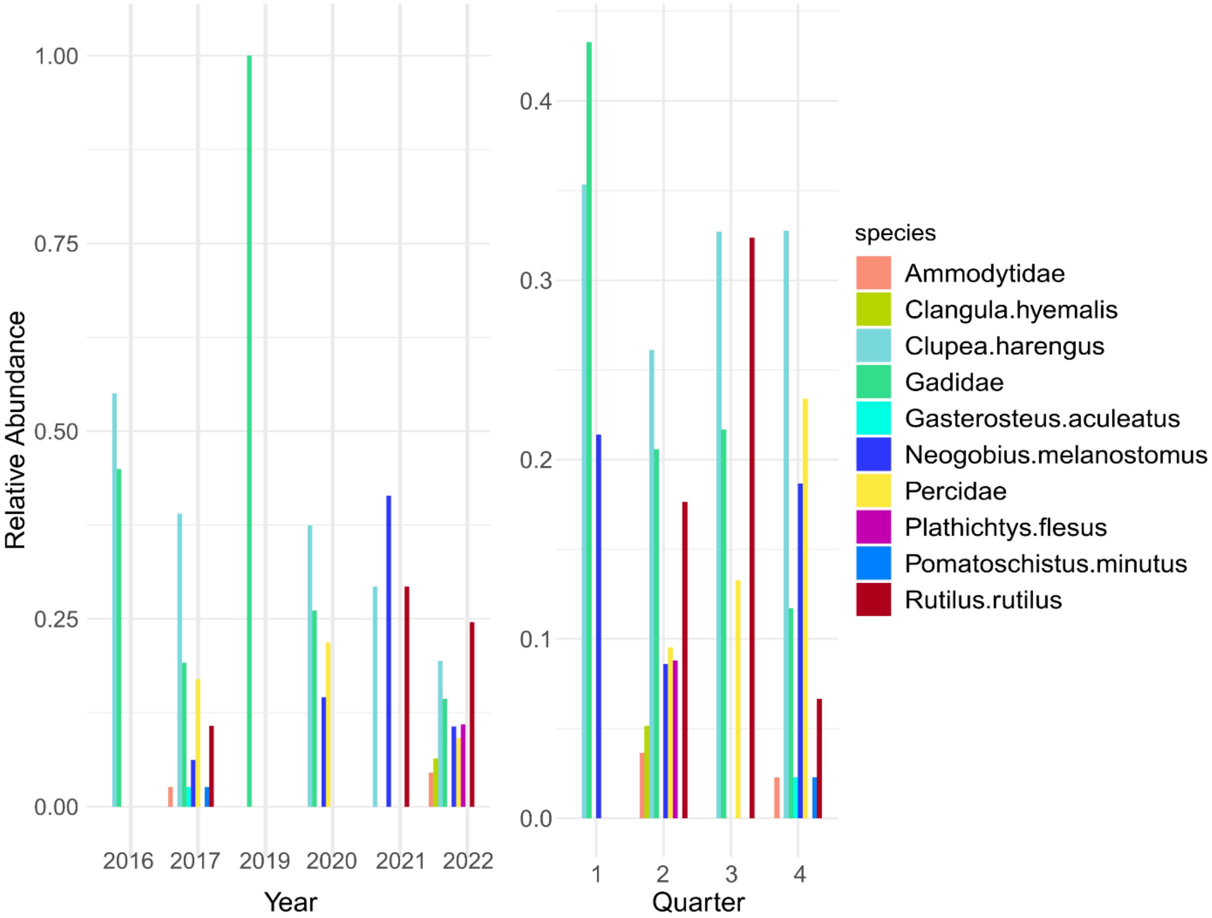

Figure 9 Relative abundance of prey species in years 2016 – 2022 and different seasons (quarter 1-4). a) Distribution of species among years (2016: n = 1, 2017: n = 8, 2019: n = 1, 2020: n = 8, 2021: n = 2, 2022: n= 15), b) Distribution of species among different seasons per quarter of the year (quarter 1: n = 4, quarter 2: n= 15, quarter 3 n = 4, quarter 4: n = 9).

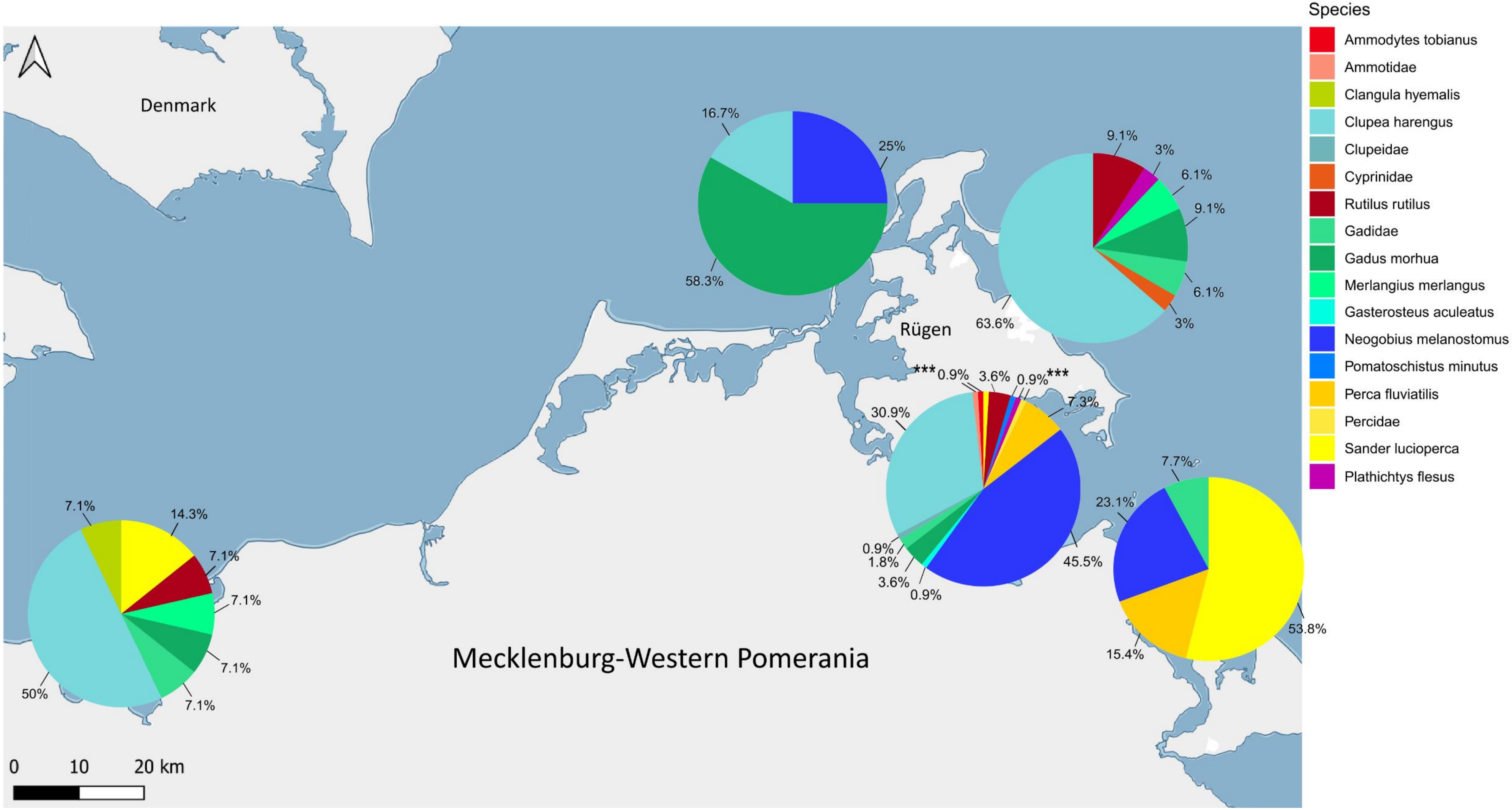

Figure 10 Prey species found within the stomachs and intestines of grey seals and identified morphologially (n = 32) in the five study areas. In Greifswald Bay (GB), six species were 0.9%; two are labelled and four are represented with ***.

The proportion of prey items in guts varied significantly among locations (chi^2 = 15.053, df = 4, *p* = 0.004592). However, an adjusted Wilcoxon analysis did not verify these significant differences. Specifically, between the Greifswald Bay and North-Western Mecklenburg (*p* = 0.15), as well as surrounding coast of Vorpommern and the Greifswald Bay (*p* = 0.17) no significance was found. Similarly, the West-coast of Rügen (*p* = 0.28) and surrounding coast of Rügen (*p*-value = 0.29) showed differences compared to species proportions within the Greifswald lagoon.

To check for potential biases, the number of seals containing each species was analysed. Round goby occurred in seals from six different locations, with three originating from the Greifswald Bay. Moreover, herring was present in 15 grey seals across the studied areas (Figure 11).

In contrast to the original counts, NFC-corrected values yielded a significant difference in the prey species composition (chi^2 = 70.799, df = 4, *p* = 1.539e-14) and further post-hoc analysis revealed significant difference in species abundance between specific locations: Greifswald Bay and NWM (*p* = 5.6e-06), GB and east coast of Rügen (*p* = 8.7e-08), GB and surrounding coast of Vorpommern (*p* = 3.7e-05) and GB and west coast of Rügen (*p* = 0.00012). However, no significant differences in species abundance were observed between all the other areas. Herring was found in seal guts in nearly every location in Greifswald Bay, the surrounding coast of eastern Rügen (SC) and Western Mecklenburg-Pomerania (n = 11, 34, 21 seals, respectively), and was present in 15 grey seals in total (Figure 11). The highest number of round gobies (48 individuals) was determined in Peenemünde. Round goby occurred in seals from six different locations, with three originating from the Greifswald lagoon. Thiessow showed highest species variability (n = 10), followed by Peenemünde and Neu Reddevitz (n = 5), both located within the Greifswald Bay (Figure 11).

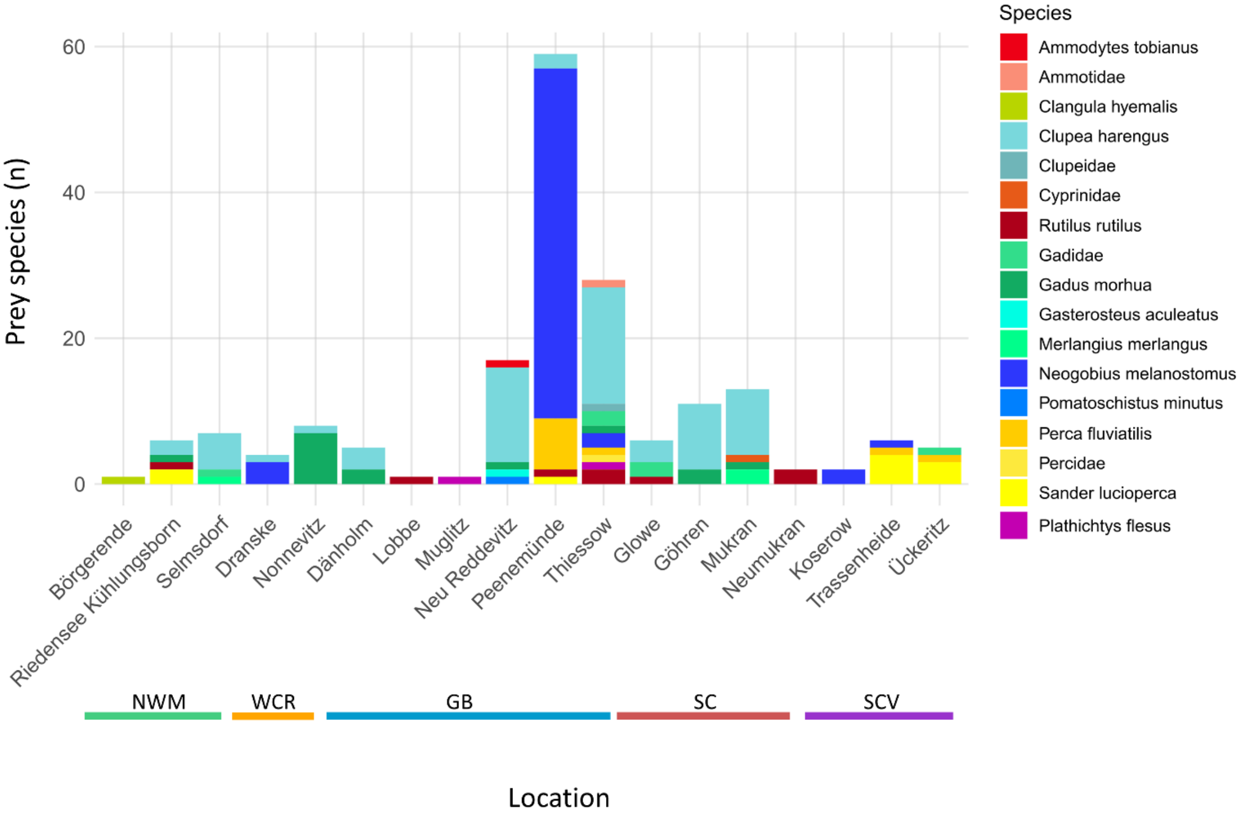

Figure 11 Distribution of prey species individuals counted in stomachs and intestines of recovered seals in specific locations within the five study sreas along the coast of Mecklenburg-Western Pomerania. Specific locations taken together into bigger areas, including NWM (North-West Mecklenburg), WCR (west coast Rügen), GB (Greifswald lagoon), SC (surrounding east coast of Rügen), SCV (surrounding coast Vorpommern) labelled below each location. Similar prey species colouration indicates relationship among species.

PERMANOVA analysis was performed to assess whether prey species composition differed according to location, decomposition stage of the seal, year, quarter of the year, and seal length. There was no statistically significant difference across these factors (Table A3, appendix). Notably, adjusting the alpha value, the year variable shows the lowest and most significant p-value = 0.09 (R^2 = 0.07, F = 2.04), suggesting a potential borderline significant effect. The NMDS scatterplot revealed a lot of dietary overlap among different areas, years and seasons, with overlapping ellipses indicating similar prey species composition (Figure 12). Further SIMPER analysis, according to different areas, presented that herring was contributing for most (23.5%) of the dissimilarities of the surrounding coast of Rügen compared to and Greifswald lagoon. Followed by percids and gadids with contributions of around 55.1% and 40.4%, respectively. Between SC and NWM, roach dominated the contributions with 26.7 %, followed by and gadids (67.4 %) and herring (47.7 %). While comparing the comparison between east coast of Rügen and west coast of Rügen, round goby led with a 41.5%

contribution, followed by herring (61.6 %) and roach (73.3 %). While comparing different years herring seemed to have the most influence in variation, with 28%, 52 % and 24 % (2017 & 2020, 2017 & 2021, 2017 & 2022, respectively). Furthermore, between 2017 and 2020 percids contributed up to 51 %, in 2017 and 2021 round goby contributed 28% cumulatively and in 2017 and 2022 roach up to 43 % to dissimilarities. Comparing different seasons, it seems that herring accounted for most of the dissimilarities, roach contributes cumulatively up to 26% and herring up to 50% between quarter 3 and 4, between quarter 3 and 1 roach contributed cumulatively up to 28% and gadids up to 56%. Between Quarter 3 and 2 roach contributed cumulatively up to 27% and herring up to 51%.

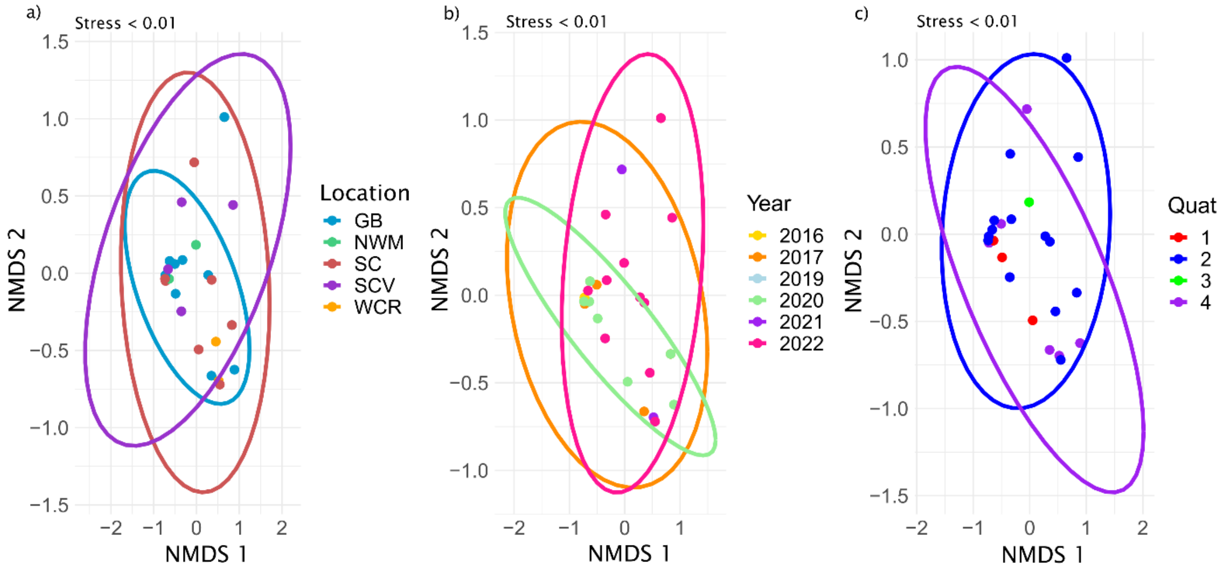

Figure 12 Non-metric multidimensional scaling graph (NMDS) graph presenting dissimilarities of Hellinger transformed species occurrences within the diet of grey seals among different areas. GB = Greifswald lagoon, NWM = North West Mecklenburg, SC = Surrounding coast east Rügen, SCV = surrounding coast Vorpommern, WCR = west coast Rügen. Circles represent 95 % CI intervals. a) Relation of location and species abundance, b) relation of species and years, c) relation of season defined as quarters and species occurrence.

To investigate the relationships between species abundances and various environmental factors, a Redundancy Analysis (RDA) was conducted. Analysing if certain patterns exist in the relation of Hellinger transformed abundance of different fish species and location, year, quarter and seal length, RDA analysis revealed no distinct relationship of variance in species abundance (Figure 13). High VIF values for variables suggest that there's multicollinearity in the dataset and some of the predictors are correlated with each other. Specifically, certain levels of the location variable demonstrated infinite VIFs, indicating perfect multicollinearity. To address this, the reference level for location was adjusted, selecting GB as the baseline. This action effectively removed the potential for multicollinearity from the dataset, allowing for a more robust RDA. RDA was carried out on the transformed species abundance data, more suitable for linear methods. Each species is represented by an arrow (Figure 13), with the direction and length of the arrow indicating the relationship strength and direction with the environmental gradients. Permutation test under the reduced model was conducted. Based on the RDA results and subsequent significance testing, there is insufficient evidence to conclude that the environmental variables (location, quarter, year, and seal length) included in the model significantly explain the variance in species abundances. It seems that round goby is related to WCR, gadids and herring are stronger related to the year 2019.

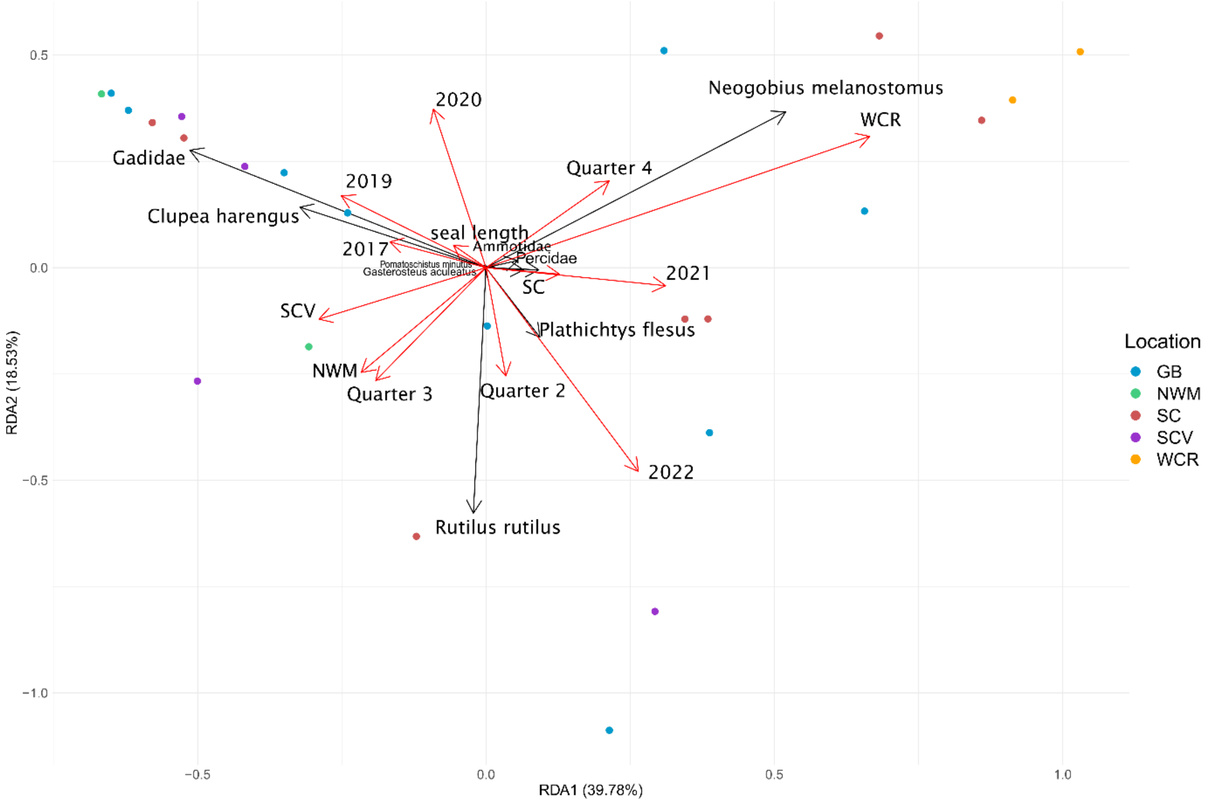

Figure 13 Relationship of species abundance and environmental variables (location, year, quarter and seal length) based on an RDA on Hellinger transformed relative abundance.

4.1.2 Biomass consumption

In the next step the biomass of prey species consumed by grey seals were calculated to check if the relative importance of different prey items, as well as to see if they are foraging preferences. Therefore, 15 grey seals hard parts such as otoliths, chewing pads or pharyngeal bones were recovered and enabled to estimate the consumed prey fish biomass. Due to degradation or missing of bone structures, only 15 animals were used in this analysis. In Table 7, the calculated biomasses (total and mean mass) are presented and notably, cod presenting the largest proportion, followed by perch. The lowest percentage of consumed biomass were sandeel and sand goby. Overall highest consumed biomass was presented by cod (42.6 %, 95 % CI = 0.21), perch (18.9 %, 95 % CI = 0.26) and herring (12.1

%, 95 % CI = 0.21). Furthermore, round goby (11.7 %, 95 % CI = 0.2) and whiting (8.9 %, 95 % CI = 0.18) biomass could be calculated. Lowest percentages were estimated over all analysed seals with by pike-perch (4.73 %, 95 % CI = 0.13), roach (1.3 %, 95 % CI = 0.07), sandeel (0.04 %, 95 % CI = 0.01) and sand goby (0.01 %, 95 % CI = 0.009).

Additionally, the biomass distribution within the five areas were also provided. Estimated biomass in Greifswald lagoon perch (m = 215.5 g, sd = 2.3) and pikeperch had highest weights (m = 204.4 g), followed by cod with 198g. Herring (m = 77.4 g, sd = 48.4). Goby (*N. mealanostomus* m = 12.7 g, sd = 11.9; *P. minutus* = 1.3g) and sandeel (2.8 g) could be estimated as well (n = 4). In NWM only one seal contained herring (m = 52.3 g). Along the Surrounding coast Rügen seals (n = 5) were found to have consumed herring (m = 78 g, sd = 38.3), cod (m = 415 g, sd = 320.2), whiting (m = 305.9 g, sd = 47.3) and roach (m = 90.9 g). Round goby (m = 40.3 g, sd = 4.9) and pike-perch (m = 19.9 g, sd = 10) could be estimated within the surrounding coast of Vorpommern (n = 3). Within the west coast of Rügen cod (m = 206.8 g, sd = 222) and round goby (m = 27.5, sd = 19.1) could be identified (n = 2).

Table 7 Estimated biomass presented as total weight, mean mass including standard deviations and frequency of occurrence of prey species within 15 analysed grey seals.

Species	Common name	Total mass (g)	Mean mass (g) (+ sd)	Frequency of occurrence by mass (%)
Ammodytes tobianus	small sandeel	2.8	2.8	3.1
Clupea harengus	herring	775.4	75.2 ± 43	12.5
Gadus morhua	cod	2890.6	263 ± 244	12.5
Merlangius merlangus	whiting	611.9		3.1
Neogobius melanostomus	round goby	801.1	15.1 ± 13.8	12.5
Pomatoschistus minutus	sand goby	1.3	1.3	3.1
Perca fluviatilis	european perch	1293.2	216 ± 62.3	3.1
Rutilus rutilus	common roach	91.0	91	3.1
Sander lucioperca	pike-perch	323.9	46.3 ± 70.3	9.4

To validate the relationship between the length of grey seals and the length of the prey fish, two statistical models were employed: a linear regression and a Generalized Additive Model (GAM). The regression model ($y=-123.2690+1.9204x$, $R^2 = 0.38$, $p = 2.72e-11$, RMSE = 69.59), assuming a straight-line relationship between the variables, indicates a statistically significant relationship between seal length and prey length. Around 38.75% of the variation in prey length can be explained by the seal length (Figure 14). The GAM fit suggests that as seal length increases, there is a general trend of increasing prey length, but this relationship isn't strictly linear. GAM, which allows for capturing both linear and non-linear relationships, shows a significant non-linear trend (F = 24.4, *p* = 1.297e-11) between seal length and prey length. While the linear regression provided a simplified, straight-line representation of the relationship, the GAM offers a more nuanced view. For data that shows clear non-linear patterns, the GAM fit is likely a more accurate representation of the underlying relationship. The diagnostic plots for the GAM, such as residuals vs. fitted values, were inspected to ensure the model's assumptions were met (Appendix A1, A3). The plots and accompanying statistical tests (W = 0.98, *p* = 0.33) support the appropriateness of the GAM for this data.

A closer analysis in the RDA analysis upon the relationship of location, year, season took previously seal length and also prey fish length into account. The RDA depending on the seal length relationship presented within the permutation test a significant influence upon the variables and seal length. With an F-statistic of 21.61 and a p-value of less than 0.001, we can confidently reject the null hypothesis that the observed relationships are due to chance. The predictors collectively explain a variance of 822.1 in the response variable. There is a statistically significant positive association between prey fish length and length of the seal. Specifically, for every unit increase in prey length, the seal length increased by an estimated 0.2 units (*p* = 5.73×10−75.73×10−7). 95% confidence interval to ascertain the range within which the true effect size likely resides. For our prey species length predictor, this interval spanned from 0.2−(1.96×0.04)0.2−(1.96×0.04) to 0.2+(1.96×0.039)0.2+(1.96×0.04), providing 95% confidence that the actual impact of prey length on seal length is captured within these bounds. Length as predictor alone accounts for approximately 12.58% of the variability in seal length (adjusted $R^2 = 0.13$). RDA results underscore the significant influence of prey species length in determining seal length. The predictors collectively explain a variance of 822.1 in the response variable, which is meaningful in the context of the model.

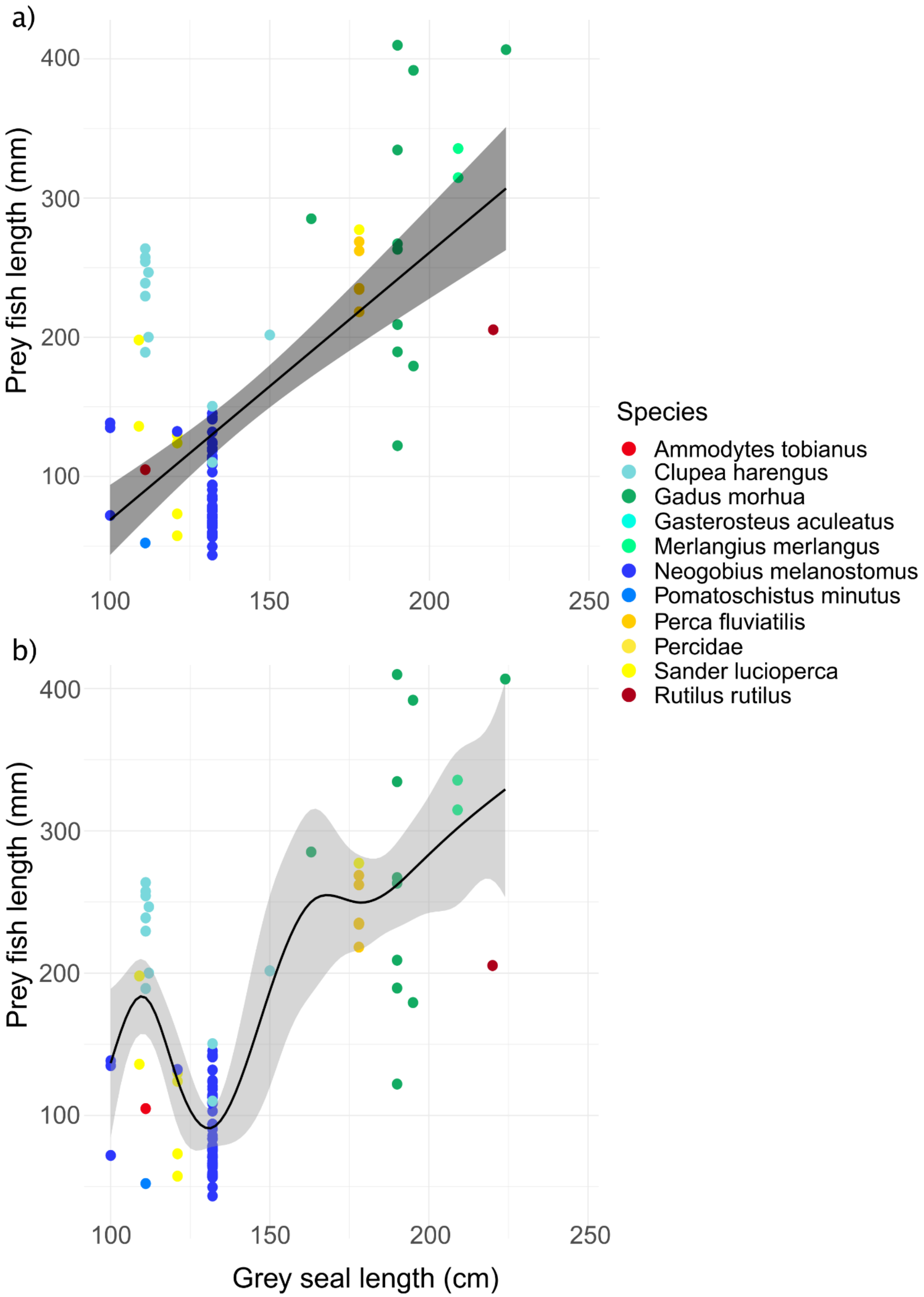

Figure 14 Estimated consumed fish length (mm) associated with the grey seal length (cm), of each consumed prey species. Within 15 samples estimations upon prey biomass were successful. a) relationship analysed in generalized additive model (GLM) b) relationship analysed in generalized additive model (GAM). Regression line and 95 % confidence interval included.

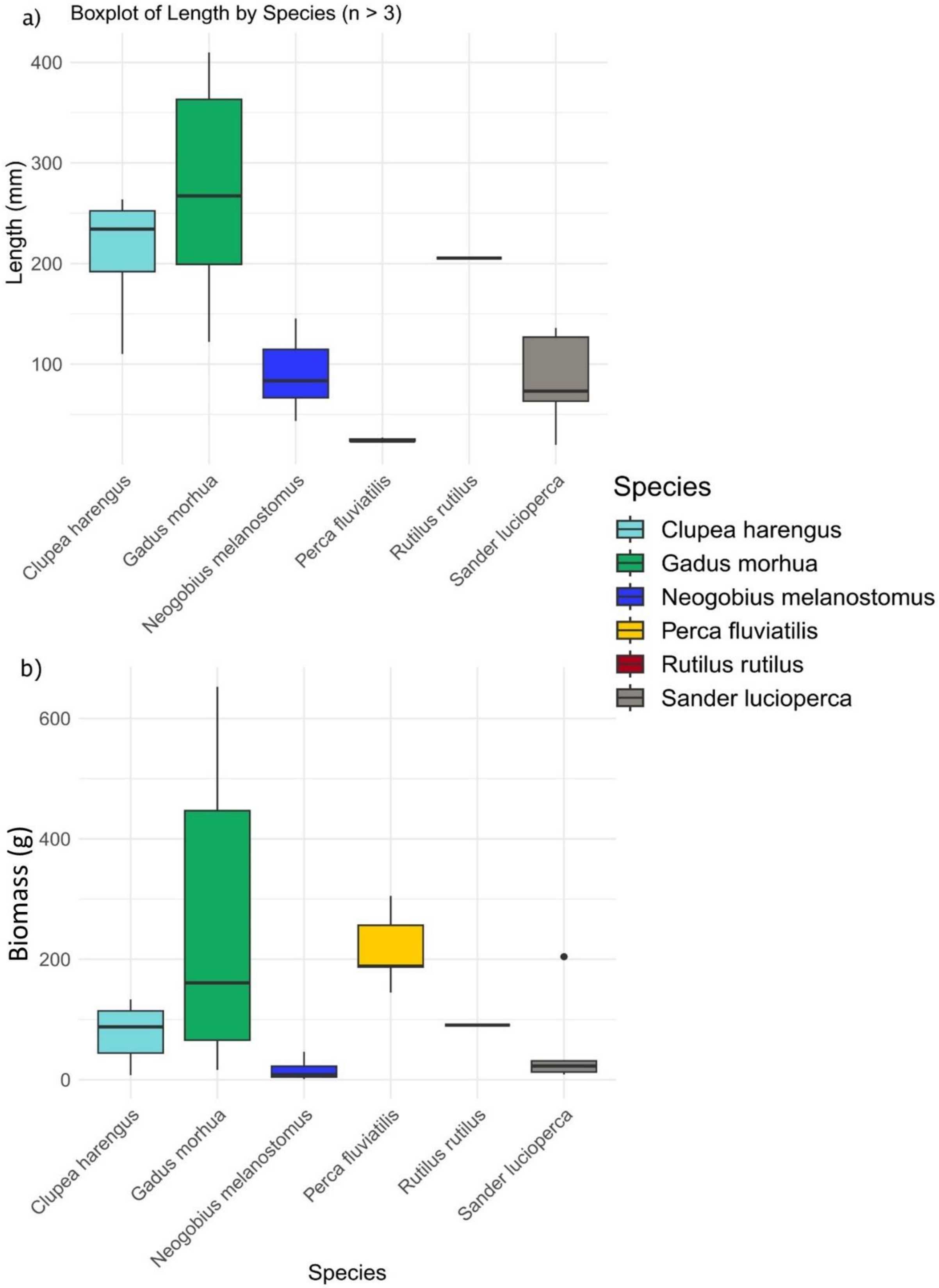

Figure 15 Boxplots including error bars and data points of estimated (a) length (mm) and (b) weight (g) of digested prey species, based on measures of otoliths and pharyngial bones including all years (2014 – 2022, excluding 2018). Outliers are depicted as black dots, bars within box show median values. (*Clupea harengus*: n = 11, *Gadus morhua*: n = 11, *Neogobius melanostomus*: n = 53, *Perca flufiatilis*: n = 6, *Sander lucioperca*: n = 6).

Estimated mass (g) and length (mm) of prey species were calculated according to regression equations (Figure 15). Starting with different length, cod showed the highest variation of prey length (m = 278 mm, sd = 98.5 mm) from a maximum length of 410 mm and to a minimum of 122 mm. Herring (n = 11) showed mean length of 214 mm +- 51,1 mm, ranging from 110.1 – 263. 7 mm. Pike-perch length rate were from 19.8 - 136 mm (m = 142 mm, sd = 75.1 mm). Perch showed a range of 218 – 268 mm (m = 142 mm, sd = 19.1 mm). Round goby ranged between 43.4 - 142.7 mm (m = 91.4 mm, sd = 29.8 mm).

For the weights, digested cod weight was displayed between 16.4 - 652.7 g (m = 263 g, sd = 244 g). Estimated weight of herring ranged from 7.9 - 133.5 g (m = 75.2 g, sd = 43 g). Pike-perch had a range between 8.6 – 204.4 g (m = 46.3 g, sd = 70.3 g). Perchs spanned between 144.8 – 304.5 g (m = 216 g, sd = 62.3 g). Estimated digested round goby weight 1.24 - 46.4 g (m = 15.1 g, sd = 13.8 g).

Additionally, *Ammodytes tobianus* (104.8 mm, 2.8 g) was only found once, as well as *Pomatoschistus minutus* (52.2 mm, 1.3 g). Furthermore, *Merlangius merlangus* (335.6 mm, 339.4 g; 314.7 mm, 272.5 g respectively) were found twice. One pharyngeal bone of *Rutilus rutilus* (205.4 mm, 91 g) could be identified.

Considering the estimated biomass and identified species abundance, herring was most abundant (42 %) in analysed grey seals when examining the IRI, followed by Gadidae (35.5 %) and round goby (9.8 %) (Table 8). When accounting for correction factors, herring was still the most abundant species in the analysed seals with 42.6 % IRI. The importance of gadids shrunk to 30.9 %, whereas the importance of round goby increased to 16 %. These results align with the numerical values presented earlier, i.e., that using NCFs results in an increase of the importance of goby.

Table 8 Calculated indices of prey taxa and family. Biomass contained corrected mean values of previous sampling data (Westphal, unpubl.). FO representing frequency of occurrence and IRI index of relative importance.

Species	**FO (n)**	**FO (%)**	**Total mass (g)**	**Mass (%)**	**Total number (n)**	**Numerical percentage (%)**	**IRI**	**IRI %**
Clupea harengus	16	28.6	3,058	21.9	65	35.9	1,178.1	41.72
Gadidae	13	23.2	6,515	46.6	24	13.3	1,389.7	35.07
Rutilus rutilus	8	14.3	781	5.59	9	4.97	151.0	3.81
Percidae	7	12.5	2,306	16.5	21	11.6	351.25	8.86
Neogobius melanostomus	6	10.7	846	6.06	56	30.9	395.5	9.98
Ammodytidae	2	3.57	9.14	0.07	2	1.1	4.2	0.1
Plathichtys flesus	2	3.57	454	3.25	2	1.1	15.5	0.39

Gasterosteus aculeatus	1	1.79	1.63	0.01	1	0.552	1.0	0.03
Pomatoschistus minutus	1	1.79	1.34	0.01	1	0.552	1.0	0.03
NCF corrected								
Clupea harengus		28.6		21.9	240.5	34.8	1,621.6	42.57
Gadidae		23.2		46.6	28.8	4.2	1,178.56	30.94
Rutilus rutilus		14.3		5.59	11.7	1.7	104.3	2.74
Percidae		12.5		16.5	37.8	5.4	273.75	7.19
Neogobius melanostomus		10.7		6.06	352.8	51	610.5	16.03
Ammodytidae		3.57		0.07	9	1.3	4.9	0.13
Plathichtys flesus		3.57		3.25	3.4	0.5	13.4	0.35
Gasterosteus aculeatus		1.79		0.01	1	0.1	0.2	>0.01
Pomatoschistus minutus		1.79		0.01	6.3	0.9	1.6	0.04

4.1.3. Species diversity

Alpha and gamma diversity was calculated in each of the specified areas. Within GB in total fourteen species could be detected, while NWM and surrounding coast of Rügen revealed seven species respectively. In SCV four and WCR three species were detected.

For both Shannon and Simpson diversity indices, the data deviated from normality across all grouping variables, as indicated by the low *p*-values from the Shapiro-Wilk test. When examining the results from the Kruskal-Wallis test, the p-values were consistently higher than the commonly accepted significance level of 0.05. This implies that there were no statistically significant differences in the diversity indices across the categories within any grouping variable—location, year, or quarter (Table 9). Shannon and Simpson indices variation are presented in Figure 16.

The table presents a detailed summary of the Shannon and Simpson diversity indices across various grouping variables (Table 10). Similar results occurred when applying bootstrapping. The difference between different areas as previously seen was quite low (Figure 12), in terms of beta diversity.

Table 9 Non-parametric analysis of Shannon and Simpson index depending on different environmental variables (location, year, quarter).

Dependent Variable	Grouping Variable	Shapiro-Wilk p-value	Test Used	Test Statistic	p-value
Shannon	location	0.0004098	Kruskal-Wallis	0.95	0.91
Shannon	year	0.0004098	Kruskal-Wallis	1.98	0.85
Shannon	quarter	0.0004098	Kruskal-Wallis	0.77	0.85
Simpson	location	8.794e-05	Kruskal-Wallis	1.07	0.89
Simpson	year	8.794e-05	Kruskal-Wallis	2.00	0.84
Simpson	quarter	8.794e-05	Kruskal-Wallis	0.82	0.84

Table 10 Statistics of Shannon and Simpson indices including the median, first quartile (Q1), third quartile (Q3), and standard deviation (SD) for each combination of diversity index and grouping variable (GB, NWM, SC, SCV, WCR, and different years and quarters).

Diversity Index	**Grouping Variable**	**Median**	**First Quartile (Q1)**	**Third Quartile (Q3)**	**Standard Deviation**
Shannon	GB	0.294	0	0.689	0.499
Shannon	NWM	0.678	0.339	1.02	0.686
Shannon	SC	0.657	0	0.668	0.334
Shannon	SCV	0.600	0.381	0.795	0.456
Shannon	WCR	0.496	0.248	0.744	0.702
Shannon	2016	0.688	0.688	0.688	NA
Shannon	2017	0.627	0	0.883	0.575
Shannon	2019	0	0	0	NA
Shannon	2020	0.618	0.294	0.662	0.315
Shannon	2021	0.347	0.173	0.520	0.490
Shannon	2022	0.600	0	0.768	0.497
Shannon	Q1	0.657	0.622	0.668	0.0475
Shannon	Q2	0.254	0	0.693	0.411
Shannon	Q3	0.668	0.334	1.02	0.686
Shannon	Q4	0.623	0	0.795	0.534
Simpson	GB	0.199	0	0.496	0.291
Simpson	NWM	0.485	0.243	0.614	0.377
Simpson	SC	0.464	0	0.475	0.238
Simpson	SCV	0.413	0.245	0.542	0.285
Simpson	WCR	0.257	0.128	0.385	0.363
Simpson	2016	0.495	0.495	0.495	NA
Simpson	2017	0.435	0	0.571	0.320
Simpson	2019	0	0	0	NA
Simpson	2020	0.427	0.199	0.469	0.221
Simpson	2021	0.25	0.125	0.375	0.354
Simpson	2022	0.413	0	0.503	0.293
Simpson	Q1	0.464	0.431	0.475	0.0455
Simpson	Q2	0.163	0	0.5	0.264
Simpson	Q3	0.475	0.237	0.609	0.376
Simpson	Q4	0.431	0	0.542	0.300

Calculated gamma diversity, based on the Simpson Index (0.82) provides a measure of diversity that considers both species richness and species evenness (abundance distribution). Overall the prey-fish community has a relatively high diversity, with no single species overly dominating the diet.

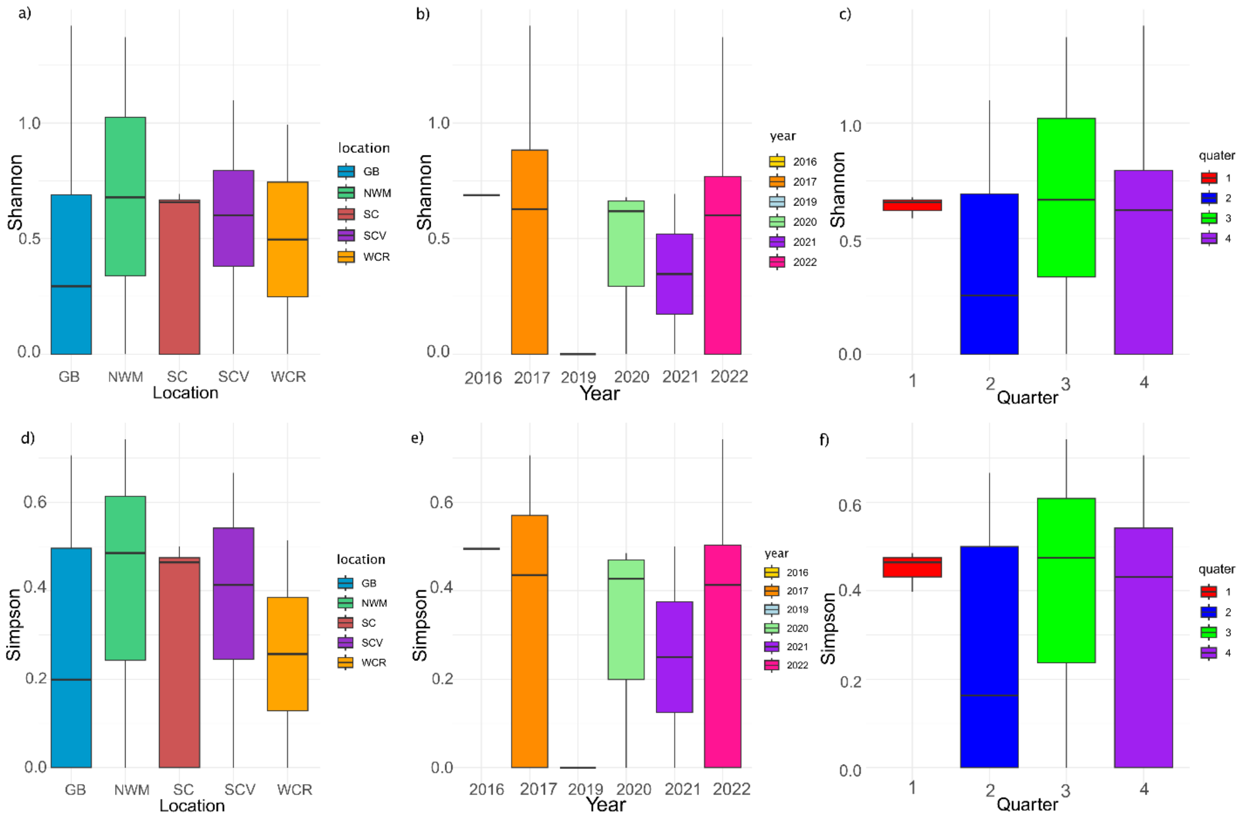

Figure 16 Calculated Shannon and Simpson index to present alpha richness within different locations, years and areas. a – c) Shannon index on location, year and quarter respectively, d – e) Simpson index on location, year and quarter respectively. GB = Greifswald lagoon, NWM = North-West Mecklenburg, SC = surrounding coast east Rügen, SCV = surrounding coast Vorpommern, WCR = west coast Rügen. Year devided into quarters (1 -4).

4.2 Metabarcoding analysis

Various PCR modifications were applied in order to obtain the best possible results for sequencing PCR products. Prior to sequencing of the samples, PCR products were checked by running them on either an agarose gel, or an electronic one. Best results were accomplished by running the PCR at 54° C annealing temperature and cycles. Including magnesium into the PCR greatly enhanced the signal of the PCR product.

After Illumina sequencing of the samples, quality checks within the dada2 pipeline, were conducted in order to verify the accuracy of the sequencing process. Within the range of the targeted base pair length (190 bp) within the 16S mtDNA region a good quality score could be maintained (> 30). The drop of the quality occurs at the distinct size end of the amplicon. Reverse reads quality was in general lower than forward reads quality, but maintaining as well a good quality score.

4.2.1 DNA isolation

Isolated DNA concentrations were measured in order to assure equimolarity between samples and thus assure a comparison of the results. Out of 107 individuals, DNA of intestinal subsamples could be successfully extracted, additionally out of 28 stomachs of the seals as well.

To match the assumption of normality, log-transformed data of the DNA concentration was used and tissues were tested separately. 107 measures of intestinal samples showed a successful extraction. Non-parametric Kruskal-Wallis test showed a significant difference of the DNA concentration among the different stages of decomposition (df = 4, p = 0,00012345). Post-hoc analysis using Dunn's test revealed state 5 to have lower concentration than states 2 (p = 0.01), 3 (p = 0.0002), and 4 (p = 0.0002) (Figure 17 a). Within degradation stage 1 only two individuals were sampled, as this stage represents freshly dead animals and is rather rare. Stage 1 showed a median value of 1.8 ng/µL (quartiles ranging from 0.9 – 2.7 ng/µL), stage 2 showed a median concentration of 5.8 ng/µL (quartiles ranging from 1.8 – 35 ng/µL), stage 3 median value of 9.8 ng/µL (2.7 - 24 ng/µL), stage 4 a median concentration of 13 ng/µL (quartiles ranging from 4.1 – 35 ng/µL), and stage 5 a median concentration of 1.4 ng/µL (quartiles ranging from 0.3 – 3.1 ng/µL). High concentrations were exceeding 100 ng/µL were measured in stage 2 - 4. High concentrations of 71 ng/µL could still be extracted from degradation stage 5. With a higher degradation state, the concentration significantly differs from lower degradation stages. Highest median values (13 ng/µL) occurred in degradation stage 4.

Two-way ANOVA analysis of stomach DNA concentrations did not show a statistically significance difference among decomposition states (p > 0.32).

For the 28 seals for which both stomach and intestinal DNA was available, high variations within each decomposition stage could be measured. The highest mean value was within degradation stage 3 (117.9 ng/µL, sd = 94.5 ng/µL), whereas stomach analysis showed a mean measured value of 21.8 ng/µL (sd = 16.7 ng/µL). In degradation state 2 intestinal concentration (38.1 ng/µL, sd = 33.5 ng/µL) was higher compared to DNA concentrations from the stomach (15.4 ng/µL, sd = 14.6 ng/µL). Within decomposition state 4 and 5 same scenarios occur (intestine state 4: 55.1 ng/µL, sd = 19.2 ng/µL; intestine state 5: 7.8 ng/µL, sd = 5.2 ng/µL; stomach state 4: 42.4 ng/µL, sd 22.8 ng/µL; stomach state 5: 4.6 ng/µL, sd = 2.5 ng/µL). Lowest values were measured within a higher stage of decay (Figure 17 b). Wilcoxon test did not show any significance within different degradation states, while comparing log transformed tissue concentrations (state 2: W = 20, *p* = 0.14; state 3: W = 12, *p* = 0.34; state 4: W = 40, *p* = 0.44; state 5: W = 73, *p* = 0.69).

Within each degradation state a DNA concentration could be extracted, while in general lower concentrations within stomach samples were measured. Highest concentrations of stomach contents were measured within degradation state 4.

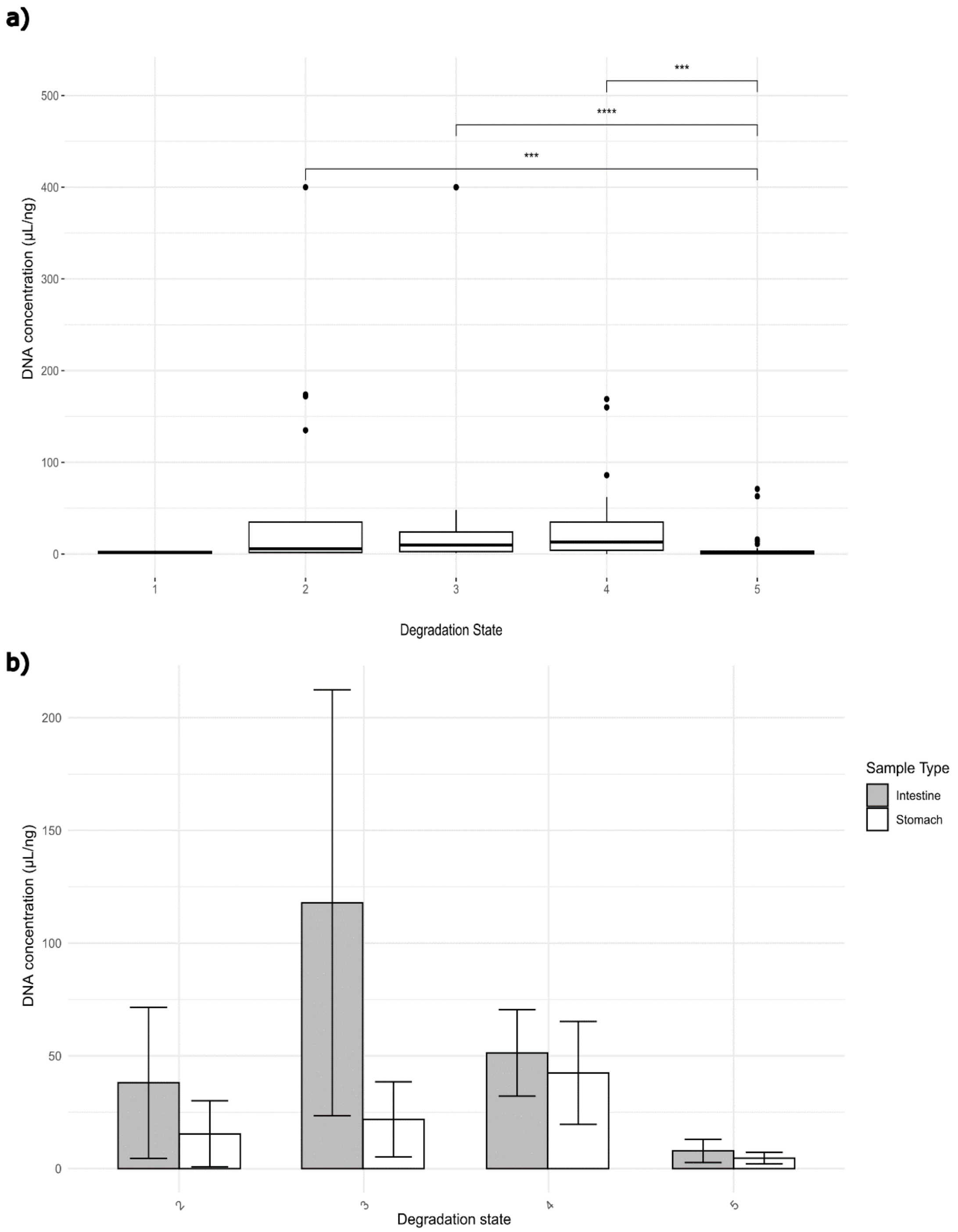

Figure 17 DNA concentration measures upon different degradation states of 107 analysed grey seals. a) Median values of different degradation stages 1 – 5 (n = 2, n = 17, n = 23, n = 29, n = 37 respectively) of dissected animals, including upper and lower limits and standard deviations. Dots representing outliers, stars significance of difference between the different analysed stages. b) Mean measured DNA concentrations intestinal and gut samples extracted from 28 grey seals (states 1: n = 5, states 2: n = 4, states 3: n = 8, states 4: n = 12), including error bars.

4.2.2 Positive controls

To control if established primers target the required amplicon and to control for accuracy of sequencing process, positive controls of extracted prey fish DNA. Extracted prey fish DNA was sequenced prior to gut or stomach samples from grey seals. 14 species were included for positive controls. Matching the sequenced mitochondrial genes to the database revealed that flatfish species (*Pleuronecta platessa* and *Platichthys flesus*) could not be distinguished between another as both were matching the 99 % of BLAST search. Therefore, plaice and flounder are for following analysis grouped together within Pleuronectes/Platichthys/Lepidopsetta, as genetic differences between these three groups were not clearly identifiable. As *Myoxochephalus scorpio* and *Microcottus* sp. are genetically very similar and an exact genetic distinguishment was not possible, these species were grouped together as Myoxochephalus_Microcottus. Sequencing of *Neogobius melanostomus* was least successful, in general maximum read counts within the sequenced sample were below 100 (41), although we have two reasons to conclude the primers successfully detect goby. First, a round goby was included as positive control in a previous sequencing round, and also showed similar read abundance of 99.8 %, >44,000 reads and mismatches being less than 100 reads within a sample. Second, because it was found in many stomach/intestine samples (Table 11). All other samples showed high read counts >2,000 (*Merlangius merlangus*) up to > 63,000 (*Sprattus sprattus*). Excluding the round goby sample showed a maintenance of high accuracy rate of matching the targeted prey species, with pike showing the lowest detection rate of 96.4 % and the highest occurring within the mackerel (*S. scombrus* and *T. trachurus*) (Table 11).

Table 11 Proportion of sequencing reads of targeted prey species within individual fish samples. Results were included for decision of threshold establishing for minimum amount of occurring reads.

Species	**Common name**	**Proportion of reads assigned to target species (%)**
Clupea harengus	herring	99.6
Esox lucius	pike	96.4
Gadus morhua	cod	99.7
Limanda limanda	dab	99.9
Merlangius merlangus	whiting	98.1
Neogobius melanostomus	round goby	25.2
Pleuronectes sp./ Platichthys sp./ Lepidopsetta sp.	flatfish (*P. platessa*)	99.5
Pleuronectes sp./ Platichthys sp./ Lepidopsetta sp.	flatfish (*P. flesus*)	99.9
Rutilus rutilus	roach	99.3
Scomber scombrus	mackerel	100
Scophthalamus maximus	turbot	99.3
Solea solea	sole	97.5
Sprattus sprattus	sprat	99.9
Trachurus trachurus	horse mackerel	100

It should be mentioned that reads occurred within target species sample not matching the targeted species, rather one of the other species. Normally these reads did not exceed 200 reads within a sample, but the highest read occurrence was noticeable for sculpin included in the sole sample, occurring with 633 reads. A high variation within the genotypes of herring could be noted while analysing the sequencing data, with 6 different sequences (ASVs) occurring. Detection of targeted species with using the existing and additionally established primers was successful.

4.2.3 Mock communities

Artificial mixed communities of fish species were designed, amplified and sequenced, functioning as well as a positive control. In total 9 equimolar mock communities were sequenced where 4 included seal DNA. If seal DNA was included in the mock communities, the sequencing of these samples seemed to fail, or rather no sequence reads or to low amounts (<100 reads) occurred, resulting in five mock community samples that could be analysed for prey fish (Figure 18). First mock communities showed similar RRA between samples. Whereas the abundance of reads within differed between different species. For the first sample read counts exceeded 1,000 whereas for the other two samples, read counts were below 1,000 reads. Kruskal-Wallis test presented no significant difference between the RRA of species in mock community (a – c), (df =2, *p* = 0.99).

While analysing the read occurrence of the two mock communities sequenced afterwards, the RRA of prey species differed within the sample. The majority >1,000 reads were found within herring, flatfish, mackerel and turbot, whereas round goby, sole and horse mackerel reads were below 500.

Limits of read abundances were set considering the mock community read abundance and the positive control. As a higher variation occurred within the MCC (Figure 18) the limit of reads for the analysis was set at 440 reads, to consider less contamination of samples. After filtering two species could not be detected within the mock community (pike and roach).

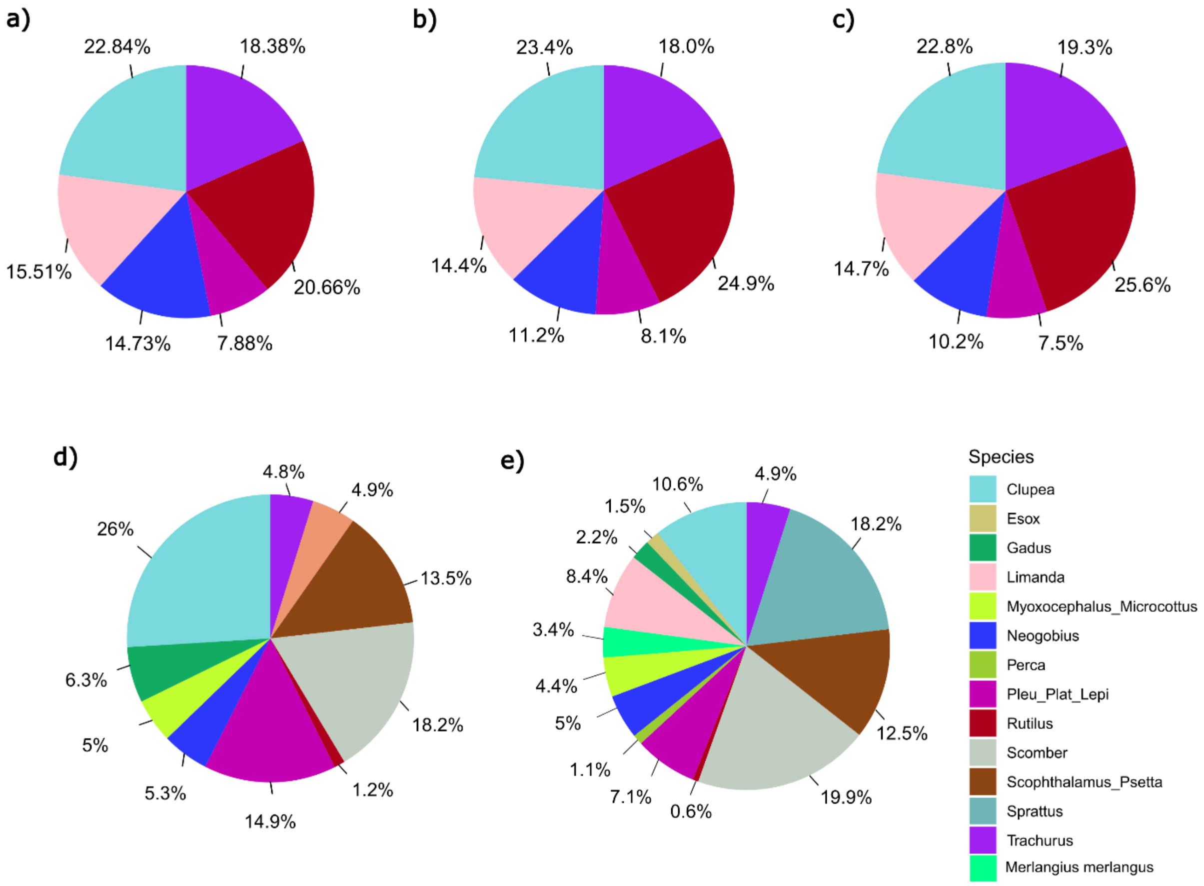

Figure 18 Species abundance within mock communities according to the relative read abundance within each sequenced sample. All samples were equimolar standardized prior to PCR. a – c) representing the same mock community (sample mockmix) that was PCR-amplified and sequenced three times (a-c were amplified using three different reverse primers), d) MCC one species was not detected (pike) and e) MCA mock community containing all 14 prey fish species in which 1 species was not detected (due to grouping of flatfish). Sculpin (Myoxocephalus_Micrococttus) was not added to the mock community and therefore is a contamination in the samples.

4.3 Species occurrence in grey seals

Several negative controls (NTC) were included in each PCR. Unfortunately, first NTCs within each PCR and sequencing round showed always a PCR product and produced sequencing reads from prey fish. For further analysis and interpretation of the data this should be considered. As in the last sequencing the NTC very similar with a stomach/gut sample, the analysed data will be pooled over all areas, since an uncertainty of the occurring contamination exists.

Noticeable was that NTC contamination always occurred within the first batch of samples, as more NTCs were included following samples were did not show a contamination. In the first PCR-and-sequencing run, only herring occurred in one of three NTCs. In the second PCR-and-sequencing run, herring (3 %), sculpin (34.8 %), perch (39 %) and roach (35 %) appeared in the same NTC but not the other two.

Including the seal blocking primer into the PCR did not block the signal of seal within the sequencing completely. Seal samples appeared within 71% of the samples (n = 25) with a total read number of 158,048 reads. For further analysis, seal sequences were excluded from the dataset. Furthermore, harbour porpoise (*Phocoena phocoena*) occurred within 16 of analysed samples (25.8 %) and a total read number of 5,333 reads, and these were also excluded from the dataset.

Overall 20 intestinal samples were sequenced and 6 stomach samples from 26 individual grey seals. First five sequenced samples are presented in Figure 19. After the filtering process, two samples only had high read ratios of seal and porpoise, whereas the DNA did not detect a fish prey species. First sequencing of five samples showed a signal within the NTC only for herring. A different diversity within the diet can be noted. As two seals showed only one species occurring in the sequence the other two seals presented a feeding on different prey species. Lower prey species abundance occurred in seals identified as juveniles. Excluding herring of the analysis still presented a high abundance of five different prey species with an almost equal read ratio between species. A genetic clear separation within clupeids could be made between herring and sprat. Freshwater species (roach and goby) could be found along with marine species (flatfish species, horse mackerel and herring). Herring did not occur within the other samples (Figure 19). The stomach sample showed metabarcoding results, whereas the DNA concentration input was too low to measure the concentration. The analysed gut sample belonged to a grey seal was found at the western coast of Rügen and was identified as a juvenile, with a measured total length of 100 cm.

When using reverse primers separately, no significant difference could be assessed while analysing the relative read abundance within the first five different samples (Kruskal-Wallis-Test: df = 2, *p* = 0.58). Also using bootstrapping method did not show a significance of difference between primers (M = 346.2, *p* = 0.5).

Overall, 306 ASVs were analysed, after filtering, 91 ASVs were used for further analysis and collapsed into distinct prey species. In total 20 species could be identified with metabarcoding. In 3 samples no prey species were detected and these were excluded from further analysis, making up a VI Index of 11,5 % for the genetic analysis.

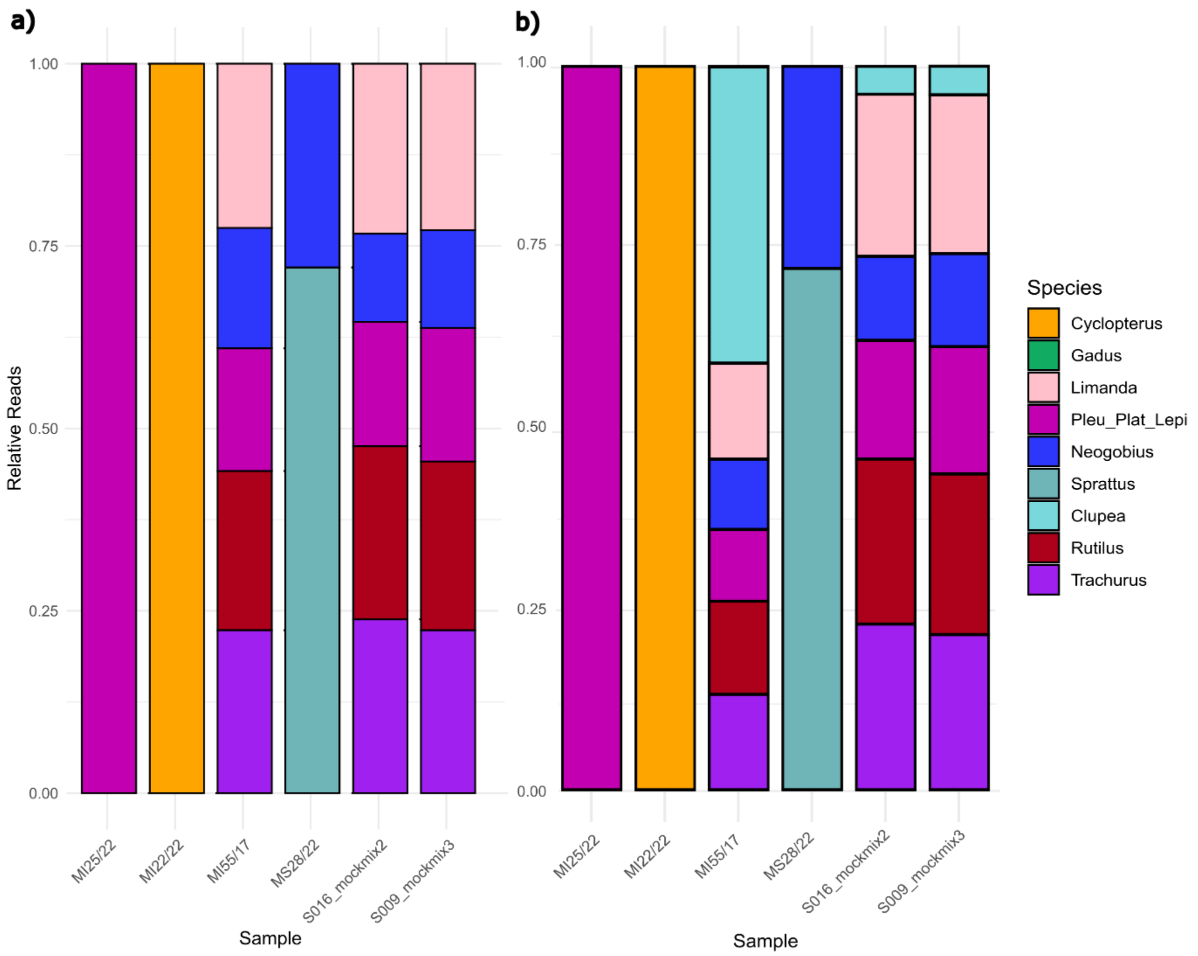

Figure 19 Relative abundance of reads presented for each sample sequenced. First sequencing of 5 samples, one was excluded for the graph as it was empty. MI indicates samples amplified from intestinal DNA and MS indicating amplification of stomach DNA. Mock communities are also presented in the graph. Similar colouration indicates relationship between families.

Main read abundances of prey species occurring within the analysed data were sculpin (making up 29 % of the whole dataset), roach (16.3 %), perch (16.9 %) and herring (23 %). While these were the species also occurring in the most of the analysed 26 samples (Table 12). Overall, perch occurred in 25 samples, herring and roach occurred in 24 samples (Figure 20). Within the 26 samples, only 4 showed an occurrence of single species with the analysis, 84.6 % showed an occurrence of at least two species (Figure 20). Negative controls showed read data from the above species, thus while the method successfully detected prey fish species, it is not possible to conclude that the species are really present in all samples in which sequences were detected. Nevertheless, there is a high certainty of the detection of dab, lumpfish, sprat, bream, garfish and eelpout, because these were never detected in negative controls.

Analysing the RRA according to the location, year and season, there is a significant difference between within the west coast of Rügen and the Greifswald lagoon (0.0007), as well as there might be a difference compared to the surrounding coast of Rügen (0.29) sprat (chi²-squared =

25, df = 4, *p* = 5.031e-05). Eelpout significantly differing in the year (chi² = 25, df = 5, *p* = 0.0001) & season (chi²-squared = 25, df = 5, *p* = 0.0001). Overall, comparing species composition within different areas, there does not seem to be a significant difference between different area for any of the prey species (*p* > 0.05).

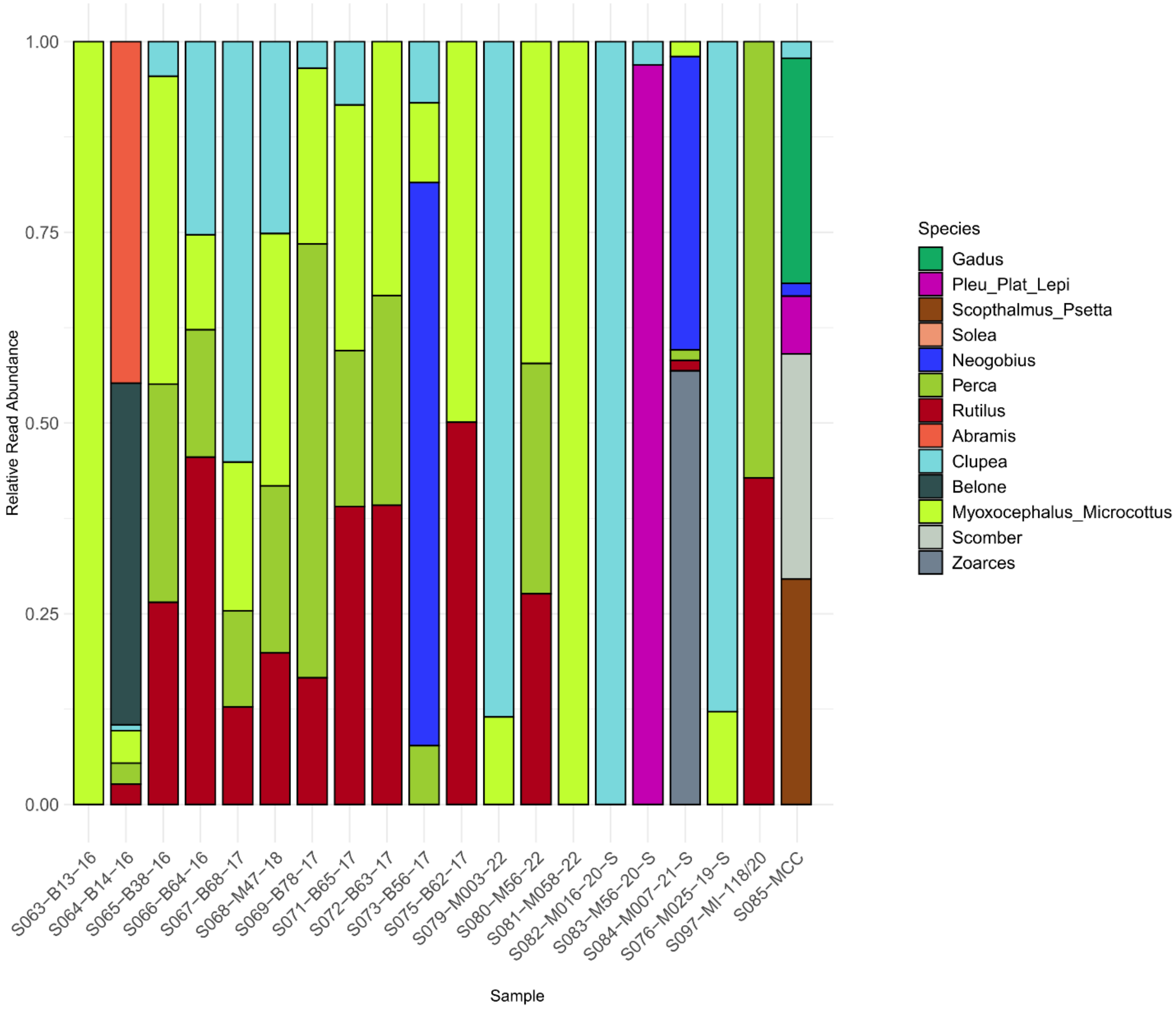

Figure 20 Filtered data, presenting the relative read abundance of each prey species of each analysed sample (n = 21). Within two samples no detection of prey species was possible and they were excluded. MCC representing the mock community 4 and samples ladled with an S represent the stomach samples.

Analysis of the relationship between species occurrence and location, year and season it appeared that there may be no correlation between different variables and RRA. The PCA analysis aimed to explore patterns and relationships within the dataset and reduce its dimensionality while retaining meaningful information. Multicollinearity previously checked by calculating Variance Inflation Factor (VIF) of species occurrence was reduced and based on the Hellinger transformation of RRA of samples, previously normalized. Analysing the variance explained by each principal component, the first five principal components together explained approximately 71.72% of the total variance in the dataset, whereas the first seven principal components accounted for about 87.94% of the total variance, also the scree plot indicated a suitable explanation of principal components. While analysing the patterns within location, year and season *p*-values were not significant (0.4; 1; 1 respectively), suggesting no significant influence of these variables according to RRA of different species.

Having a closer look into the biplot, a relatively even distribution suggests that each variable (RRA) provides a unique aspect of variance that isn't strongly correlated with the others, hence they spread out in different directions (Figure 21). Samples are clustered based on their location, indicating that samples from similar locations have similar relative read abundances for these RRAs. The length of an arrow indicates the importance of that variable in the PCA. Longer arrows (Figure 21) are more influential in the projection, presenting dab, sculpin, horse mackerel and roach being most influential within the projection. Direction of an arrow indicates the relationship between the original variable and the principal components (PCs). Two arrows close to each other or have a small angle between them, indicates that those two original variables tend to vary together in the dataset. Such as horse mackerel and dab, as well as bream and garfish (Figure 21d). If a point is close to an arrow, it suggests that for that sample, the corresponding variable has a high value. Seeming perch is important within the diet of seals from the Greifswald lagoon, as well as sprat and round goby. Overall, the overlap of diets from seals is reflected as the 95 % CI intervals of year quarter and location overlap. Based on the current data and the PCA representation, there isn't strong evidence to suggest that the samples from different locations have distinct multivariate profiles (Figure 21).

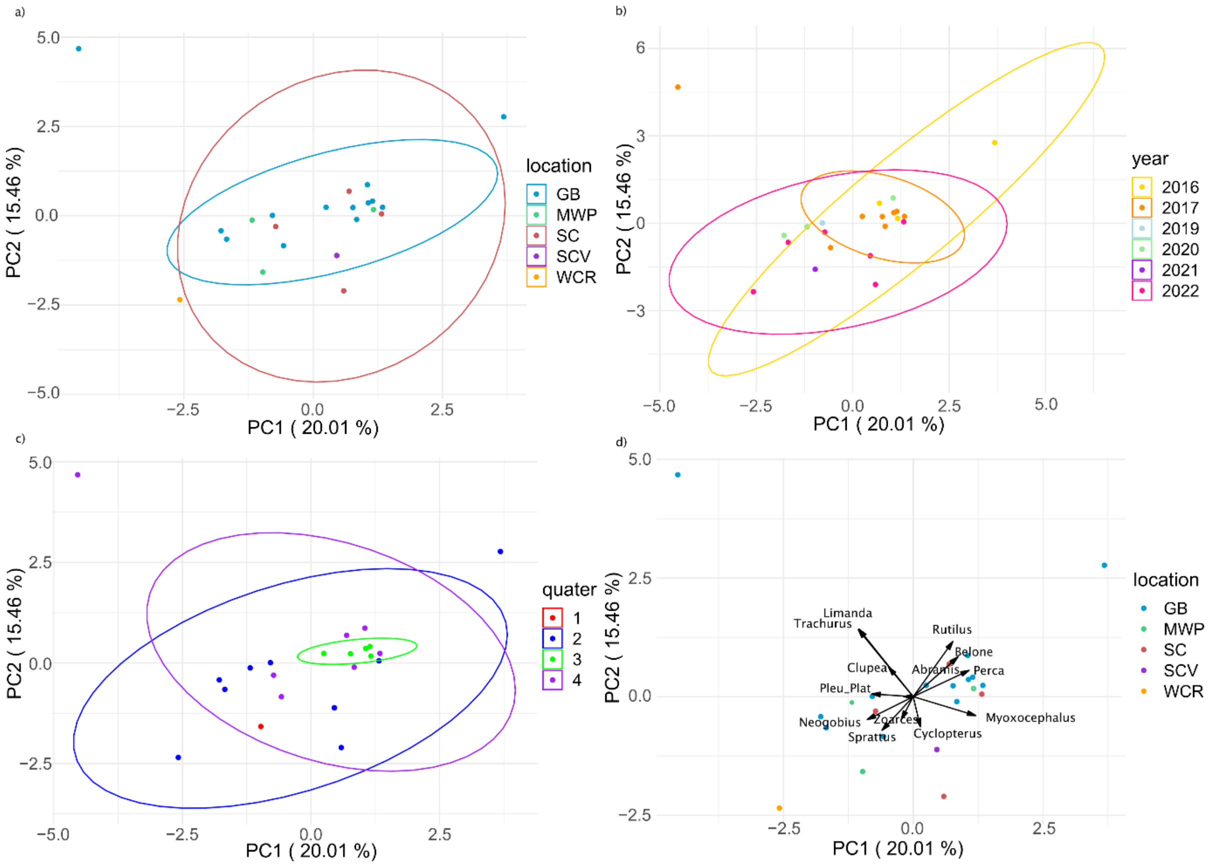

Figure 21 PCA plots of normalized Hellinger transformed RRA from metabarcoding samples. a) PCA in the context of different locations, b) RRA plotted by year and c) RRA in relation to the season. d) represents the biplot of the PCA analysis.

Table 12 Prey species occurrence within 26 samples of analysed grey seal gut and stomach contents. Frequency of occurrence including CI (95 %) from metabarcoding analysis.

Species	Common name	Frequency of occurence (%)	95 % Confidence Interval
Abramis brama	bream	3.8	0.2 – 21.5
Belone belone	garfish	3.8	0.2 – 21.5
Clupea harengus	herring	50	32.1 - 67.9
Cyclopterus lumpus	lumpfish	3.8	0.2 – 21.5
Limanda limanda	dab	3.8	0.2 – 21.5
Myoxocephalus scorpio/ Microcottus sp.	sculpin	65.4	44.4 – 82.1
Neogobius melanostomus	round goby	15.4	5.0 – 35.7
Perca fluviatilis	perch	46.2	27.1 - 66.3
Pleuronectes sp./Plathichthy sp./Lepidopsetta sp.	flatfish	11.5	3.0 – 31.8
Sprattus sprattus	sprat	3.8	0.2 – 21.5
Rutilus rutilus	roach	53.8	33.7 – 72.9
Trachurus trachurus/Lepidorhombus sp.	mackrel	26.9	12.4 - 48
Zoarces viviparus	eelpout	3.8	0.2 – 21.5

Having a look on the raw data, one species was found to not with a longer DNA fragment (89 bp) of the 16S mtDNA region. Sequencing reads did not match the criterion of high amounts to be considered in the analysis. Still European eel (*Anguilla anguilla*) was detected using the reverse Primer R3 and outstanded within the results. The eel sequence could be found within the one intestinal sample of a male adult grey seal, in Thiessow (GB) with an amount of 241 reads.

For each variable alpha and beta richness according to RRA were calculated. Shannon and Simpson indices did not show a significant difference in either of the variables (Figure 22). Species richness did not differ significantly (SHI: GB: 0.49 ±0.66, SC: 0.17 ± 0.34; SI: GB: 0.28 ± 0.36, SC: 0.12 ± 0.24). Within different years also no significant variation could be analysed (SHI: 2016: 0.23 ± 0.39, 2017: 1.37± 0.002, 2022: 0.14 ± 0.31; SI: 2016: 0.1 6± 0.29, 2017: 0.74 ± 0.001, 2022: 0.09 ± 0.22). Neither a significance according to the season could be determined (SHI: quarter 2: 0.17 ± 0.32, quarter 3: 0.69 ± 0.97, quarter 4: 0.34 ± 0.6; SI: quarter 2: 0.12 ± 0.23, quarter 3: 0.37 ± 0.52, quarter 4: 0.18 ± 0.37 (Figure 22). As comparing the beta diversity, the PCA plot presents overlap between RRA and location, year and quarter (Figure 21). Overall gamma diversity resulted in 0.67 ± 0.3.

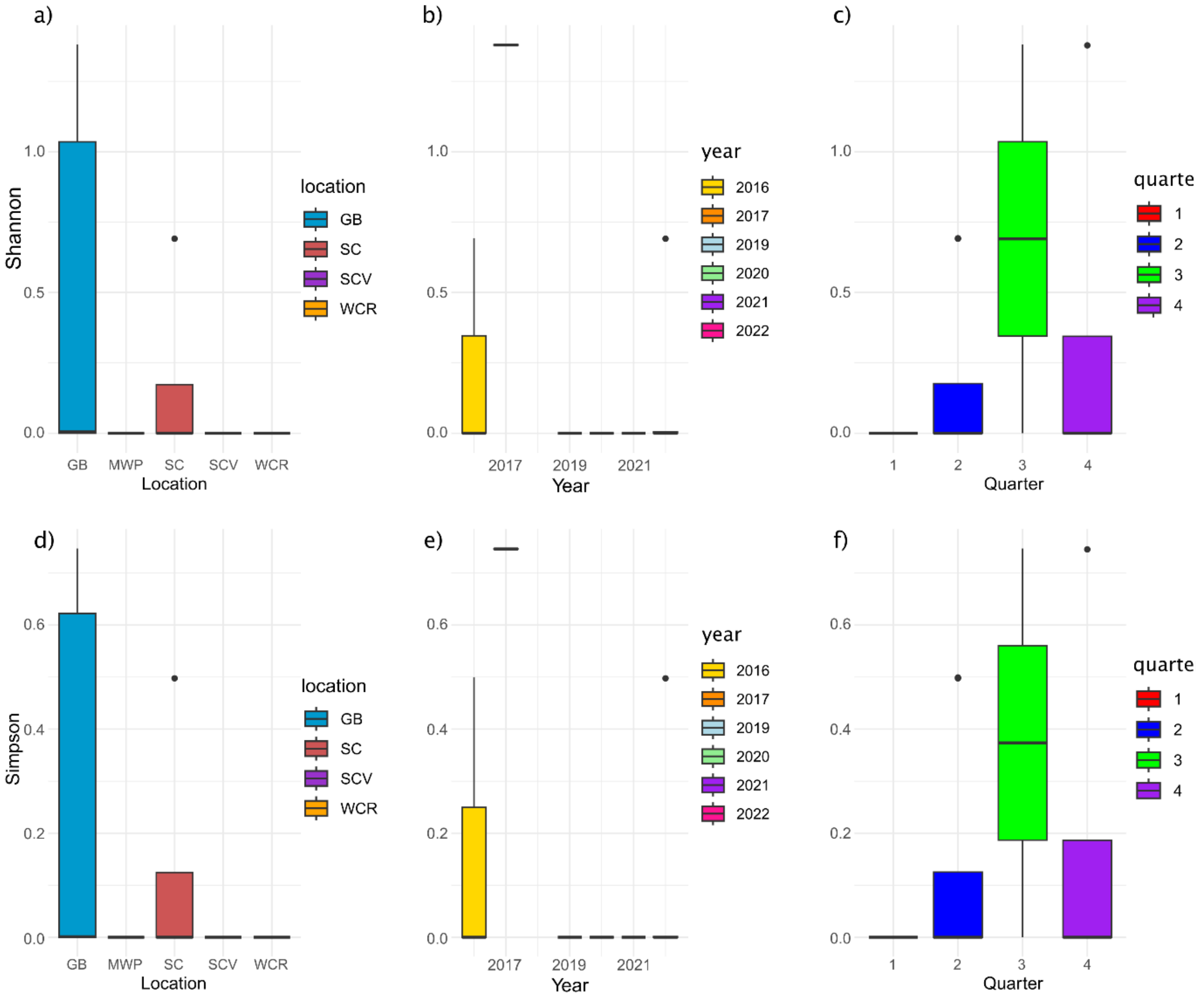

Figure 22 Shannon and Simpson index of log transformed RRA presented by a & d) location, b & e) year and c &f) quarter.

When analysing the influence of the decomposition state of the seal and the detection of prey species within the metabarcoding data, *p*-values indicated decomposition state may not be strongly related to the dependent variable across different prey species (> 0.05). Cross validation of log transformed relative read abundance among different models was applied, to find the best fitting model suitable for the data. Most suitable model was evaluated to be multivariate regression, but still R^2 values varied over different species (Table 13). Whereas, only round goby showed a marginally significance within the decomposition variable (p = 0.07), the intercept of decomposition showed a significance (p = 0.02). So as the intercept provides an estimate of this expected value, it suggests that even in the absence of decomposition influence, one would still expect to find a certain average RRA for that species in your samples. All the other species indicated no significance between decomposition and read abundance.

Furthermore, due to the low sample size bootstrapping was applied, which resulted in almost same results. GLM was run depending on the relative read abundance and the decomposition state as it fitted best the data (Table 14). The GLM analysis revealed that for round goby, the estimated coefficient for the decomposition variable was 0.2 (SE = 0.0007127, t = -1.09, *p* = 0.28), may indicating a relationship between decomposition and read abundance. For all other species decomposition did not seem to be a significant predictor for read counts (*p*-values >0.07). AIC and deviance were overall low, indicating a good fit of the model, for different species the goodness of fit varied.

With rising decomposition stages still high relative read abundances could be sequenced. For herring RRA, perch and flatfish (herring: median decomposition 2 = 0.3, decomposition 3 = 0.2, decomposition 4 = 0.3, decomposition 5 = 0.6; perch: median decomposition 2 = 0.1, decomposition 3 = 0.2, decomposition 4 = 0.2, decomposition 5 = 0.23; flatfish: median decomposition 2 = 0.5, decomposition 5 = 0.8) abundance was even higher with rising decay state (Figure 23). The interquartile range was highest for herring in decomposition state 4 and 5 (0.76, 0.4 respectively) and flatfish in stage 2 (0.46) and sculping in decomposition state 4 (0.39). Within decomposition state 3 and 5 overall five species could be detected. For decomposition state 2 and 4, eleven and eight species could be identified respectively when analysing sequencing data. Additionally, the width of the boxes varied among decomposition states, reflecting differences in read variability. Furthermore, depicting the detactability of prey fish within decomposed animals.

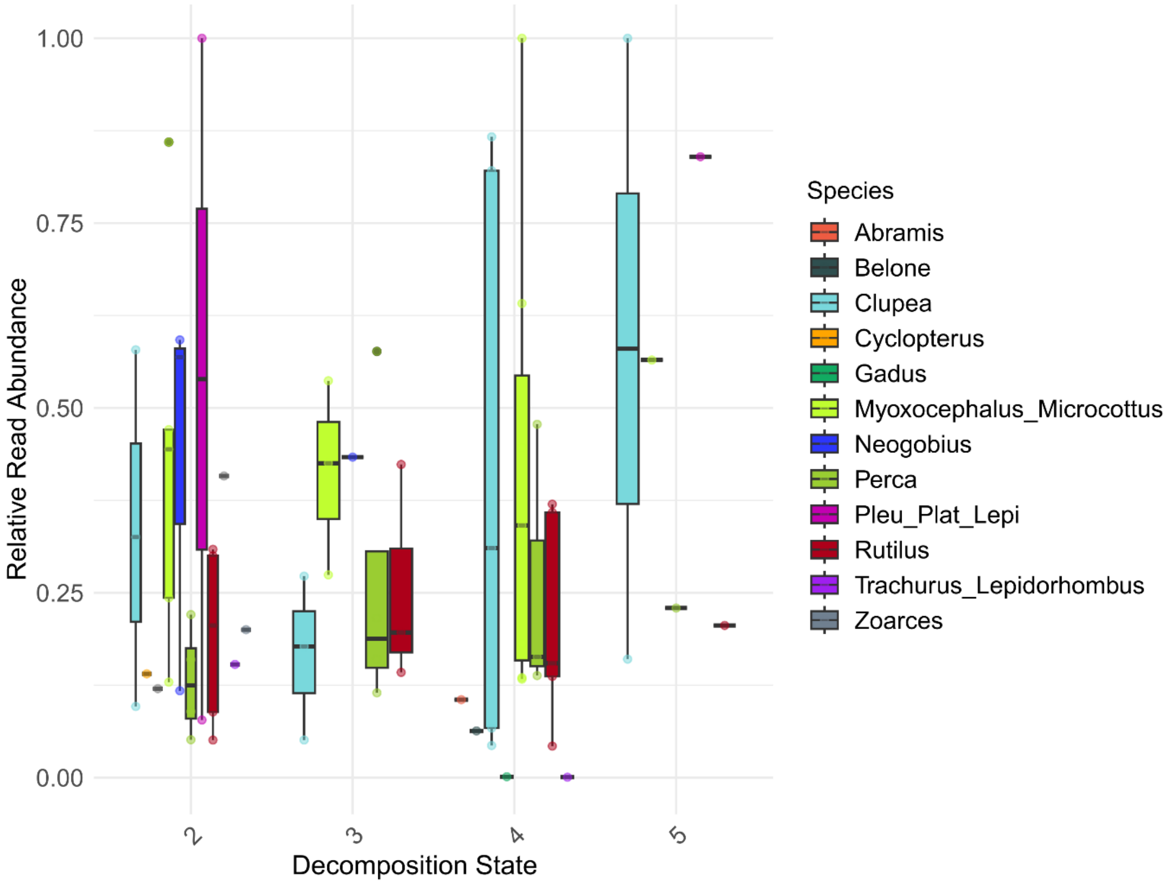

Figure 23 Measured RRAs for different prey species of sequencing data depending on different decomposition states (2-5) of analysed grey seals (n = 26). Single points represent measured values whereas bars represent median and boxes as well error bars the interquartile ranges. Rising numbers of decomposition represent higher decay stages stage 2 representing fresher analysed animals (n = 8), 3 (n = 4), 4 (n = 7) and 5 (n = 3).

Table 13 Summary of multivariate regression model of all prey species depending on the decomposition and log transformed RRA. Significant values are marked with a star.

Species	**Coefficient (Intercept)**	**Std. Error**	**t-value**	**p-value**	**Coefficient (Decomposition)**	**Std. Error**	**t-value**	**p-value**	**Residual SE**	**Multiple R-squared**
Abramis	-0.003841	0.012289	-0.313	0.757	0.002354	0.003565	0.660	0.515	0.019104	0.01785
Belone	-0.002348	0.007511	-0.313	0.757	0.001439	0.002179	0.660	0.515	0.011677	0.01785
Clupea	-0.009137	0.133023	-0.069	0.946	0.046643	0.038586	1.209	0.239	0.2067956	0.05739
Cyclopterus	0.022574	0.015809	1.428	0.166	-0.005359	0.004586	-.169	0.254	0.02558	0.05384
Gadus	-4.631e-05	1.481e-04	1.274	0.757	2.838e-05	4.297e-05	0.660	0.515	0.0002303127	0.01785
Myoxocephalus_Microcottus	0.18504	0.14528	2.439	0.215	0.01575	0.04214	0.374	0.712	0.225843	0.00579
Neogobius	0.20037	0.08215	1.123	0.0225*	-0.04498	0.02383	-.888	0.0712	0.1329	0.1293
Perca	0.0882370	0.0785437	-0.313	0.272	0.002354	0.0227834	-.013	0.990	0.1271	7.365e-06
Pleu_Plat_Lepi	0.044574	0.111738	0.399	0.693	0.002575	0.032412	0.079	0.9373	0.9373	0.000263
Rutilus	0.128369	0.075382	1.703	0.102	-0.008194	0.021866	-0.375	0.7111	0.7111	0.005818
Trachurus_Lepidorhombus	0.024462	0.017147	1.427	0.167	-0.005799	0.004974	-1.166	0.2551	0.2551	0.05361
Zoarces	0.031338	0.021946	1.428	0.166	-0.007440	0.006366	-1.169	0.254	0.254	0.05384
Limanda	0.019520	0.013671	1.428	0.166	-0.004634	0.003965	-1.169	0.254	0.254	0.05384
Sprattus	0.05879	0.04117	1.428	0.166	-0.01396	0.01194	-1.169	0.254	0.254	0.05384

Table 14 Summary of GLM (generalized linear model) statistics of bootstrapped log transformed RRA for each prey species according to the decomposition state. Fisher iterations were 2.

Species	Coefficient	Std. Error	t-value	p-value	Deviance	AIC
Abramis	0.0002478	0.0011660	0.213	0.833	0.009937	-182.25
Belone	0.0001515	0.0007127	0.213	0.833	0.003712	-207.85
Clupea	-0.00687	0.02584	-0.266	0.793	1.289255	-19.707
Cyclopterus	-0.0005833	0.0015508	-0.376	0.710	0.016942	-168.29
Gadus	2.988e-06	1.406e-05	0.213	0.833	1.444e-06	-412
Myoxocephalus_Microc	0.01575	0.0011660	0.374	0.712	1.326132	2.4132
Neogobius	-0.010501	0.0007127	-1.094	0.285	0.506812	-70.447
Perca	-0.004259	0.015104	-0.282	0.780	0.404423	-6.802
Pleu_Plat_Lepi	-0.002751	0.011721	-0.235	0.816	0.837011	-3.248
Rutilus	-0.006178	0.020030	-0.308	0.760	0.359272	-4.017
Trachurus_Lepidorhombus	-0.0006193	0.0016821	-0.368	0.716	0.019929	-64.06
Zoarces	-0.0008097	0.0021528	-0.376	0.710	0.032648	-51.23
Limanda	-0.0005044	0.0013410	-0.376	0.710	0.012668	-75.85
Sprattus	-0.001519	0.004039	-0.376	0.710	0.114906	-18.51

4.4 Direct comparison of methods

As comparing the two approaches, presence absence of filtered data of each prey species was used. Seven samples were included in this analysis. Genetically 9 different species could be identified, plus an additional group comprising 3 possible flatfish species. Morphologically, 4 species could be identified. For the comparison a stomach samples were excluded, as the DNA was taken from the intestines. Gut samples showed 2 more species. Additionally, within the morphological analysis it should be noted that due to degradation only an identification a determination to family level could be made. For further analysis they were grouped to family level. PERMANOVA results showed that the metabarcoding approach has a significant effect on detecting species (p = 0.019, R^2 = 0.21). NMDS shows a significant difference when taking the sampling methods into account (Figure 25). Post-hoc SIMPER analysis showed sculpin explaining most of the variation (51.9 %), followed by roach (63.7 %) and flatfish species (51.9

%). Least species causing variation were herring (38.5 %) and cod (21.1 %).

While comparing directly 7 samples using RRA data, overall more species could be detected whereas one sample no species occurred within the genetic sampling, but herring could be identified morphologically. Gadids (cod) could only be identified morphologically. As the total read counts were below filtering limit (Figure 24).

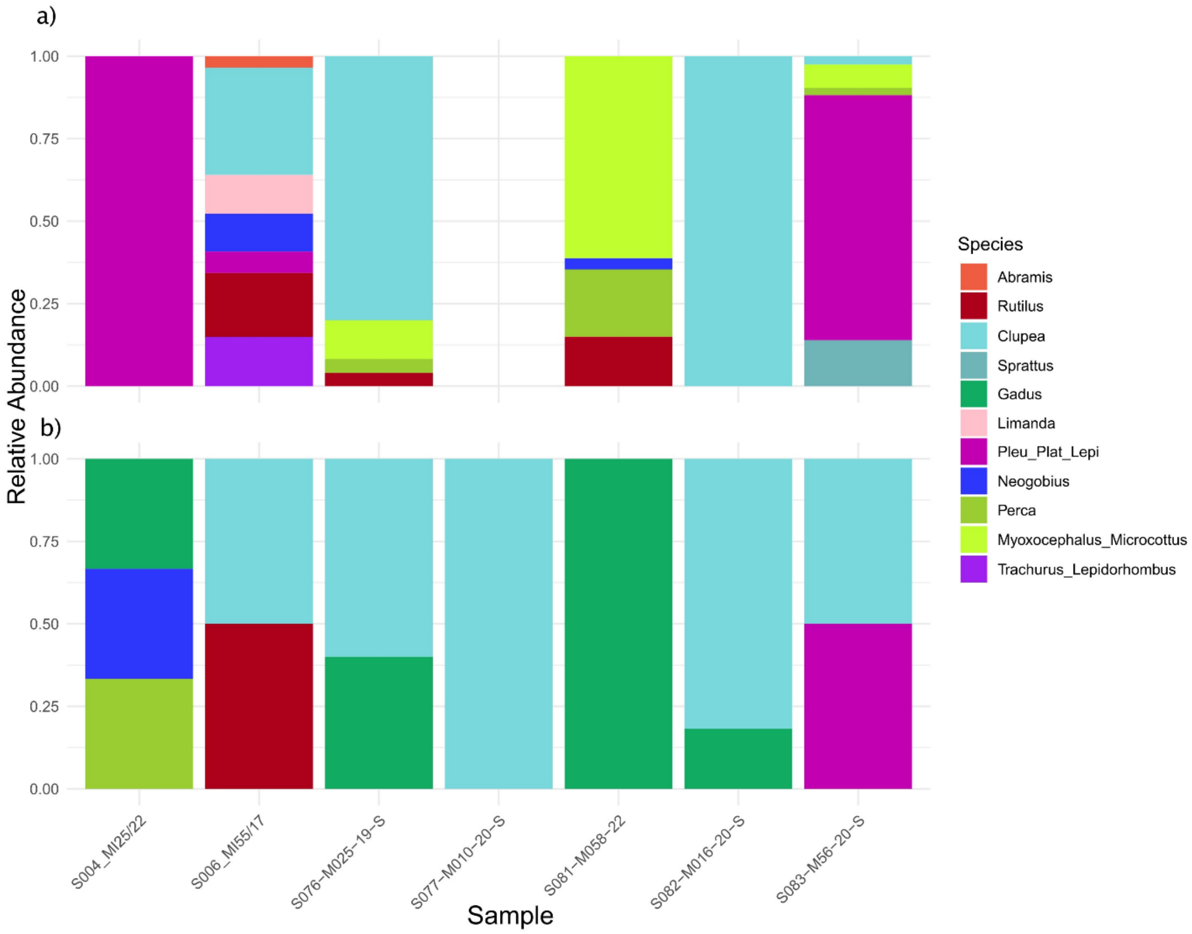

Figure 24 Direct comparison of RRA within seven distinct grey seal samples. S indicating stomach samples and similar colouration indicating relationship among species. a) presenting genetic data and b) morphological species relative abundance.

PERMANOVA results on Hellinger transformed relative abundance data to analyse where dissimilarities occur, indicated that the distinction between the genetic and morphological methods was approaching statistical significance (F = 2.1692, p = 0.09). Although this p-value is slightly above the conventional threshold for statistical significance, it suggests a trend that might be of biological or ecological importance. The variation explained by the method difference (genetic vs. morphological) was approximately 16.47% (R^2 = 0.16). SIMPER analysis was employed to discern which species were primarily responsible for the observed dissimilarities between the two methods. Herring showcased the highest average dissimilarity contribution of 0.17. Following, cod and flatfish had considerable contributions with average dissimilarities of 0.13 and 0.13, respectively (Figure 25b). These species, among others in the top 10 list, cumulatively represent the primary sources of dissimilarity between the genetic and morphological data. In summary, while there's an observable difference between the two methods, both in terms of overall community composition and the contribution of specific species to these differences, the statistical significance of these differences is marginal.

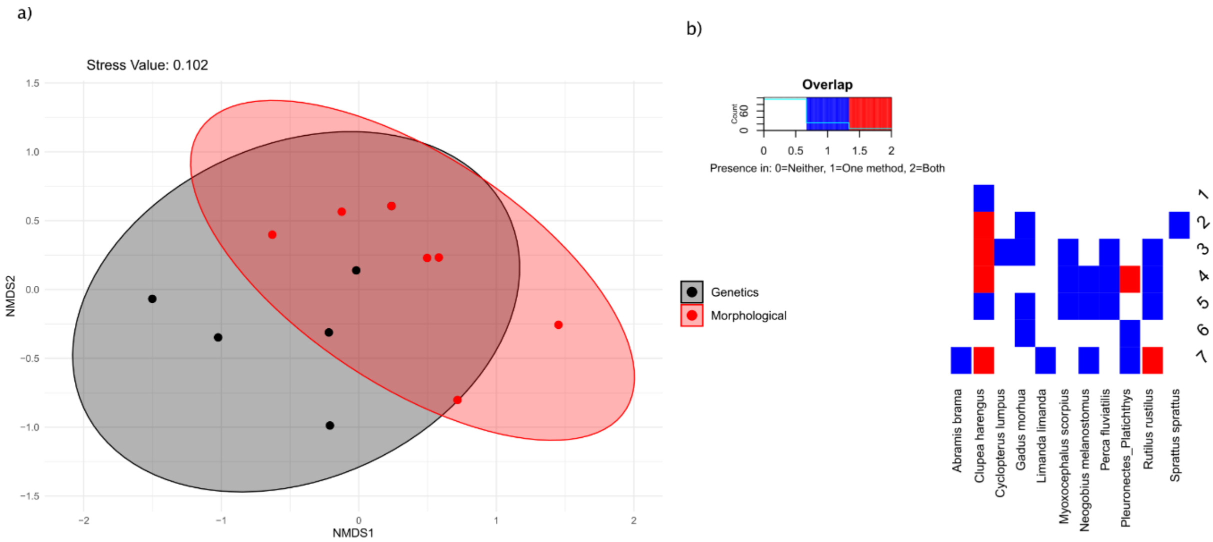

Figure 25 Direct comparison of genetical and morphological analysis of 7 analysed grey seals. Elipses depict 95% confidence interval. a) Hellinger transformed NMDS plot, presenting Bray-Curtis dissimilarity b) species detected using genetic and metabarcoding approach.

Overall a higher amount of species was detected using the metabarcoding approach, even with a low sample size. While comparing the species present within the samples. Species richness curves of linear regression fitted well to the genetic samples ($R^2 = 0.93$), whereas a plateau was not reached, indicating a small sample size (Figure 26). Furthermore, morphological species richness curve fit the regression model less well ($R^2 = 0.84$). Species richness curve displays the same picture as nearly the same amount of samples was analysed, the species richness is higher within genetic samples compared to morphological analysis. Morphological species richness analysis reached a plateau, indicating a good sample size for the analysis (Figure 26).

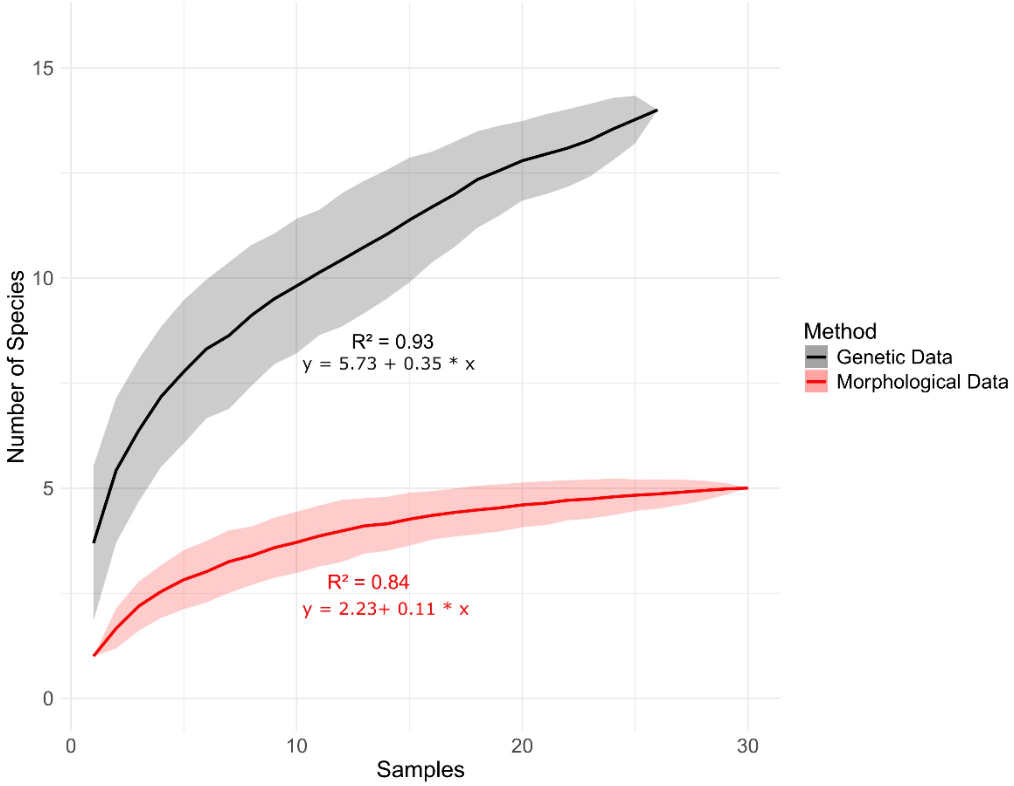

Figure 26 Calculate cumulative species richness curves, for metabarcoding analysis (black) and morphological analysis (red) of the prey species within the grey seal diet depending on the sampling effort. Shades around the curves presenting confidence intervals.

5. Discussion

5.1 Prey species abundance

5.1.1 Comparison of genetic metabarcoding and morphological analysis for detecting prey species abundance

Overall, 40 % more species could be detected using the genetic metabarcoding approach compared to the morphological approach. Even though sample size was low, analysis indicated that marginal significance of these methods mainly due to the different ability of the methods to detect herring, cod and flatfish species. The direct comparison was also limited because of the possible contamination of metabarcoding data and also the grouping of species to family level in morphological analysis. Interestingly, while genetic methods detected more species, morphological analysis could detect herring where genetics metabarcoding could not identify any prey species. In one sample, genetic methods could identify flatfish, and morphological identification was performed identifying percids, gadids and round goby. Having a look into the raw data, perch sequencing reads occurred, but did not reach read minimum limits (<100 bp). It may therefore be that certain species may not be detectable due to setting limits too high or when DNA occurs in low concentrations. Previous studies on bacterial metabarcoding indicated a reproducible overamplification of specific templates and PCR products in 16S ribosomal RNA, especially of those with a GC-rich template that were amplified with 25 % higher efficiency (Polz & Cavanaugh, 1998). Within metabarcoding studies on cats, universal primer biases presented a rate of false negatives is partly linked to DNA integrity (and so to food item categories), meaning metabarcoding is efficient detecting specific food items, but certain items in the diet may remain highly underestimated (e.g. 46 % for pet food and 70 % for out-of-date raw fish and meat) (Forin-Wiart et al., 2018). For prey fish species it could be proven that RRA is dependent on community composition marker, and DNA concentration and rare target species, may present low DNA concentration and thus may be proportionally under amplified. Species presence was lower than 0.6 % of the input DNA within low concentrations of mock communities (Hilário et al., 2023). The ability of primers to detect species in mock communities varies (Sard et al., 2019; Duke & Burton, 2020) and depends on the presence of both target species and non-target species in the DNA mixture (McLaren et al., 2019).

This thesis presents on the one hand the reproducibility and effectiveness of primers used, as sequencing the same mock communities multiple time resulted in comparable results. Results were not ideal, in that flatfish and round goby were slightly underrepresented. Studies using mock communities in various fields seem to range from there being no correlation between mixture and sequence reads to good correlations (Edgar, 2017; Kimmerling et al., 2018; Hilário et al., 2023).

Interesting, with increasing species abundance in the mock communities, the misrepresentation of species appeared more dramatic. Our data suggest that when fewer species are occurring, the proportional estimate of species abundance in the diet might be more accurate. Previous studies also suggested that RRA are more accurate when mean number of food taxa in samples is small (Deagle et al., 2019). As cod and pike were very low when using them in a mock mix or in samples, it could suggest that when grey seals feed on a variety of prey species, some might be unlikely to be detected, or might occur in such low read counts that it does not reach read limits due to recovery biases and could be overlooked.

5.1.2 DNA concentration in connection to the decomposition state related to prey species abundance

As the correlation of input DNA and RRA was presented by Hilário et al. (2023), having a closer look on the DNA concentration of feces and gut contents from this thesis, it appears that the concentration was lower within stomach contents than intestinal contents, although there was no significant difference. This contratst with other studies, suggesting compromised detectability of animal DNA when it reaches the lower sections of the gastrointestinal tract, as it will be already further degraded. Within mice, soft-bodied prey items contributed the most to differences between gut sections, as they are probably already mostly absorbed when they reach the intestines, leading to a lower detection of its DNA in the lower parts of the gastrointestinal tract (Tomé, 2013; Pinho et al., 2022). Within seaside sparrows, stomach contents produced about 2.5 times greater DNA concentrations than faecal samples and stomach contents produced significantly more read identifications than faecal samples (Snider et al., 2022). These differences did not influence the description of diet, as similar measures of richness and diversity were found in both sample types. As our results does not seem to differ significantly and prey species were detectable, this influence might be not strongly different within grey seals but should be considered when DNA concentrations correspond with target DNA. As a standardized protocol was used, with the same sample input, incomparable content should not be an issue. There might be the possibility that a lot of gut epithelia might have been sampled or the low pH of the stomach could influence extraction process. High throughput sequencing techniques (HTS) are commonly used also for invertebrates and fish, but Deagle et al. (2019) suggested that faecal material contains a more consistent signal and might be more meaningful for qualitive RRA signal compared to stomach contents within marine mammals (Nakahara et al., 2016; Thomas et al., 2016).

The significant influence upon the state of degradation of the seal and the extractable DNA concentration was found here, as the DNA concentration decreased with increasing decomposition state. When apoptosis sets in, DNA strands breakage rapidly begins to occur as a result of endogenous endonuclease activity and spontaneous depurination (Lindahl, 1993). Further strand breaks, oxidative damage and molecular crosslinks accumulate will damage DNA according to the environmental conditions (Höss et al., 1996; Morin et al., 2001; Mitchell et al., 2005). Deagle et al. (2006) estimated the frequency of polymerase blocking

DNA damage using a model of random degradation on real-time quantitative PCR from fragments of various sizes on captive sea lion faeces samples fed herring. The analysis revealed a rapid decrease with degraded DNA template, as the amount of amplifiable DNA was inversely related to PCR product size and that predator DNA is more prevalent than prey DNA. There was no clear relationship between the amount of sea lion DNA and herring DNA purified from individual samples. Furthermore, the estimated frequency of damage was always, 2-fold less in average, in predator DNA than in prey DNA.

As faeces analysis is an effective approach of monitoring, previous studies on woodland caribou and swift fox revealed high concordance between total and target DNA estimates from faecal extracts by comparing amplification products, where 10 % of the samples relatively lower target-to-total DNA (Ball et al., 2007). Within wolves scats the number of prey sequence reads and the quantity of DNA to be sequenced did not vary between fresh and degraded scats (Massey et al., 2021). Applying multivariate regression and GLM on bootstrapped data revealed that decomposition state of the seal might not be a strong predictor for prey species detection via metabarcoding. Significance of the intercept for decomposition (p = 0.02) implies that, irrespective of decomposition's influence, there's an inherent expected value (baseline) of relative read abundance (RRA) for species in the samples. GLM reinforces this hint at a potential relationship between decomposition and read abundance. While decomposition might have a nuanced influence on the detection of specific prey species, the overall impact seems limited. The robustness of metabarcoding techniques allows for consistent species detection across varying decomposition stages, emphasizing its utility in ecological applications and suggesting that metabarcoding is sensitive enough to determine prey assemblages in degraded scats. Within marine mammals Tollit et al. (2009) measured a decline DNA was recovered from 87 % of scats, although the recovery rate declined to 52 % in scats considered “old” at the time of collection. Accounting for the possible contamination of the samples, less species were detected within degradation state 5, compared to 2, 3 and 4.

While DNA damage should correlate with age of template, the connection is often somewhat unclear (Thomas & Gilbert et al., 2003; Mitchell et al., 2005; Gilbert et al., 2006). The process of DNA degradation is sample specific, but within any sample, damage that prevents PCR amplification will be caused by a large variety of mechanisms (Deagle et al., 2006). According to high DNA concentrations within degradation state 3 and 4 it seems there is a great variation of DNA concentration, but showing a tendency that with increasing decomposition state the DNA concentration decreases and therefore also the amount of detectable species DNA. But the sequencing results also represent a high sensitivity with rising decay state, as various species are still detected and no significant difference occurs.

A great advantage of the PERMANOVA is that is non-parametric, can handle unbalanced sample sizes among groups and is a distance or dissimilarity measure. The stress value (0.1) of the NMDS presents an acceptable representation of the data and presents the overlap between the two methods. While having a closer look on the RRA of species in correlation to the degradation state of the seal, cross-validation on log-transformed relative read abundance was employed to discern the optimal model. The multivariate regression emerged as the best-fitting model, implying that this method was most suitable for predicting the dependent variable given the predictors in your dataset. AIC and deviance values suggest a satisfactory fit of the GLM model to the data. However, the goodness-of-fit varied among species, meaning the model's explanatory power is not consistent across all species. Bootstrapping was used to increase data based on the low sample size. But these results assume that the given data represents the population of grey seals and should be also therefore taken with caution.

5.1.2 Effect of indices calculations on prey species analysis

In terms of overall species richness, higher R^2 values of genetic samples seem to imply a better fit and a possible underestimation of species richness, compared to morphological identification reaching a plateau, due to the small sample size. According to previous studies, a higher taxonomic resolution was expected within metabarcoding approach. Increased species identification was expected due to various previous studies on seal diets (Purcell et al., 2004; Deagle et al., 2005; Deagle & Tollit, 2006; Matejusová et al., 2008; Méheust et al., 2015; Granquist & Hauksson, 2016). Méheust et al. (2014) revealed a combined approach, increased the identification of prey items by around 32 % for grey seals and Granquist and Sigurjonsdottir (2014) presented and increase of 38 %. Recent study showed an increase of detections of 13 prey taxa, with 32 prey taxa identified overall combining methods increased (McCosker et al., 2023). Tverin et al. (2019) presented that DNA analysis provides firm identification of many prey species, which were neglected or identified only at species group level by morphological analysis.

The assessment of species diversity using both morphological and genetic indices offers a holistic view of biodiversity across different variables. Morphologically, variables like GB and SCV displayed moderate species diversity with Shannon values of 0.294 and 0.600, respectively. However, when comparing species evenness using the Simpson index, the GB samples indicated relatively low evenness at 0.199, suggesting potential dominance by a few species. On the contrary, the SCV samples displayed a higher and more even species distribution with a Simpson value of 0.413. Genetically, the data portrays substantial yearly variations in species diversity. For instance, the Shannon index for 2016 was 0.23 ± 0.39, indicating moderate diversity, while 2017 showcased a much higher diversity with a value of 1.37 ± 0.002. This contrast is dedicated to selective choice of sampling. Comparing the two methods, the gamma diversity derived from the genetic data was 0.67 ± 0.3, slightly lower than the 0.82 value observed morphologically using the Simpson Index. This disparity underscores the nuances and intricacies inherent in each method, suggesting that while morphological data might offer a broader view of diversity, genetic data can delve deeper into the specifics of species composition and evenness. Thus, integrating insights from both methodologies provides a comprehensive understanding of biodiversity across different variables and timeframes.

5.1.3 Limitations of both methods

There are certain limitations within each method. Due to degradation of morphological analysis it was only possible to determine species to family level. This was especially the case for intestinal samples. Previous in-vitro digestion studies of artificial seal stomachs presented, the complete digestion of smaller fish such as sprat (within 30 min) and herring (18 - 35 g; after 180 - 240 min) was faster, compared to larger fish such as cod (within 540 - 660 min) (Hoffmann, 2019; Klemens et al., 2022). Using the genetic approach a limited resolution in the identification of flatfish species in contrary to other studies using cytochrome b, 12S and COI primer, respectively (Céspedes et al., 1998; Comesaña et al., 2003; Paracchini et al., 2017) was similar to findings from (Boyi et al., 2022). However, the cytochrome b or 12S mitochondrial markers used in such studies present notable challenges, especially in terms of conserved regions (Miya et al., 2015; Peters et al., 2014; Zhang et al., 2020) and the identification of flatfish using metabarcoding (Valentini et al., 2016). In environmental sample metabarcoding, there's often a balancing act between achieving detailed taxonomic resolution and amplifying the shortest possible DNA fragment (Taberlet et al., 2012). As working with degraded DNA, existing in smaller DNA fragments, targeting the 16S mtDNA region seemed to be most suitable. However, this also implies that the resultant sequence might hold limited information, which was evident in the confidence levels when identifying flatfish and sculpin.

Due to the findings, both metabarcoding and morphological methods offer unique and invaluable insights into species diversity, with each presenting distinct strengths and disparities. To achieve a comprehensive understanding of biodiversity, it's paramount to leverage the advantages of both approaches synergistically.

5.2 Prey species occurrence by ecological variables

To understand the dietary habits of grey by the influence of different ecological variables, the investigation involved an examination of diet variations concerning different geographic areas, distinct years, and quarterly seasons (referred to as quarters). Our goal was to determine if we could detect noticeable differences in the seals' diets metabarcoding and morphological results in relation to the variables. Upon a closely examination of dietary patterns across varying geographical regions, year and quarterly seasons there was no significant difference observed, employing morphological sampling and metabarcoding. This observation aligns with previous studies on stomach content in the Baltic Sea, which confirmed opportunistic feeding behaviour of grey seals depending on region and prey availability (Lundström et al., 2007, 2010; Winkler et al. 2011; Olsen et al., 2018; Mehtonen, 2019; Hoffmann, 2019).

5.2.1 Investigating grey seal diets across the variables area, year and season

While the general absence of distinct patterns in the RDA plot makes it challenging to draw concrete conclusions, few noteworthy associations have emerged from the analysis. As previously mentioned, the coast of Mecklenburg-Western Pomerania includes different habitats, ranging from ranging from moderately saline (mesohaline) to slightly saline (oligohaline) conditions (Thiel, 1990; Winkler & Schröder, 2003; Rittweg et al. 2023). This diversity in environmental conditions naturally leads to variations in fish communities. Consequently, it was suggested that such habitat diversities might manifest as differences in the dietary of grey seals inhabiting these regions. However, the data revealed a clustering of samples and the overlapping ellipses in both genetic and morphological analyses suggest a similarity in prey species composition across these areas (Figure 12. The few observed differences could be attributed to the higher prevalence of cod and herring along the east coast of Rügen compared to the Greifswald Bay, where percids are more prominently present. These variations appear to be influenced by the mesohaline conditions prevalent in the respective areas, such as freshwater species distribution is limited to lower PSU.

Notably, round goby appeared to be somewhat associated with the WCR location. However, permutational testing did not reveal certain significant differences. In terms of genetic analysis, the first five principal components account for approximately 71.72% of the total variance. Including two more components this proportion increased to 87.94%. *P*-values associated with location suggest that this factor do not significantly influence the relative read abundances (RRA) of different prey species. This trend is further emphasized in the PCA plot (Figure 21), where samples tend to cluster based on their respective location, highlighting that similar locations yield similar RRAs. In the PCA plot, the proximity between a point (sample) and an arrow implies a high value of the corresponding variable for that particular sample. For instance, the PCA suggests that perch plays a significant role in the diet of seals from the Greifswald lagoon, along with the importance of sprat and round goby. Overall, despite the absence of conspicuous patterns, the multidimensional analysis provides valuable insights into the nuanced relationships between seal diet, location, and prey species composition. Freshwater species are present in the western part of the Baltic Se as well as they increase in abundance the eastern part of Mecklenburg-Western Pomeranian coast (Winkler & Schröder, 2003). The distribution is tied to the salinity gradient, which, in turn, influences the composition of fish assemblages in these habitats. This salinity-driven shift in prey availability is a well-documented phenomenon. Studies in Sweden, for example, have presented that large predator fish like pike is an important prey species in the inner and central regions of the archipelago, contributing up to 20% of the diet biomass, ranking third after perch and herring (Svensson, 2021). In contrast, the outer archipelago records a lower representation of pike in the diet, accounting for less than 5% of the biomass (Lundström et al., 2007; 2010; Sjödberg et al., 1992; Hansson et al., 2018). Interestingly, pike was not identified in the diet irrespective of the used analysis method. However, the limitations of this study must be taken into the account. Firstly, the sample size for both genetic and morphological analysis was relatively low. Furthermore, most of the seals were found in the central and outer coast of the Greifswald Bay and may not depict seals preying on large prey fish. At this point it should be mentioned that the analysis was constrained by the certain bias, that deceased and stranded grey seals were analysed, without knowledge of their previous feeding locations. Additionally, as a previous study show, targeting large prey species could be a specialized feeding behaviour, known to occur in especially adult male grey seals (Königson et al., 2013; Tverin et al., 2019). Indicating also ripped of parts and leaving lots of flesh behind, which on the other hand can contribute valuable energy to the ecosystem (Cederholm et al., 1999; Watkinson, 2000).

Investigating the temporal dynamics by looking into the relationship of years, RDA analysis (Figure 13) presents that there appears to be a stronger affinity between gadids and herring with the years 2019 and 2017. It is worth noting that in 2019, there was only one sample available and no herring could be detected in that particular sample. Additionally, flounder appears to be correlated to 2022. This relationship is further visualized in the NMDS plot (Figure 12), which shows that in 2022 samples seem slightly more dissimilar compared to samples from 2020. Analysing the relative abundance of prey species across the years, it becomes evident that herring is present all over the year with a declining trend. Herring seemed to have the most influence in variation when comparing 2017 to the subsequent 3 years (2020-2022). Comparing 2020 with 2017, percids and round goby increased in relative abundance. While in 2022 roach caused for cumulative dissimilarities. While there is no statistically significant influence of the year on the abundance of various prey species, subtle patterns are still discernible within the data. Assuming that dietary preference reflects species assemblage present at specific locations and times, these findings try to shed a light on some trends in fish stocks. It is shown that certain fish stocks have declining trends, such as overexploited western Baltic cod stock (SD22-24) (ICES, 2023b). Similarly, in the central Baltic Sea, the herring stock has experienced a decrease by more than 80 percent compared to levels in the 1980s. Over the past four years, this trend has accelerated and the stock has declined by 40 percent, reaching a critical stage (ICES, 2023). In addition to these declines, invasive species, such as round goby, have increased quantities in all coastal waters in Germany since about 2005 and have now become an important part of the lagoon food web (Winkler et al., 2014; Rothe et al., 2016; Oesterwind et al., 2017). In the late 1980s and early 1990s the regime shift in the open-sea pelagic ecosystem was evident by a transition from a cod-dominated system to one dominated by sprat and herring (Michalsen et al., 2013). A transition that was reflected in a shift of grey seal diet (Sjödberg et al., 1992; Lundström et al., 2007; Strömberg et al., 2012). The high abundance of herring in the diet of grey seals could be verified in this study and freshwater species are gain greater importance in lagoon areas. Additionally, the data indicates an increase in round goby consumption in the past years, while herring and cod seems to decline in importance. To further validate these trends and the patterns at the population levels, it is recommended that additional samples should be included in future analysis of grey seals in the area.

The PCA based on genetic analysis did not reveal significant differentiation based on the year (Figure 21). The 95% CI intervals for different years overlapped, suggesting consistent diet profiles across years. However, it should be mentioned that the year factor was somewhat biased, as most samples were originated from 2017, resulting in narrower Cis for this particular year. Overall, the year 2016 displayed higher variation, probably caused by the detection of bream and garfish in the diet.

When comparing the quarters of the year, the analysis did not find significant differences in prey species abundance. The RDA showed no clear correlation between species abundance and the quarter variable. It is worth noting, that goby seemed to be linked to the fourth quarter, while roach showed a stronger link to the second quarter. Morphological analysis revealed that the mean species diversity in the fourth quarter was lower in comparison to the other quarters of the year. The genetic data also could not reveal a distinct relationship between the quarter and prey species RRAs. It appeared that quarter 3 seems to have a narrower range of species in these samples when compared to quarters 2 and 4, as evidenced by the relatively less wide confidence intervals.

As previously mentioned, in the Greifswald lagoon, the highest sighting frequencies of grey seals occur between February and May, coinciding with the herring spawning season around the island Greifswalder Oie, located east of Greifswalder Bodden (Buschhaus & von Rönn, 2022; von Nordheim et al., 2019). It was expected that spring spawning movements might be noticeable in prey species diversity and abundance, but the analysis did not support this hypothesis. This underlines the importance of certain species, such as herring and perch (within genetic analysis), throughout the entire year. It is important to acknowledge again that our study faced limitations related to sample size, that the results only provide a small insight in the whole context. Furthermore, the warming winters induced by climate change, provides a present-day stressor that affects the reproductive capacity recruitment of species, e.g. herring (Polte et al., 2021). Meaning that spawning time may be shifting to earlier months, which could also contribute to the absence of a significant pattern in our findings.

5.2.2 Using RDA and PCA to investigate the species-environment relationships

The Redundancy Analysis (RDA) was implemented to explore potential relationships between fish species abundances and environmental variables. The analysis suggests that the drivers of species abundance in this study may be a complex interplay and may not be adequately represented by the examined variables. The presence of elevated VIF values indicates multicollinearity, implying strong correlations among certain predictor variables. Specifically, the issue of multicollinearity related to location was addressed by designating GB as the reference level, enhancing the RDA's robustness. However, it is essential to highlight that the permutation test under the reduced model did not identify significant relationships between the species abundances and the environmental variables. Thus, while certain species my display tendencies towards specific environmental variables in the RDA plot, the statistical evidence supporting these relationships is not robust. Consequently, while some species seem to have associations with particular variables, it must be looked at it with caution when drawing definitive conclusions from these findings. PCAs primarily served the purpose to reduce the dimensionality of the data while preserving as much of the variance as possible. Furthermore, it was employed to discern patterns within the dataset concerning species occurrence and the variables of location, year, and season. From the analysis, the first five principal components account for approximately 71.72 % of the total variance, while the inclusion of two more components increases this proportion to 87.94 %. This strong representation of variance is captured by the initial components, as supported by the scree plot, indicates that these components are instrumental in representing the data's structure. The biplot (Figure 21), a valuable component of the PCA, provides additional layers of supplementary insights. Arrows in the biplot represent different variables (RRAs in this context), and their distribution suggests that these variables each offer a unique facet of variance, uncorrelated with the others. The length of an arrow reflects its importance in the PCA. Notably, species such as dab, sculpin, horse mackerel, and roach appear to be pivotal within this projection. Arrow directionality reveals the relationship between the original variables and the principal components: closely aligned arrows, such as those for horse mackerel and dab, or bream and garfish, suggest these species' abundances vary in tandem. Neither RDA nor PCA revealed strong patterns or significant relationships between species data and the variables under consideration: location, year, and quarter. However, it is important to note that these two methods employed different approaches to analyse and visualize these variables. While RDA focused on direct relationships but faced issues like multicollinearity, where on the other hand, PCA identified patterns based on variance without considering any external variable during the computation. Despite these differences in approach, both methods suggested a high degree of overlap and consistency in the dietary profiles across the different categories of these variables.

5.2.3 Size dependent prey preferences in grey seal diets

In the context of using the RDA, seal length did not demonstrate a significant relationship with prey species they consumed. However, when recalculated the estimated lengths of prey fish using the otoliths a non-linear relationship appeared, indicating bigger seals may tend to prey on larger fish. To investigate this relationship, both a linear regression and the GAM were performed, indicating that there is a significant relationship between the length of seals and the prey length they consume. The linear model provides a simplified view of this relationship, suggesting a constant rate of increase in prey length with every unit increase in seal length. In contrast, the GAM plot illustrated a more detailed understanding of the relationship between seal length and prey length and revealed the tendency that larger seals consume larger fish. Given the observed patterns in the data, it appears that the GAM fit captures the underlying trends more accurately than the linear regression, especially when considering the evident significant non-linear trend between seal length and prey length. The plot suggests that while larger seals generally tend to eat larger fish, the rate at which prey size changes is not consistent across all seal sizes (Figure 14). Given the observed patterns in the scatterplot and the results from both models, it suggests that while larger seals generally prefer larger prey, the relationship is not linear and varies depending on the seal size. Still, it is important to not, that grey seals are opportunistic foragers, so this non-linear trend might be better reflected within the GAM. Similar patterns have been observed in other studies, such as in Faroese waters, where juveniles' grey seals diet consistent most frequently on sandeels, pre-adults on sandeels and saithe and adults on cod (Mikkelsen 2002). Lundström et al. 2007 also reported a similar pattern, where younger seals prey on smaller sized fish compared to larger adult seals.

In has to be mentioned that in the analysis, read counts of grey seal and harbour porpoise DNA was excluded. The primary reason for this was the limited efficiency of blocking primer and sampling was performed within necropsies of the marine mammal stranding network in the Oceanographic museum in Stralsund. During these necropsies, it was challenging to ensure sterile working conditions, which could introduce noise and potential contamination into the genetic data. Nevertheless, as earlier mentioned grey seals are opportunistic foragers and recent studies present that cannibalism and predation on other mammals is present (van Neer et al., 2015). A recent study by Westphal et al. (2023) document this behaviour for the first time also in the Baltic region. This predatory behaviour highlights that specialised individuals can feed on large prey items (>1m). These specialized seals typically focus on consuming soft tissues, leaving characteristic lesions on the carcasses of their prey Due to the footage of lesions on carcasses of large predatory fish induced by grey seals, this might indicate that specialization of grey seal individuals occurs at the inner archipelagos of the lagoons.

5.2.4 Predatory impact on fish stocks in the southern Baltic Sea

The daily food requirement for adult grey seals can varying between 4 and 8 kg of prey fish (Wendt, 2018; Nestmann & Harder, 2014). Predation effects can be substantial and affect fish abundance and size-structure of fish populations (Arlinghaus et al., 2021; Klimaszyk & Rzymski, 2016; Veneranta et al., 2020).

In this analysis of 15 grey seals, certain prey species emerges as particularly important. These included herring, cod and round goby. Pike-perch played also an important role, whereas total consumption of perch was rather high. The IRI, considering the weight proportion reflects the importance of herring, cod and round goby within the diet of analysed grey seals.

Due to the low sample size, it was not possible to estimate total consumptions of species by grey seals comprehensively. This was further limited by the fact that the samples were collected across different years and did not cover the whole spectrum of the year. The opportunistic feeding behaviour and preference to prey species occurring at haul-out sites, depict a possible pressure on fish stocks that already occur in low natural abundance, such as pike.

In the southern Baltic Sea, various natural predators coexist alongside anthropogenic-induced abiotic pressures. Fish stocks, especially in brackish inshore ecosystems, face exploitation challenges (Basan 1988; Winkler, 1989; Döring, 2001; Larsson et al., 2015; Koemle et al., 2021). In the central and southern Baltic, seals and birds consume about as much flatfish as is caught by the fishery. Birds and seals consume 2-3 times as much coastal fish as is caught in the fisheries, what lead to a possible competition (Hansson et al., 2018). Representing the urge of carefully considering predation in ecosystem analyses and stock assessment models. Furthermore, poor status and bad nutritional status of the grey seal in assessments by HELCOM (2018), it is suggests that grey seal in the Baltic Sea may be food limited and indicates the possibility they are forced further into the archipelago (Kauhala et al., 2018), expanding their forage grounds.

6. CONCLUSION

The hypotheses explored in this study have yielded first insights into the dietary habits of Baltic grey seals. While the genetic analysis detected a higher number of species compared to morphological sampling, it is important to exercise caution when interpreting these results. Furthermore, the decomposition of the seals significantly affects DNA concentration and potentially hamper the detectability of prey species. The identification of a non-linear relationship between seal length and prey species indicates that larger seals may prefer to prey on larger prey species. However, the findings of this study emphasize the need for further research using a larger sample size, as environmental variables such as area, year and quarter did not have a significant effect on abundance of prey species. Future investigations with more extensive data collection could enhance the resolution of our understanding.

This work elucidates another effective approach in analysing the dietary composition of Baltic grey seals (*Halichoerus grypus*). This work presented an innovative application of metabarcoding as a tool to detect multispecies consumption patterns in grey seals in the southern Baltic Sea. Although contamination was a challenge, the study successfully detected distinct prey species. Genetic analysis presents a potential to unravel the full assortment of taxa consumed by grey seals and improve knowledge on foraging ecology of the target species. Presenting the well-functioning and effectiveness of primers used to detect prey species, there might be the possibility of missing species in the_genetic detection, as more species are present in the diet. Up to date morphological analysis remains essential for estimating consumed biomass, as RRAs are not directly linked to biomass. The results of this study emphasize, to consider a holistic approach when estimating the diet composition of grey seals, leveraging the strengths of both genetic and morphological methods. This study enhances the knowledge of prey species diversity and abundance, and was trying to estimate the significance of distinct prey fish in the diet of grey seals along the coast of Mecklenburg-Western Pomerania.

For future studies a useful addition would be sampling of grey seals haul-out areas, as they show a certain side fidelity and also it could provide valuable insights into the reflected fish assemblages within their diets. Short-term dietary analysis only reflects the past 24 – 48h, making it essential to investigate these haul-out areas where prey species interactions are likely. Interactions with grey seals and fisheries are of increasing concern along the coast of German Baltic coastline, therefore diet analyses are becoming a necessary tool to determine actual overlap between targeted fish species and marine mammal prey. Understanding their diet is vital for conservation and management efforts, because recovering populations of predatory wildlife could result in stronger top-down control on their ecosystems and lead to potential increase of human-wildlife conflicts. Furthermore, feeding studies with a diverse prey species selection could provide additional insights into DNA primer efficiency.

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Abbreviations

Abbreviation	**Description**
AIC	Akaike Information Criterion
Bp	Base pair
CI	Confidence Interval
GB	Greifswald lagoon / Greifswalder Bodden
HELCOM	Helsinki Commission
ICES	International Council for the Exploration of the Sea
LALLF	Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei
NCF	Numerical correlation factors
NMDS	Non-Metric Multi-Dimensional Scaling
NWM	North-West Mecklenburg
MWP	Mecklenburg-Western Pomerania
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
RDA	Redundancy analysis
RRA	Relative read abundance
SC	Surrounding coast east Rügen
SCV	Surrounding coast Vorpommern
SIMPER	Similarity Percentage Analysis
VIF	Variance Inflation Factor
WCR	West coast Rügen

Appendix

Tables:

Table A1: Regression equations used to estimate consumed prey length and weight.

Study	**species**	**Regresssion (TL)**	**Regression (g)**
Härkönen et al. (1986)	Sander lucioperca	FL = -124.80 + 52.49 OL r^2 = 0.928	FW = 0.05852 * OL$^{4.0007}$ r^2 = 0.908
Leopold et al. (2001)	Clupea harengus	FL = -6.36 + 15.5*OW r^2 = 0.95	FW = (1.42*OW)$^{4.46}$ r^2 = 0.96
	Gadus morhua	FL = -5.51 + 7.84*OW r^2 = 0.93	FW = (0.92*OW)$^{3.82}$ r^2 = 0.97
	Perca fluviatilis	FL = -3.21 + 7.3*OW r^2 = 0.89	FW = (0.9*OW)$^{4.07}$ r^2 = 0.91
	Rutilus rutilus	CL = 0.24+ 1.5*CW r^2 = 0.84	FW = (0.9*OW)$^{2.31}$ r^2 = 0.91
Azour et al. (2015)	Neogobius melanostomus	OL = 0.026 × TL + 0.97 r^2 = 0.97	log(W) =log(0.128)+3.25log(OL) r^2 = 0.97

Table A2: GLM and GAM statistical analysis

Metric	Linear Regression	GAM
Model Formula	length∼length_seallength∼length_seal	length∼s(length_seal)length∼s(length_seal)
Coefficient (Intercept)	-123.2690	- (Non-linear model)
Coefficient (length_seal)	1.9204	- (Non-linear model)
Coefficient p-value	2.72e-11	2.2e-16 (overall)
R-squared	0.3875	- (Use deviance explained)
Adjusted R-squared	0.3807	- (Use deviance explained)
Model Significance (p-value)	2.722e-11	2.2e-16
Residual Standard Error	70.36	- (Use RMSE)
AIC	- (If available)	1008.62
Shapiro-Wilk Normality Test	- (If conducted)	0.3357

Morphological Ananlysis

Table A3: PERMANOVA results of Hellinger transformed relative abundance of prey species from morphological analysis and additional ANOSIM analysis.

Factor	Df	Sum of Squares	R^2	F	Pr(>F)
further_location	4	1.3315	0.13735	0.9969	0.489
Decomposition	1	0.1676	0.01728	0.5018	0.772
year	1	0.6828	0.07043	2.0448	0.073*
quater	1	0.3905	0.04028	1.1694	0.343
length_seal	1	0.1101	0.01136	0.3299	0.900
Residual	21	7.0122	0.72331	-	-
Total	29	9.6947	1.00000	-	-

* Significance codes: 0.001 '*' 0.01 '' 0.05 '.' 0.1 ' ' 1

Analysis	R	Significance
ANOSIM on rearranged_data1.52 vs further_location	-0.07933	0.882

SIMPER analysis of Hellinger transformed relative abundance of species:

Table A4: Influence of Location on Species Composition.

Comparison	Top Contributing Species	Cumulative Contribution (%)
SC_GB vs SC_NWM	Rutilus rutilus	26.7
	Clupea harengus	47.7
SC_GB vs SC_WCR	Neogobius melanostomus	42.5
	Clupea harengus	60.5
SC_GB vs SC_SCV	Gadidae	25.3
	Clupea harengus	50.3
SC_NWM vs SC_WCR	Neogobius melanostomus	41.5
	Rutilus rutilus	62.0
SC_NWM vs SC_SCV	Rutilus rutilus	30.3
	Gadidae	58.2

Table A5: Influence of quarter on species composition.

Comparison	Top Contributing Species	Cumulative Contribution (%)
Q3 vs Q4	Rutilus rutilus	26.0
	Clupea harengus	49.6
Q3 vs Q1	Rutilus rutilus	28.3
	Gadidae	55.8
Q3 vs Q2	Rutilus rutilus	27.1
	Clupea harengus	51.1

Table A6: Influence of year on species composition

Comparison	Top Contributing Species	Cumulative Contribution (%)
2017 vs 2020	Clupea harengus	28.2
	Percidae	51.2
2017 vs 2022	Clupea harengus	24.4
	Rutilus rutilus	43.4
2017 vs 2021	Neogobius melanostomus	27.8
	Clupea harengus	52.0

Direct method comparison

Table A7: PERMANOVA Results direct comparison of morphological and genetic analysis.

Factor	Degrees of Freedom (Df)	Sum of Squares (SumOfSqs)	R^2	F-value	p-value
Combined Factor	1	1.3608	0.26768	4.0207	<0.001
Residual	11	3.7231	0.73232	-	-
Total	12	5.0839	1.00000	-	-

Table A8: Top Influential Species from SIMPER Analysis (top 10 direct comparison)

Rank	Species	Average Dissimilarity	Standard Deviation (SD)	Ratio	Cumulative Sum (cusum)	p-value
1	Clupea	0.3594	0.4066	0.8839	0.7926	0.589
2	Pleu_Plat_Lepi	0.2992	0.4119	0.7263	0.9936	0.003
3	Myoxocephalus_Microcottus	0.1335	0.2212	0.6034	0.9261	0.001
4	Rutilus	0.0640	0.0794	0.8060	0.9994	0.229
5	Perca	0.0446	0.0737	0.6055	0.9740	0.423
6	Trachurus_Lepidorhombus	0.0249	0.0562	0.4419	1.0000	0.001
7	Neogobius	0.0247	0.0424	0.5834	0.9508	0.949
8	Sprattus	0.0232	0.0524	0.4419	1.0000	0.001
9	Limanda	0.0196	0.0443	0.4419	0.9012	0.001
10	Abramis	0.0058	0.0132	0.4419	0.3597	0.001

Figures:

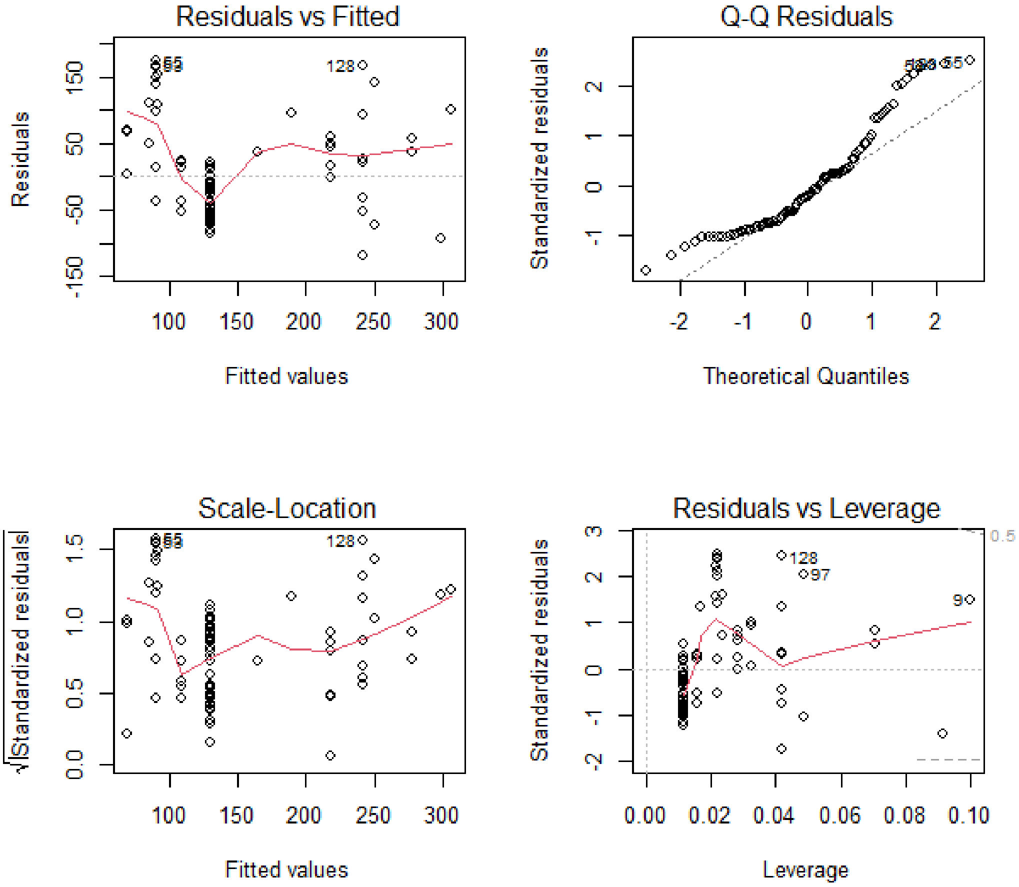

Figure A1: Investigation of the correlation between grey seal length and prey fish length by using GAM Model

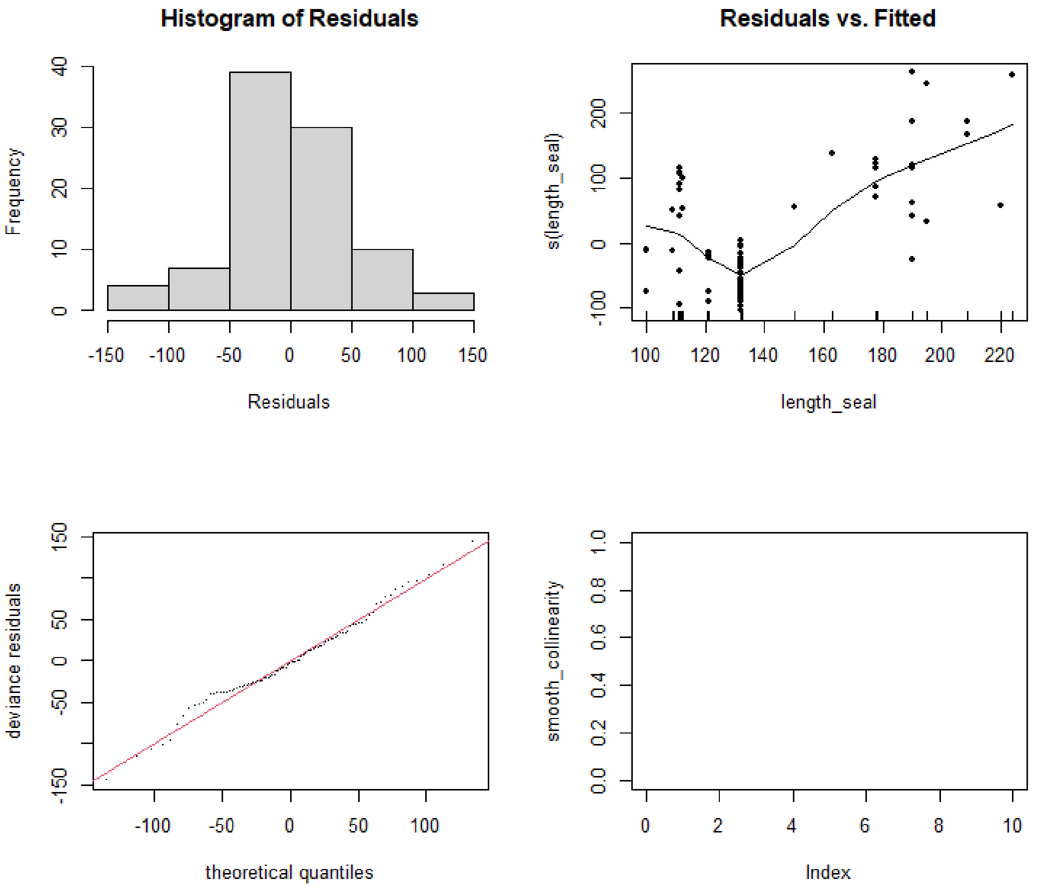

Figure A2.: Investigation of the correlation between grey seal length and prey fish length by using GAM Model

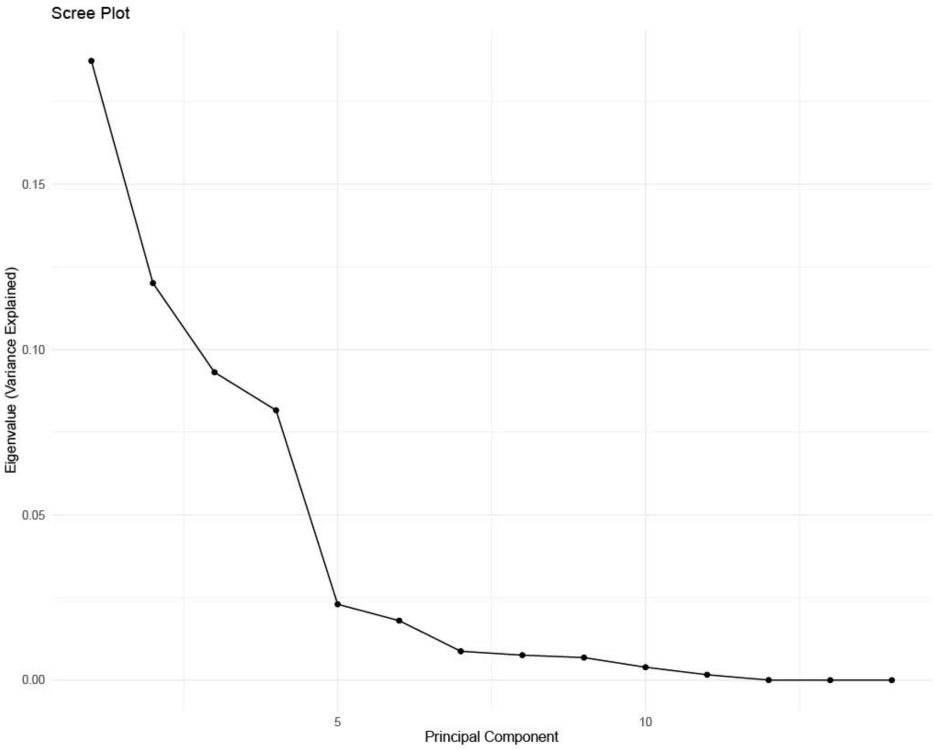

Figure A3: Scree plot of Hellinger transformed RRA from metabarcoding PCA analysis.

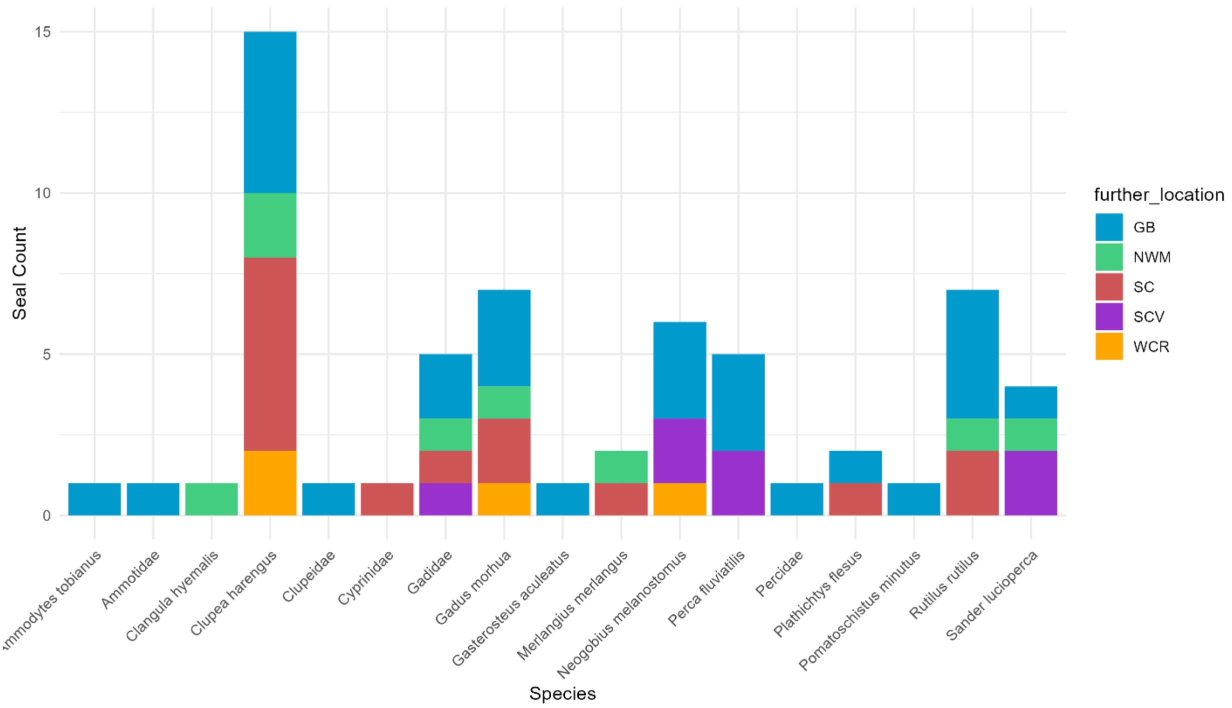

Figure A4: Count of grey seals (n = 32) in which each prey species was found. Different colours present different areas NWM (North-West Mecklenburg), SCR (surrounding coast Rügen), SC (surrounding east coast of Rügen), GB (Greifswald lagoon), SCV (surrounding coast Vorpommern).

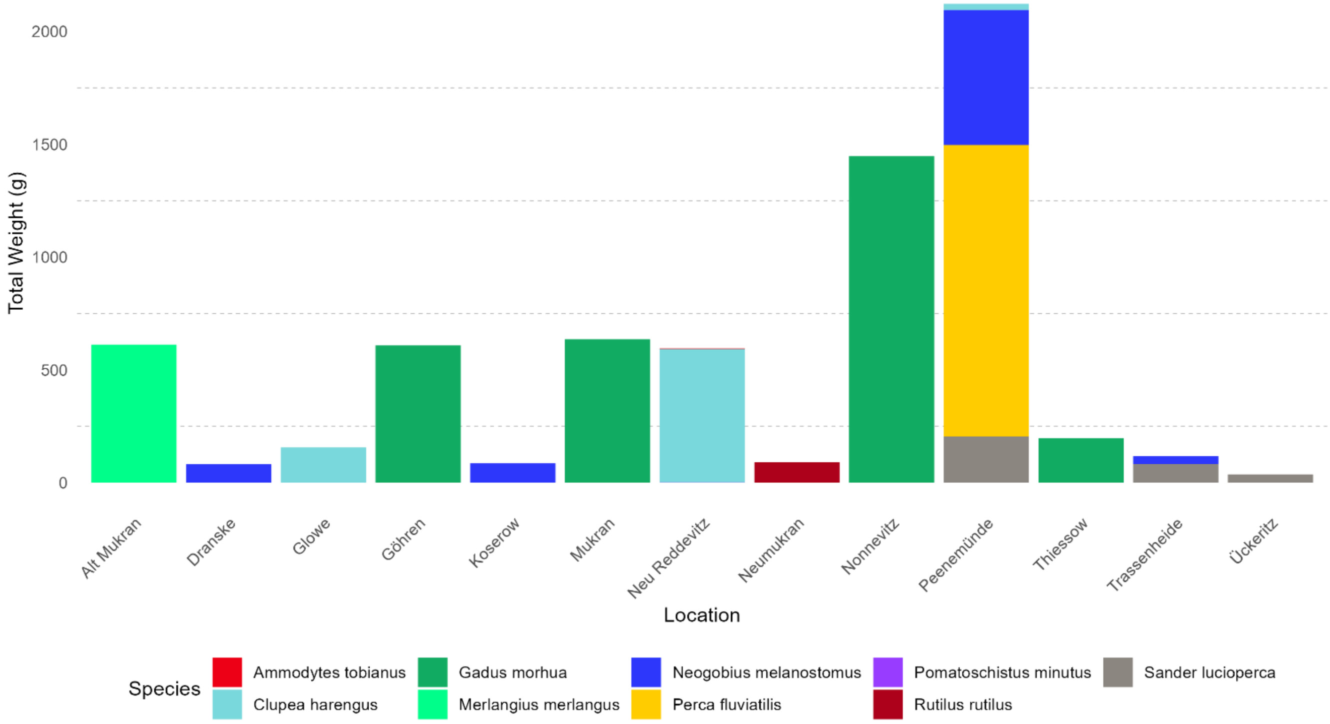

Figure A5: Estimated prey biomass of analysed grey seal stomachs.

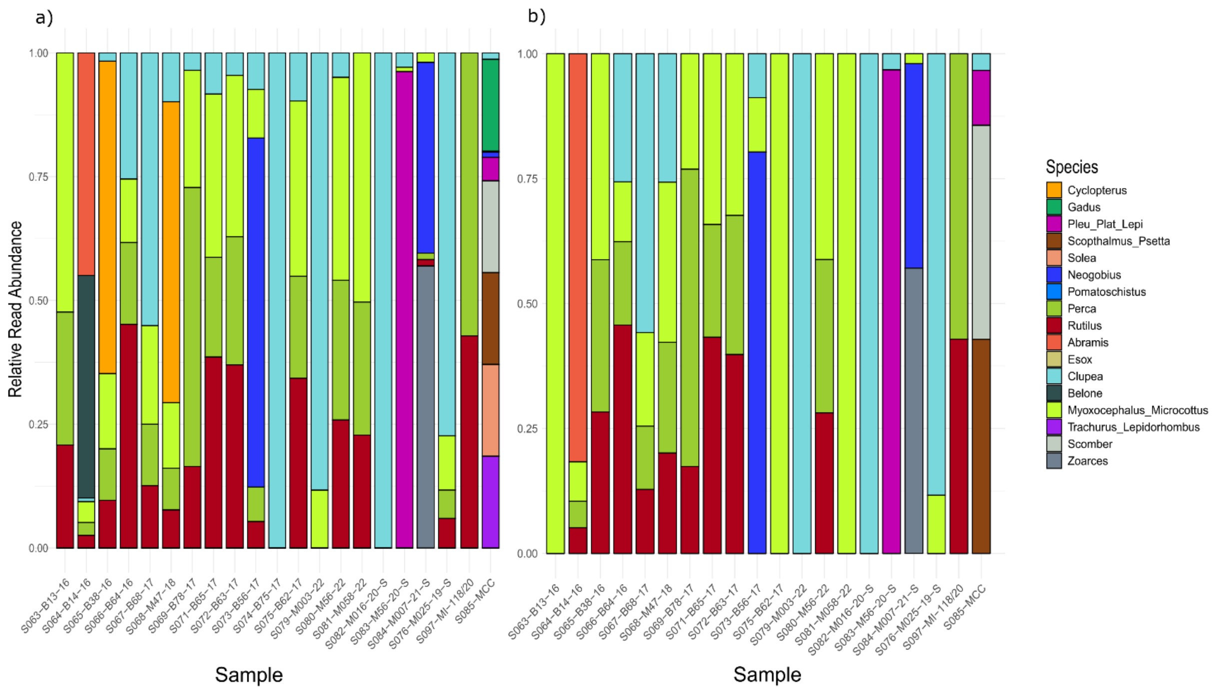

Figure A6: Realtive read abundances of each individual Illumina sequenced metabarcoding sample (n = 21). Empty samples were excluded and additionally mock community is depicted. a) showing lowest limit of filtered data (excluding <200 reads) and b) presenting highest filtering (<633 reads).