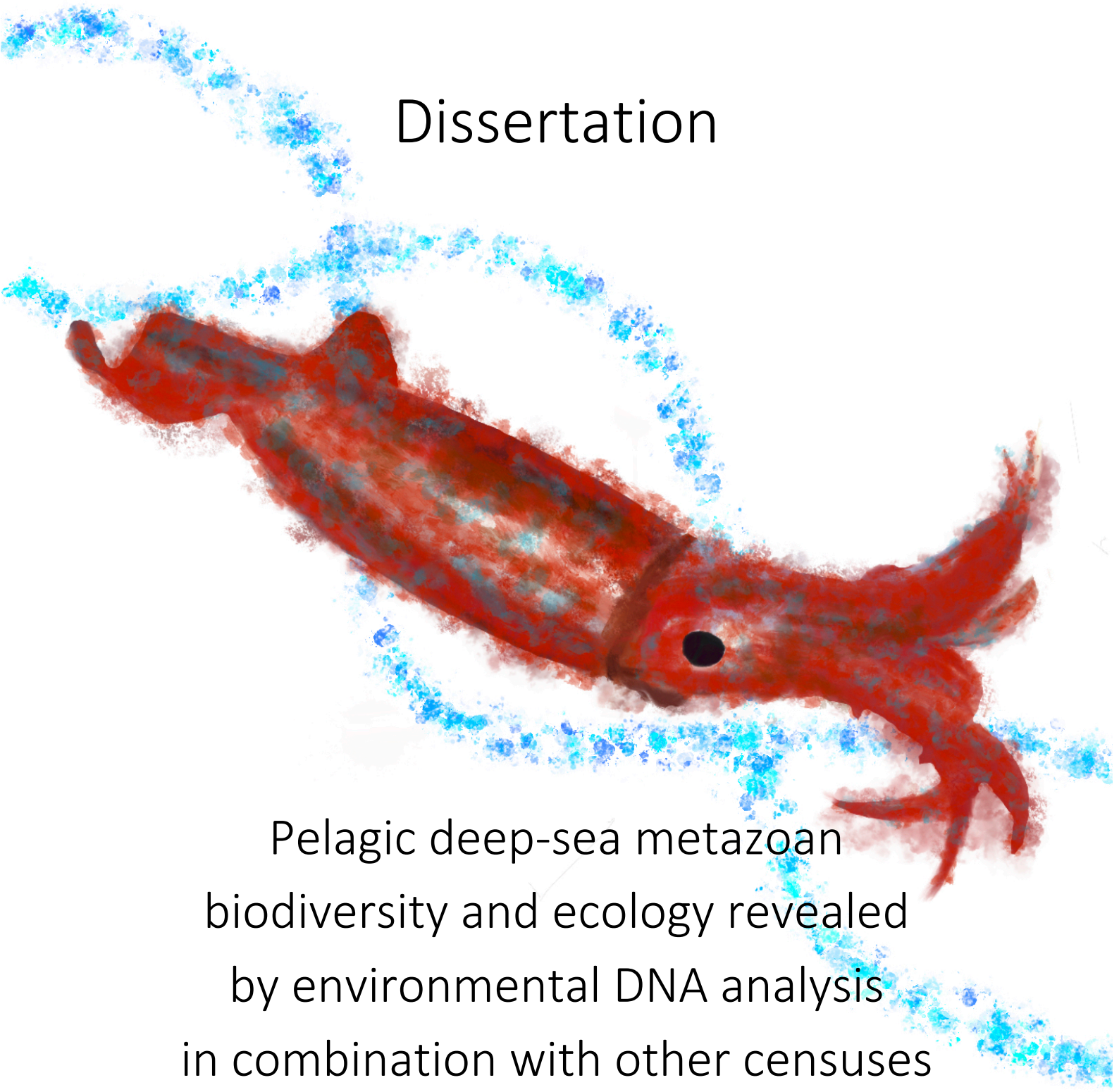
Dissertation



Pelagic deep-sea metazoan biodiversity and ecology revealed by environmental DNA analysis in combination with other censuses

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“There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.”

— Charles Darwin, The Origin of Species, 1859

“The sea, once it casts its spell, holds one in its net of wonder forever”

- Jacques-Yves Cousteau

Table of Contents

Summary

The deep sea (> 200 m) not only represents the largest habitat on earth, but also has the highest faunal biomasses and greatest number of individual organisms. While the deep sea provides humans with substantial services, its ecosystems remain poorly studied. Logistical and technical challenges to sample deep-sea ecosystems as well as organisms' avoidance behavior to underwater gear stress the need for alternative techniques. In this thesis, I focused on a relatively novel tool in deep-sea biology; environmental DNA (eDNA) analysis. Environmental DNA is genetic material that organisms shed into their environment. This eDNA can be assigned to a specific taxon and provides information on species presence, diversity and distribution without the need to encounter or capture the source animal.

The first objective of this thesis was to develop a pipeline to collect diversity and distribution data on deep-sea cephalopods with eDNA analysis from water and sediment samples. A metabarcoding approach was developed and optimized to detect eDNA of cephalopods (Chapter 1-3) using a novel primer that targets the nuclear 18S rRNA gene (Chapter 4). The workflow resulted in detection of cephalopod eDNA in seawater from different oceanic regions, but not in sediments, probably due to low cephalopod DNA concentrations. Due to the limitations of general primers, I developed species-specific primers for the deep-sea squid *Taningia danae* (Chapter 2) and the pelagic tunicate *Pyrosoma atlanticum* (Chapter 5).

The second objective was to establish biodiversity baselines and distribution patterns of key organisms in the deep sea and to put these patterns into an ecological context. The developed eDNA pipeline was first applied to investigate deep-sea cephalopod diversity off the Azores (Chapter 1). A total of 39 cephalopod taxa were detected. This diversity and distribution data was then used to reconstruct the prey community composition of the foraging zones of two top-predators (*Ziphius cavirostris* and *Grampus griseus*). These cetaceans hunt in the same geographic region off the Azores in the North Atlantic, but at different depths and distances from the coast (Chapter 1). Using a novel combination of biologging of predators and analysis of preyscapes via eDNA, we tested the hypothesis that cetacean niche differentiation is driven by difference in prey diversity. The cephalopod diversity and hence the cetacean prey community composition overlapped between the different foraging habitats and depths of the whales. This led to the new hypothesis, that both cetaceans feed on similar species, but on different ontogenetic stages. Combining eDNA analysis, net sampling and *in situ* observations from video surveys and existing literature records, I established a cephalopod species list for the archipelago of Cabo Verde and identified Cabo Verde as cephalopod hotspot with 64 species (Chapter 2). I also tested the hypothesis that Cabo Verdes' cephalopod diversity is distinct from the Canary Islands and Azores. The cephalopod diversity was very similar between Cabo Verde, Azores and Canary Islands, but the benthic octopus' diversity differed. These findings support the hypothesis that Cabo Verdes' benthic and coastal biodiversity is biogeographically disconnected from other Macaronesian archipelagos, but not for pelagic species. The recent increase in Atlantic water inflow to the Arctic Ocean through the Fram Strait

results in “Atlantification”, which is expected to change the Arctic species community composition. To test this hypothesis for mobile nekton, cephalopod and fish diversity and distribution patterns were analyzed with eDNA metabarcoding of seawater and sediment samples. With eDNA, I detected the most abundant cephalopod taxa in this area*, Gonatus*. As the applied primer detected only part of the cephalopod community, additional primers are needed with a focus on cirrate octopuses. For fish, 27 taxa were detected in water and 18 in sediment. Capelin (*Mallotus villosus)* was the most frequently detected taxa followed by Zoarcidae, Liparidae, *Sebastes* sp., and Atlantic herring (*Clupea harengus*). Range expansions of capelin from the subarctic to 79 ° North could here be confirmed with eDNA data supporting the hypothesis that some abundant nekton species migrate further North.

The third objective of this thesis was to identify cephalopod and fish taxa that potentially contribute to the vertical transport of carbon. Off Cabo Verde, *T. danae* and *Sthenoteuthis pteropus* were identified as potential foodfall species due to their frequent detections, abundance, size and life history (Chapter 2). Using *in situ* observations and net sampling, high densities of the pelagic tunicate *P. atlanticum* were identified in midwater off Cabo Verde, in particular in mesoscale eddies (Chapter 5). Their extensive vertical migration from ~10 to 360 m result in active carbon flux via respiration and fecal pellet production. Pyrosome carcass deposition was documented on the islands slopes (213-500 m) and these carcasses were scavenged by decapods, sea anemone and gastropods. Specific primers detected pyrosome eDNA at 400, 600 and 1000 m, which is beyond their migration range. This eDNA could originate from either feces or dying individuals. In the Arctic, eDNA of the pelagic fishes’ capelin and barracudina (*Arctozenus risso)* were detected in water and sediment samples (Chapter 3). The presence of eDNA in both environmental samples, combined with their high abundance and life history, suggests that the carcasses of those two fishes contribute to carbon flux to the seafloor in the Arctic ecosystem.

This thesis showed that eDNA can be used successfully in the assessment and monitoring of deep-sea pelagic metazoans in hotspots of diversity and climate change. Especially when eDNA analysis is analyzed in different kinds of samples and used in combination with other techniques, it can help to answer ecological questions and ultimately contribute to aid in conservation of deep-sea habitats.

Zusammenfassung

Die Tiefsee (< 200 m) ist nicht nur der größte Lebensraum der Erde, sondern auch derjenige mit der höchsten Faunenbiomasse und der größten Anzahl von Organismen. Obwohl die Tiefsee dem Menschen wichtige Dienste leistet, sind ihre Ökosysteme nach wie vor kaum erforscht. Logistische und technische Herausforderungen bei der Beprobung der Tiefsee, sowie das Vermeidungsverhalten pelagischer Tiere gegenüber Unterwassergeräten, machen den Bedarf an alternativen Techniken deutlich. In dieser Arbeit habe ich mich auf ein relativ neues Instrument in der Tiefseebiologie konzentriert: die Analyse von Umwelt-DNA (eDNA). Umwelt-DNA ist genetisches Material, das Organismen an ihre Umwelt abgeben. Diese eDNA kann einem bestimmten Taxon zugeordnet werden und gibt Aufschluss über das Vorhandensein, die Vielfalt und die Verbreitung von Arten, ohne dass die Tiere, von denen sie stammen, beobachtet oder gefangen werden müssen. Das erste Ziel dieser Arbeit war die Entwicklung einer Pipeline zur Erfassung von Diversitäts- und Verbreitungsdaten von Tiefsee-Tintenfischen mittels eDNA-Analyse von Wasser- und Sedimentproben. Es wurde ein eDNA-Metabarcoding-Ansatz entwickelt und optimiert, um Tintenfische nachzuweisen (Kapitel 1-3), wobei ein hier entwickelter Primer verwendet wurde, der das nukleare 18S rRNA-Gen amplifiziert (Kapitel 4). Der Tintenfisch-Primer funktionierte erfolgreich in Meerwasser, aber nicht in Sedimenten. Dies war wahrscheinlich auf die ungleichmäßige Verteilung der Tintenfische und die schnelle Umsatzrate von Tintenfischkadavern zurückzuführen. Auch die Unempfindlichkeit des 18S-Primers für Oktopusarten und die geringe Artenvielfalt in der Arktis könnten eine Rolle spielen. Aufgrund der Einschränkungen allgemeiner Primer entwickelte ich artspezifische Primer für den Tiefseekalmar *Taningia danae* (Kapitel 2) und den pelagischen Tunikaten *Pyrosoma atlanticum* (Kapitel 5). Das zweite Ziel bestand darin, Basiswerte der biologischen Vielfalt und der Verbreitungsmuster der wichtigsten Organismen in der Tiefsee zu ermitteln und diese Muster in einen ökologischen Kontext zu stellen. Die entwickelte eDNA-Pipeline wurde zunächst zur Untersuchung der Tiefsee-Tintenfischvielfalt vor den Azoren eingesetzt (Kapitel 1). Es wurden insgesamt 39 Tintenfisch-Taxa nachgewiesen. Diese Diversitäts- und Verbreitungsdaten wurden dann verwendet, um die Zusammensetzung der Beutegemeinschaft in den Nahrungsgebieten von zwei Top-Prädatoren (*Ziphius cavirostris* und *Grampus griseus*) zu rekonstruieren. Diese Wale jagen in der gleichen geografischen Region vor den Azoren, jedoch in unterschiedlichen Tiefen und Entfernungen von der Küste (Kapitel 1). Mithilfe einer neuartigen Kombination aus der Besenderung von Walen und der Analyse von Beutetieren mittels eDNA haben wir die Hypothese getestet, dass die Nischendifferenzierung der Wale durch Unterschiede in der Beutevielfalt bedingt ist. Die Vielfalt der Kopffüßer und damit die Zusammensetzung der Beutegemeinschaft der Wale überlappte sich in den verschiedenen Nahrungshabitaten und -tiefen der Wale. Dies führte zu der neuen Hypothese, dass sich beide Wale von ähnlichen Arten ernähren, aber diese sich in unterschiedlichen ontogenetischen Stadien befinden. Durch die Kombination von eDNA-Analysen, Netzproben und In-situ-Beobachtungen aus Videoerhebungen und bestehenden Literaturaufzeichnungen erstellte ich eine Liste von Tintenfischarten für das Archipel von Kap Verden und identifizierte

Kap Verden als Tintenfisch-Hotspot mit 64 nachgewiesenen Arten (Kapitel 2). Ich testete auch die Hypothese, dass sich die Vielfalt der Kopffüßer auf den Kap Verden von der auf den Kanarischen Inseln und den Azoren unterscheidet. Die Vielfalt der Tintenfische war zwischen Kap Verden, den Azoren und den Kanarischen Inseln sehr ähnlich, aber die Vielfalt der benthischen Oktopoden unterschied sich. Diese Ergebnisse stützen die Hypothese, dass die benthische und küstennahe Artenvielfalt der Cabo Verde biogeografisch nicht mit der anderer makaronesischer Inselgruppen verbunden ist. Der Anstieg des atlantischen Wasserzustroms in den Arktischen Ozean durch die Framstraße führt zu einer "Atlantifizierung", die die Zusammensetzung der arktischen Artengemeinschaft verändern dürfte. Um diese Hypothese für Nekton zu testen, wurden die Diversität und Verteilungsmuster von Tintenfischen und Fischen mit eDNA-Metabarcoding von Meerwasser- und Sedimentproben analysiert. Mit eDNA konnte ich das abundanteste Tintenfisch-Taxon in diesem Gebiet, *Gonatus*, nachweisen. Da der verwendete Primer nur einen Teil der Tintenfisch-Gemeinschaft nachweisen konnte, werden weitere Primer benötigt, die sich vor allem auf Oktopus Arten spezialisiert haben. Bei den Fischen wurden 27 Taxa im Wasser und 18 im Sediment nachgewiesen. Die Lodde (*Mallotus villosus*) war das am häufigsten nachgewiesene Taxon, gefolgt von Zoarcidae, Liparidae, *Sebastes* sp. und dem Atlantischem Hering (*Clupea harengus*). Die Ausdehnung des Verbreitungsgebiets der Lodde von der Subarktis bis 79° Nord konnte hier mit eDNA-Daten bestätigt werden. Dies stützt die Hypothese, dass einige häufige Nekton Arten weiter nach Norden wandern. Das dritte Ziel dieser Arbeit war die Identifizierung von Tintenfischen und Fischen, die potenziell zum vertikalen Transport von Kohlenstoff als „Foodfalls“ beitragen. Vor Cabo Verde wurden *T. danae* und *Sthenoteuthis pteropus* aufgrund ihrer Häufigkeit, ihres Vorkommens, ihrer Größe und ihrer Lebensgeschichte als potenzielle Foodfalls identifiziert (Kapitel 2). Durch In-situ-Beobachtungen und Netzproben wurden hohe Dichten des pelagischen Tunikaten *P. atlanticum* im mesopelagial vor Cabo Verde, insbesondere in mesoskaligen Wirbeln, festgestellt (Kapitel 5). Ihre ausgedehnten vertikalen Wanderungen von ~10 bis 360 m führen zu einem aktiven Kohlenstofffluss durch Atmung und die Produktion von Fäkalienpellets. Die Ablagerung von Pyrosomen-Kadavern wurde an den Hängen der Insel (213-500 m) dokumentiert, und diese Kadaver wurden von Dekapoden, Seeanemonen und Gastropoden gefressen. Spezifische Primer wiesen eDNA von Pyrosomen in 400, 600 und 1000 m Tiefe nach, was außerhalb ihres Migrationsgebiet liegt. Diese eDNA könnte entweder aus Fäkalien oder von sterbenden Individuen stammen. In der Arktis wurde eDNA der pelagischen Fische Lodde und Barrakudina (*Arctozenus risso*) in Wasser- und Sedimentproben nachgewiesen (Kapitel 3). Das Vorhandensein von eDNA in beiden Umweltproben in Verbindung mit dem häufigen Vorkommen der beiden Fischarten und ihre Lebensstrategien lässt vermuten, dass die Kadaver dieser beiden Fische zum Kohlenstofffluss zum Meeresboden im arktischen Ökosystem beitragen. Diese Arbeit hat gezeigt, dass eDNA erfolgreich für die Bewertung und Überwachung von pelagischen Tiefsee-Metazoen in Hotspots der Diversität und des Klimawandels eingesetzt werden kann. Vor allem, wenn eDNA in verschiedenen Arten von Proben analysiert und in Kombination mit anderen Techniken eingesetzt wird, kann sie helfen, ökologische Fragen zu beantworten und letztlich zur Erhaltung von Tiefseelebensräumen beitragen.

Introduction

The deep sea

Earth's largest biome, the deep sea, is home to one of the greatest organism diversities (Robison, 2009). It also has the highest faunal biomass and greatest number of individual organisms (Ramirez-Llodra et al., 2010; Robison, 2004). Yet, an estimated 91% of marine species are not described, with many likely occurring in biodiversity hotspots and within less explored areas of the oceans (Mora et al., 2011). The deep sea provides essential ecosystem functions and services such as nutrient cycling, climate regulation and fisheries (Armstrong et al., 2012; Thurber et al., 2014). Organisms store and recycle nutrients within ecosystems that support commercial fish stocks, but also reduce CO_2 in the atmosphere (Costanza et al., 1997). Without the oceans fixing CO_2 in surface layers through photosynthesis and exporting it to deeper layers and eventually to the ocean floor, the CO_2 concentration in the atmosphere would be ~50% higher (Parekh et al., 2006). The services provided by the deep sea can be affected by anthropogenic disturbances. These disturbances can be temporary such as oil spills or dredging operations or long-term such as ocean acidification and ocean warming. Overfishing and human-induced habitat loss are major reasons for many animals now facing extinction (Burney and Flannery, 2005) and the significant increase in biodiversity loss over the last decades (IPBES, 2019).

Ecological role and importance of pelagic Metazoan

The majority of deep-sea studies focus on the seafloor, despite the fact that the water column above the seafloor comprises 90% of the living space in the deep sea habitat of which less than 1% has been explored (Robison, 2009). The pelagic deep sea (> 200 m) is vertically structured in zones, each with their own physical, chemical and biological characteristics (Figure 1). These zones harbor a great diversity of fishes, invertebrates and gelatinous zooplankton and also serve as foraging grounds for air-breathing predators including cetaceans and pinnipeds (Robison, 2009).

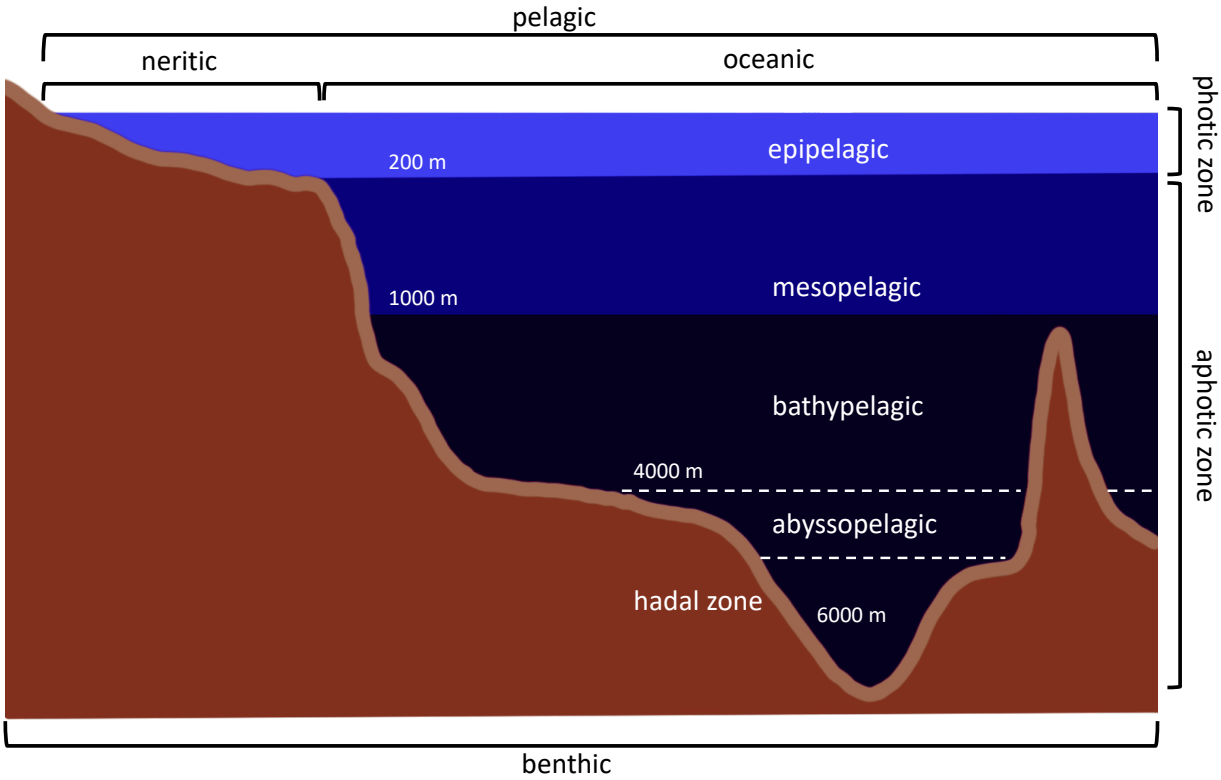

Figure 1| Ocean zonation. The seafloor is defined as the benthic zone and the water column the pelagic zone. The epipelagic zone supports primary production by photosynthesis and covers depths from the surface to around 200 m. The deep sea commences below 200 m. The zone between 200 and 1000 m is called the mesopelagic or twilight zone. From 1000 m to 4000 m stretches the bathypelagic zone, the largest ecosystem on earth. This zone is characterized by a complete lack of sunlight. The abyssopelagic zone stretches from the bottom of the bathypelagic to the seafloor. The hadal zone or hadopelagic zone only occurs in certain regions of the oceans. It lies within deep-sea trenches.

The mesopelagic species community is dominated by myctophids (lanternfishes) associated with crustaceans and cephalopods as well as gelatinous zooplankton such as cnidarians, ctenophores and salps (Robison, 2004). Myctophids and deep-sea cephalopods have been identified as potentially valuable and yet unexploited resources in the future (FAO, 2001, 1997; Gjoesaeter and Kawaguchi, 1980; Valinassab et al., 2007). However, uncontrolled harvesting from this community is associated with eminent risks. The organisms of the mesopelagic community perform diel vertical migration between the epipelagic and deeper water layers. By transporting organic carbon during this migration, they play an important role in carbon sequestration and thus climate regulation (Hidaka et al., 2001; Hudson et al., 2014). Estimates suggest marine animal migration to represent between 10 and 50% of the total export of

carbon to deeper layers (Aumont et al., 2018). These migrating individuals also serve as prey for higher trophic levels such as marine mammals, sharks, tuna, billfish and other commercially captured pelagic fish species (Brophy et al., 2009; Drazen and Sutton, 2017; Fanelli et al., 2009; Naito et al., 2013; Potier et al., 2007), therefore influencing and maintaining biodiversity and human fishery stocks. Changes in community composition and abundances of species may directly affect the entire food web and functioning of ecosystems.

High biodiversity contributes to ecosystem resilience, as greater morphological, genetic und functional diversity allows communities to quickly respond to changes in the environment (Loreau, 2008; Thurber et al., 2014). Our current lack of knowledge on species distribution, life history and ecology impedes conservation and sustainable management planning, and results in ongoing changes in this remote ecosystem unrecognized (Fisher et al., 2016). Only with improved information on species diversity and distribution, especially on key species that shape marine food webs, may changes in community structures due to climate change and anthropogenic stressors be optimally detected and ecosystems can be preserved (Glover et al., 2018; Hidalgo and Browman, 2019; Oliver et al. 2015, St. John et al., 2016; Webb et al., 2010).

This thesis is focused on the pelagic deep-sea and aims to investigate species diversity and distribution of pelagic metazoans in relation to their ecology.

Glossary

Biodiversity: The variety of life on Earth from genes to ecosystems. Genetic diversity describes the variation of genes among individuals in a population. Species diversity includes the number of different species in an environment (**species richness**) and relative abundance of each of these species. Ecosystem diversity is defined as the diversity among ecosystem within a geographical area. (Swingland, 2001). This thesis focuses on species diversity and the term “diversity” is used in that context.

Ecosystem function: Biological, geochemical and physical processes that occur within an ecosystem. Those processes control the fluxes of energy, nutrients and organic matter in an ecosystem. Ecosystem functions are directly linked to biodiversity. Changes in biodiversity ultimately change how ecosystems function and reduced biodiversity leads to lower and less efficient ecosystem functions. This is attributed to the fact that diverse communities are more likely to have a greater variety of functional traits and environmental adaptations leading to more efficient resource use and stability in the face of habitat disturbance and climate change (Loreau, 2008).

Ecosystem service: Basic services provided by ecosystems that are necessary to sustain human life on earth. In the marine realm, these include fishing, climate regulation, carbon fixation or oxygen production by phytoplankton (Armstrong et al., 2012).

Gelatinous taxa

Gelatinous zooplankton comprise a polyphyletic group of organisms including the medusae, siphonophores, ctenophores and tunicates. They are a major component of the deep pelagic fauna, as they are diverse and abundant. Their role and significance in marine ecosystems have been underestimated for a long time, largely as a result of their bodies containing at least 95% water, having typically low nutritional value and most containing no hard parts (Pugh, 1989; Robison, 2009). When food is abundant, these fauna can often maintain high growth rates. Gelatinous zooplankton span three trophic levels, and can act as significant consumers in pelagic communities as well as important prey for a variety of organisms (Alldredge, 1984). As consumers, they have been demonstrated to substantially reduce food stocks and to compete with fish (Arai, 1988; Opdal et al., 2019; Purcell and Arai, 2001). Gelatinous zooplankton are consumed by turtles (Hetherington et al., 2019), fish such as rockfishes, walleye pollock, grenadiers (Brodeur et al., 2021), tuna, swordfish (Cardona et al., 2012), and cephalopods (Hoving and Haddock, 2017). Various gelatinous zooplankton species channel energy from lower trophic levels to higher trophic levels (Llopiz et al., 2010), but this pathway is often neglected in food web models (Jaspers et al., 2015). Mass depositions of dead gelatinous zooplankton potentially contribute to the vertical flux of carbon to the deep sea. They have been reported to occur on the seafloor of the Arabian Sea (Billett et al., 2006), Atlantic Ocean of West Africa (Lebrato and Jones, 2009), the Madeira Abyssal Plain (Roe et al., 1990) and other regions of the worlds' oceans (Lebrato et al., 2012). Those mass depositions transport carbon from upper water layers to the seafloor.

This thesis is focused in part on one of the most abundant, but least studied groups of gelatinous zooplankton: pyrosomes. Pyrosomes are pelagic colonial tunicates which can grow to lengths up to 20 m. They occur from the warm tropics to cold-temperate regions (Décima et al., 2019) in epi- and mesopelagic layers (van Soest, 1981). They filter-feed on phytoplankton and have high clearance rates of up to 35 L colony^{-1} h^{-1} (Schram et al., 2020). Due to these high clearance rate, they are capable of reducing plankton standing stocks by more than 50% in surface waters (Drits et al., 1992). The species *Pyrosoma atlanticum* contains up to 35% of dry weight carbon content, which is one of the highest carbon contents measured in gelatinous zooplankton (Lebrato and Jones, 2009). When individuals die and sink to the seafloor, they can

transport substantial amounts of carbon to the deep sea (Archer et al., 2018; Lebrato et al., 2013; Lebrato and Jones, 2009) and provide food for benthic organisms such as sea anemones, sea urchins and crabs at least as deep as 2102 m (Archer et al., 2018; Lebrato and Jones, 2009). Pyrosomes also actively contribute to the carbon cycle by excreting of fecal pellets (Drits et al., 1992) and undergoing diel vertical migration from surface layers to water depths of 900 m (Andersen et al., 1992; Andersen and Sardou, 1994; Angel, 1989). Despite the potential ecological significance of pyrosomes, they are the least studied of all pelagic tunicates. Information on their ecology and trophic function is scarce and if present, mostly focused on the Pacific Ocean (Henschke et al., 2016; Madin and Deibel, 1998), while studies on the Atlantic are few.

Fishes

To date, around 16,722 fish species have been described (Appeltans et al., 2012) of which an estimated 10 to 15% occur in the deep sea (Hoar et al., 1997). Deep-sea fishes appeared early in the evolution of modern fishes. As a consequence, they are highly specialized and adapted to the particular environment and ecological conditions of the deep sea (Hoar et al., 1997). For example, they have evolved specialized eyes, complex bioluminescent organs, elaborate gas glands and swim bladders and often sophisticated jaws and teeth. Many deep-sea species have long generation times, low fecundities and mature very late (Hoar et al., 1997). In the North Atlantic, around 80 species (28 families) are reported to occur in the epipelagic, of which 89% inhabit only this depth zone (Merrett, 1994). Meso- and bathypelagic fishes are more speciose with 509 species (66 families), of which 79% are found exclusively at those depths (Merrett, 1994). Important deep-sea families are lanternfish (Myctophidae), silver hatchetfishes (Sternoptychidae), gonostomids (Gonostomatidae), viperfish (Chauliodontidae) and black stomiatoids (Stomiatoidei). Mesopelagic fishes are the most abundant vertebrates on earth (specifically the gonostomid *Cyclothone* sp.) (Nelson et al., 2016) and, they form the deep scattering layer together with crustaceans, gelatinous zooplankton and cephalopods at depths of between 200 and 1000 m (Marshall, 1951). Many deep-pelagic fishes undergo diel vertical migration, that is, they migrate from deeper waters to the surface at night to feed and back down again during the day to digest and hide from predators (Brierley, 2014; Marshall, 1979). This movement may be the largest animal migration on earth, substantially moving carbon and

nutrients from surface layers to the deep (Brierley, 2014). Carbon can also be distributed to the deep seafloor in form of sinking fish carcasses, although reports are few. On the Angola continental margin, carcasses of a whale shark and three mobulid rays were reported (Higgs et al., 2014) and another fish carcass reported at 1280 m depth west of Svalbard (Soltwedel et al., 2003). Mesopelagic fish are one of the least investigated components of the open ocean. Their biomass is estimated to be 10-fold higher than previously suggested, which has major implications for our understanding of carbon fluxes in the oceans and food web dynamics (Irigoien et al., 2014).

Recent warming of Arctic marine ecosystems has led to poleward shifts in distribution of fishes (Cheung et al., 2013; Richardson et al., 2012). In eight years, the distribution of bigeye sculpin (*Triglops nybelini*), Greenland halibut (*Reinhardtius hippoglossoides*) and snailfish (*Liparis* spp.), which are all Arctic species, has shifted 159 km northward. As the Arctic fish community retracted, their abundance also decreased. Simultaneously, the North Atlantic community dominated by cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) expanded its distribution northwards as well, "taking over" former Arctic species habitats (Fossheim et al., 2015). The poleward shifts of boreal fish species has the potential to alter Arctic marine food web dynamics and impact ecosystem functioning (Kortsch et al., 2015). One part of this thesis addresses fish diversity and distribution in the Arctic Ocean to identify range expansions and to predict fish species contributing to the regional carbon cycle.

Cephalopods

Members of the molluscan class of Cephalopoda are found in all habitats of the world's oceans (except the Black Sea) from the Arctic to the Antarctic, in coastal areas and the deep sea. Cephalopods include octopuses, squids, cuttlefishes, vampire squid and nautiluses. They have evolved 490 million years ago and include ~800 extant species known today (Figure 2). Cephalopods have an advanced and well-organized nervous system with a complex brain. With most also having extremely well-developed eyes that are an example of convergent evolution with vertebrates (Hanlon et al., 2018). They exhibit highly diverse behaviors and life history strategies (Figure 3). Many live only one year or less, though some species, particularly those from within the deep-sea species likely have extended life spans (Hoving et al., 2014). For

example, a female of the deep-sea octopus *Graneledone boreopacifica* has been shown to protect and care for her eggs for 53 months (Robison et al., 2014). As a result, they have to become older than 4.5 years. All cephalopods are carnivorous with the exception of the vampire squid *Vampyroteuthis infernalis* feeding on detritus (Hoving and Robison, 2012). Cephalopods are important predators with high growth rates and wet weight food conversion efficiencies of 30 – 60% (Clarke, 1996; Hanlon and Messenger, 2018). They mostly feed on small crustaceans as juveniles and switch to fishes and other cephalopods as they grow (Merten et al., 2017; Villanueva et al., 2017). Despite their opportunistic feeding behavior, myctophid fishes are suggested to be an important food source for oceanic cephalopods.

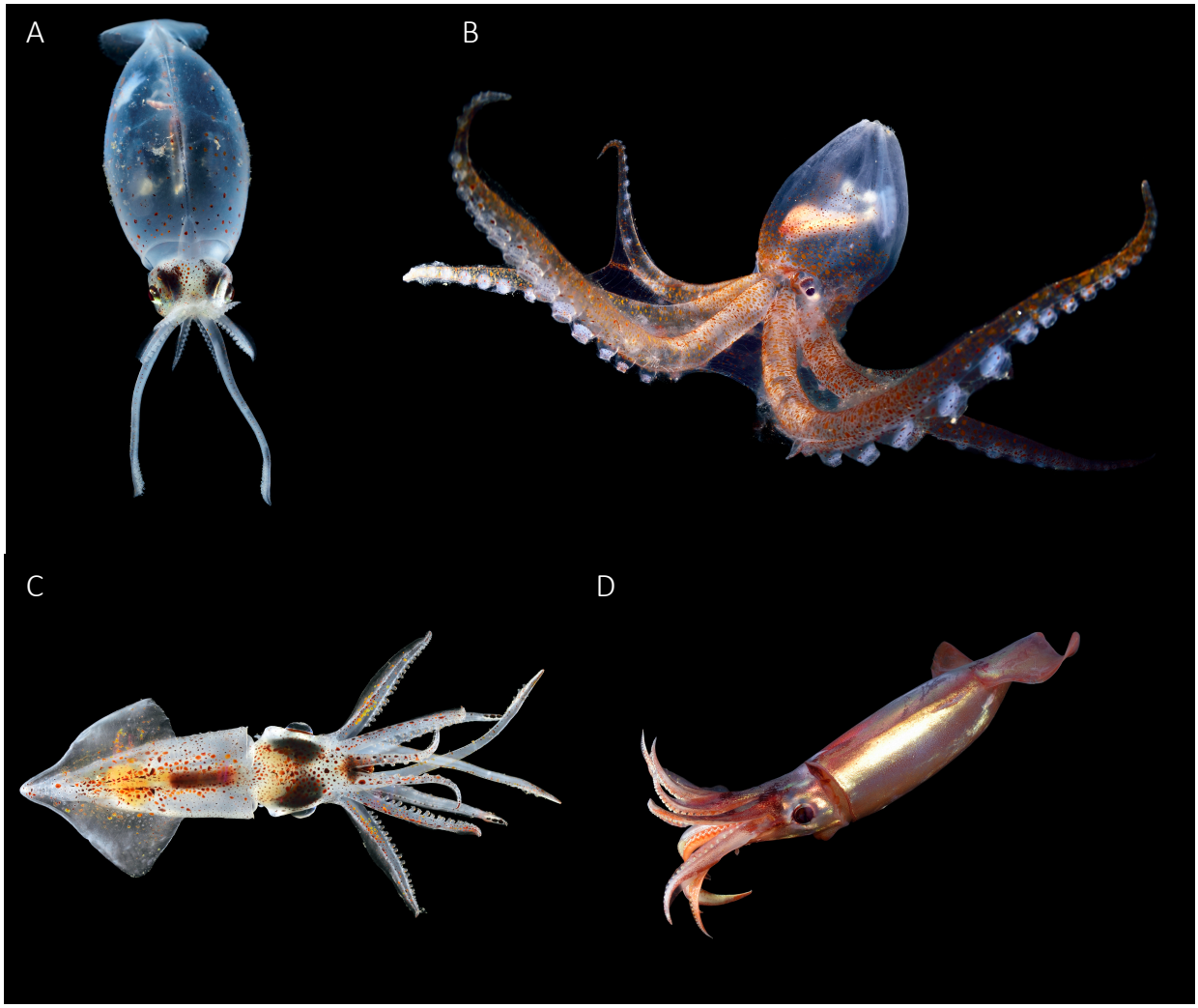

Figure 2| Representatives of the Cephalopoda. A) *Liocranchia reinhardtii* (Cranchiidae) **B)** The pelagic glass octopus *Vitreledonella richardi* (Vitreledonellidae). **C)** The Atlantic firefly squid *Abraliopsis atlantica* (Enoploteuthidae) **D)** The orangeback flying squid *Sthenoteuthis pteropus* (Ommastrephidae)
®Pictures by Solvin Zankl

The total cephalopod biomass is estimated to be 0.05 Gt carbon and the annual global consumption by cephalopods is estimated to be 2.09 – 4.03 x 10^9 t (Bar-On et al., 2018; Rodhouse et al., 1996). However, it is difficult to estimate the global role of cephalopods as predators due to limited data availability on stock size and predatory behavior for species that are not commercially exploited. Most data from cephalopod biomass in the meso- and benthopelagic ocean stem from predator stomach content analysis (Clarke, 1980). Besides being important predators transferring energy from lower to higher trophic levels in marine food webs, cephalopods are also important as prey. Many species are highly muscular and rich in protein, making them a valuable food source. Combined with their extreme abundances, cephalopods play an important role in the diet of seabirds, seals, toothed whales and fishes (Clarke, 1996; Smale and Clarke, 1996). Squid are estimated to constitute to 95% of the diet of sperm whales (Clarke, 1996).

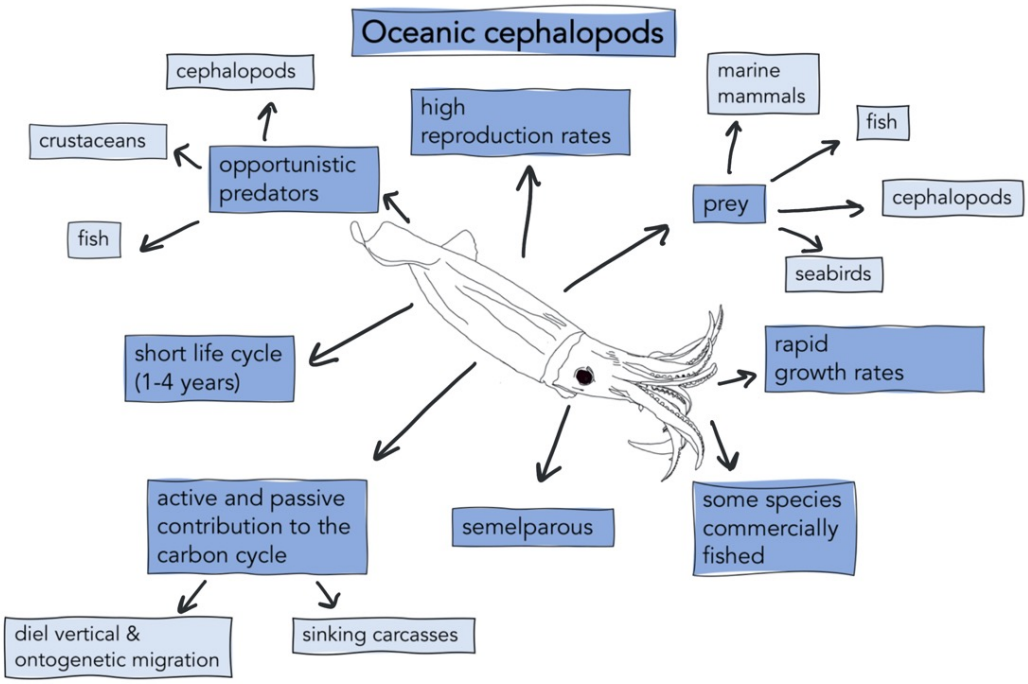

Figure 3| Main characteristics of oceanic cephalopods. Oceanic cephalopods are defined by high reproduction and growth rates, short life cycles and opportunistic predation on mostly other cephalopods, crustaceans and fish. They are also important prey for e.g., marine mammals, fish, other cephalopods and seabirds. Semelparity is a reproductive strategy characterized by a single reproductive event before death.

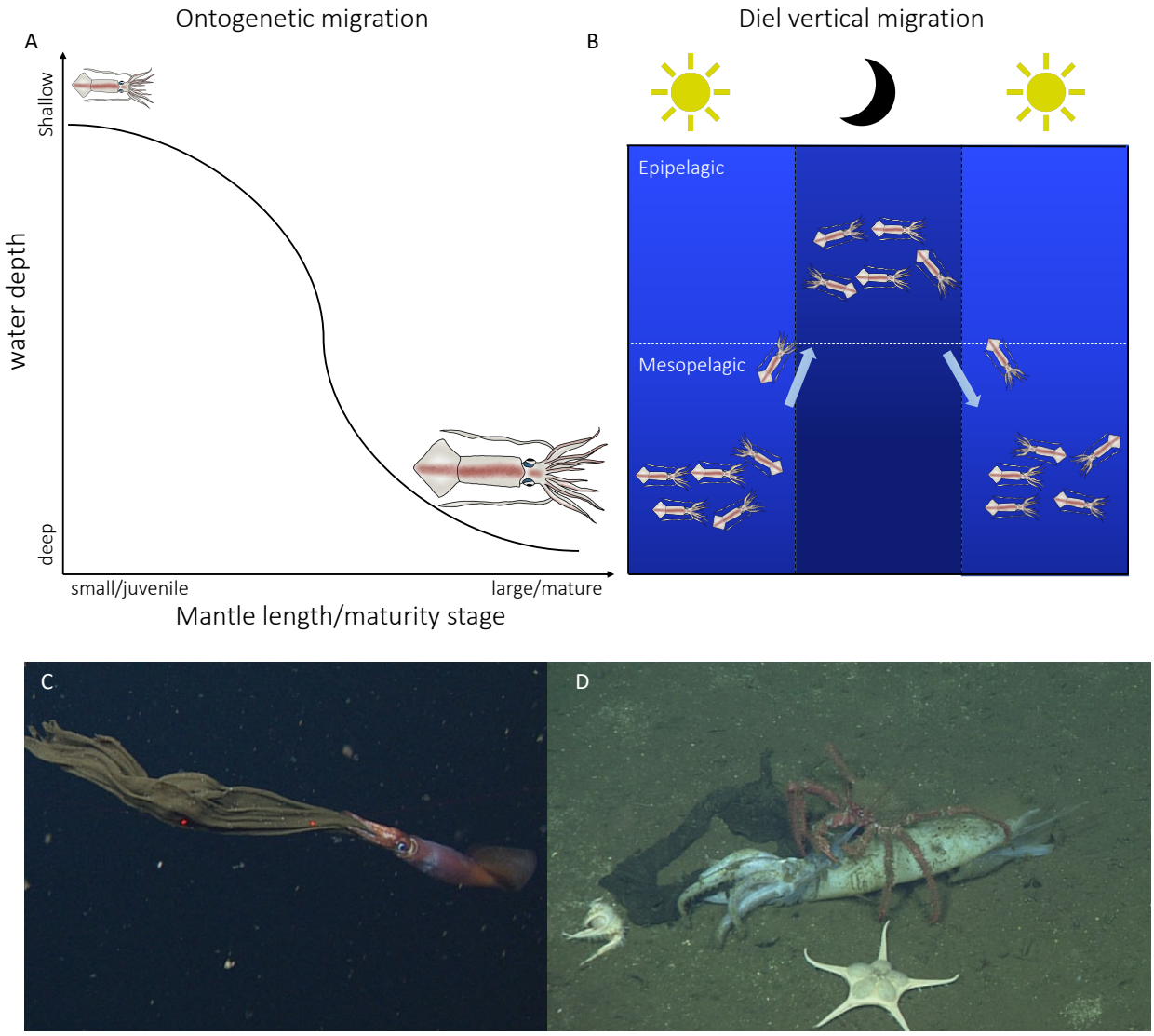

Figure 4| Active and passive vertical distribution of organic carbon by cephalopods. **A)** Ontogenetic migration of cephalopods. As paralarvae and juveniles, many cephalopod species reside in surface layers to profit from increased primary productivity for feeding. As they grow, they descend into deeper layers to mature and reproduce. This transports carbon from the surface into deeper waters, where the cephalopods eventually die and either sink to the seafloor where they might nourish benthic organisms or float to the surface where e.g., seabirds feed on them. **B)** Diel vertical migration of cephalopods. Many cephalopod species (together with fish and zooplankton) reside in deep layers of the mesopelagic during the day to hide from visually attuned predators. During the night, they ascend to surface layers to feed. By diel vertical migration, cephalopods and other nekton species transport carbon from surface layers to deeper layers where they also excrete feces. **C)** *In situ* ROV observations of a brooding gonatid squid. **D)** Squid carcass with egg-sheet on the seafloor scavenged upon by starfish (*Nymphaster diomedeae*) and a lithodid crab (*Paralomis multispina*) at 1246 m. Pictures C and D are from Hoving et al. 2017

Most species that live the majority of their lives below the euphotic zone (below 200 m) belong to the families Cranchiidae, Histioteuthidae, Octopoteuthidae, Mastigoteuthidae, Chiroteuthidae and cirrate octopuses (Hoving et al., 2014).

They spend their paralarval phase in surface waters to profit from increased primary productivity and descend to deeper waters as they grow to avoid predators. This behavior is called ontogenetic migration (Figure 4A). Another type of vertical migration in cephalopods is diel vertical migration (Figure 4B). Cephalopods that perform diel vertical migration typically stay at the surface at night to feed on epipelagic prey and descend to depth at dawn to avoid visual predators and to rest (Lampert, 1989; Zaret and Suffern, 1976). They mostly feed at the surface, but they respire, excrete and produce fecal pellets at depth. The active transport of carbon by diel vertical migration of squid and other groups represented in the mesopelagic is suggested to contribute substantially to the biological carbon pump (Buesseler and Boyd, 2009). Besides the active transport of carbon by diel or ontogenetic migration, cephalopods are suggested to passively contribute to the vertical transport of carbon by carcass deposition on the seafloor (foodfalls) when they die (Hoving et al., 2017) (Figure 4C+D). Especially squid are predicted to be important foodfall species (Hoving et al., 2017), as they are extremely abundant, support large industrial fisheries (Arkhipkin et al., 2015) and a variety of predators. For instance, sperm whales alone are estimated to feed on the same quantities of squid that are commercially fished worldwide (including all fisheries) (Whitehead, 2003). As for fish, foodfall detections of deep-sea cephalopods are extremely rare, with reports of carcasses of *Brachioteuthis* in the Atlantic Ocean (Roper and Vecchione, 1996), squid carcasses in the Gulf of California (Hoving et al., 2017) and Catalina Basin (Smith, 1985) and a squid carcass on the seafloor of the Indian margin (Gooday et al., 2010) thus far published.

Cephalopods in a changing ocean

The human demand for cephalopods is increasing steadily, as shown by the rise in global cephalopod catches from 3.4 million tons in 1999 to 4.7 million tons in 2015 (Hanlon et al., 2020). Traditionally fished fish species are depleted and it is expected that fisheries will shift progressively towards the still abundant cephalopods (Anderson et al., 2011; Caddy and Rodhouse, 1998; Rodhouse et al., 2014). The very short life cycle of many cephalopod species and high fecundity make them a promising human food source. These attributes may enable them to quickly respond to environmental change including climate change. (Jackson and

O'dor, 2001; O'Dor and Webber, 1986; Rodhouse et al., 2014). It has been shown that cephalopod populations are increasing in size (Doubleday et al., 2016). Reasons for this are suggested to be ocean warming, that can be preferentially beneficial for some cephalopod species in accelerating their life cycle, the depletion of fish stocks and therefore the reduction of competition and predatory pressure (Caddy and Rodhouse, 1998) and cephalopods' high adaptive potential and plasticity in adapting quickly to changing ocean environments (Figure 5). These traits are reflected in range expansions observed in cephalopod species. The Humboldt Squid (*Dosidicus gigas*) has expanded its distribution from the eastern North Pacific as far north as to Alaska and south to southern Chile (Zeidberg and Robison, 2007). The cause for this range expansion is debated, but possible explanations are responses to thermal changes and oceanographic conditions as well as overfishing of competing top predators (Zeidberg and Robison, 2007). A range expansion due to Arctic warming has been observed in the bobtail squid *Sepietta oweniana (Golikov et al., 2014).* This species inhabits the eastern Atlantic Ocean and Mediterranean Sea and was first recorded in the Barents Sea in 2014. It is suggested that the species has been transported into the Arctic by warm currents. The same may have happened with *Teuthowenia megalops* and *Todaropsis eblanae*, which were found 1000 and 2500 km outside their known range in the Arctic, respectively (Golikov et al., 2013). The native Arctic species *Gonatus fabricii* has increased its range into the eastern parts of the Barents Sea and to the adjacent areas of the Kara Sea (Golikov et al., 2013). Further range expansions of cephalopods are expected as a result of ongoing ocean warming. The impacts of these changes are difficult to predict and will likely lead to ecosystem changes. Some changing environmental factors can be detrimental for cephalopods, such as changing current systems leading to the potential dispersal of paralarvae into less suitable environments, eutrophication, ocean acidification and habitat modification (Halpern et al., 2008).

This thesis is focused on deep-sea cephalopod diversity and distribution to evaluate current community composition and enable detection of changes in these communities, to reconstruct potential prey spectra in predator habitats and to predict what species may contribute to the local carbon fluxes.

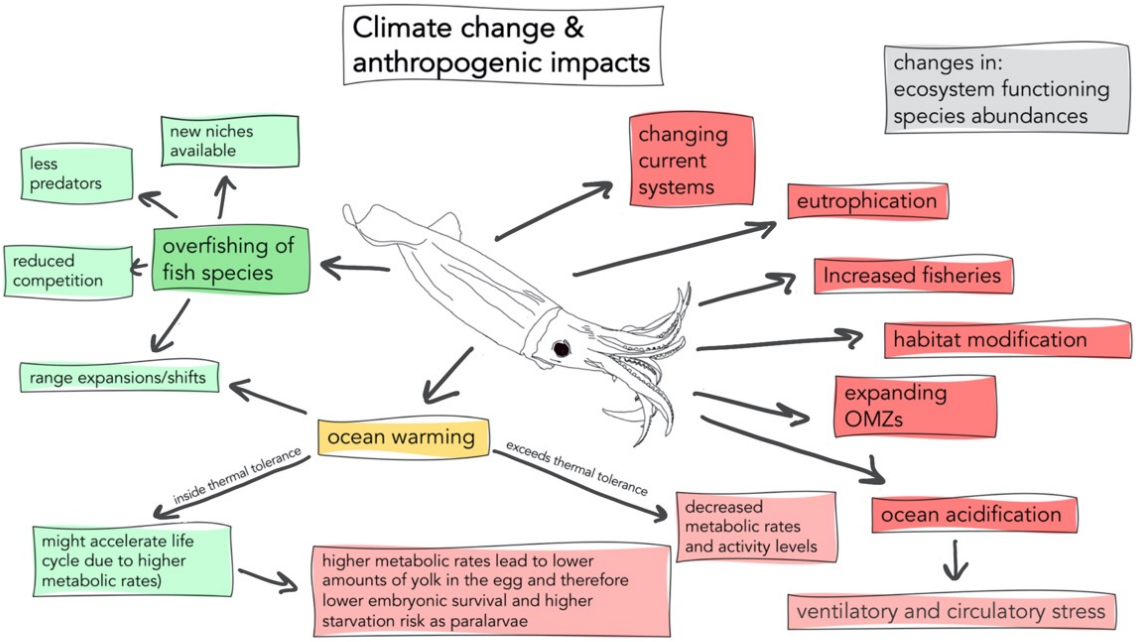

Figure 5| Climate and anthropogenic impacts on cephalopods. The green boxes depict climate change and anthropogenic impacts that may increase cephalopod abundance, while the red boxes depict impacts that might influence cephalopod populations negatively. The orange box (ocean warming) is suggested to have negative and positive effects on cephalopods, depending on whether ocean warming is inside or outside of a species thermal tolerance.

BOX 1: The biological carbon pump

The deep sea lacks the sunlight that can be used for photosynthesis. Therefore, most of the deep sea is heterotrophic and relies on sinking or advected food from the euphotic zone by the biological carbon pump (BCP). The BCP is a process fueled by the transportation of photosynthetically fixed CO_2 into the deep ocean. The major pathways of the BCP include 1) organic carbon particles or carcasses sinking to the seafloor, 2) the transport of organic carbon by currents or mixing and 3) the active transport of carbon by animal migrations such as fishes and cephalopods. Only 0.5 to 2% of the net primary production generated in the euphotic zone reaches the seafloor below 2000 m (Buesseler et al., 2007). Hence, benthic organisms are among the most food-limited groups (Smith et al., 2008), resulting in low faunal biomass and productivity (Michael A. Rex et al., 2006; Rowe et al., 2008). However, habitat spots of high productivity do exist on the deep seafloor. They occur when organic material from the surface becomes concentrated by e.g. canyons, food falls, wood falls, hydrothermal vents and oxygen minimum zones (Levin, 2003; Smith, 2006; Smith and Baco, 2003; Vetter et al., 2010) and are able to support highly diverse communities. Simultaneously, many oceanic carbon budgets are imbalanced as the measured amount of carbon sinking to the seafloor does not match the amount of carbon required by deep-sea benthic communities to survive (Rabouille et al., 2009; Smith and Kaufmann, 1999). Medium-sized organisms (> 1 cm) including gelatinous zooplankton are suggested to locally nourish benthic communities (Christiansen and Boetius, 2000; Higgs et al., 2014; Lebrato and Jones, 2009; Robison et al., 2005; Smith et al., 2014; Sweetman and Chapman, 2015) and are missed by sediment trap analysis. The role of medium-sized foodfalls is largely unexplored and natural observations are scarce (Higgs et al., 2014; Klages et al., 2001; Smith, 1985; Soltwedel et al., 2003). Reasons for that might be logistical and technological challenges to observe deep-sea ecosystems, temporal variability of foodfall events, spatial aggregation and fast consumption rates of foodfalls (Stockton and Delaca, 1982).

Scientific sampling methods of pelagic metazoans in the deep sea

Sampling the deep sea is not only accompanied by logistical and technological challenges, but also by avoidance behavior of the organisms of interest. Sampling possibilities of the deep sea range from trawling with nets and visual observations, to acoustic and genetic techniques. Each method has its own advantages and disadvantages. To receive the most holistic picture of the species diversity and abundance of a given habitat, different methods should be applied that complement each other.

Mid-water trawls

It is well known that trawling for cephalopods provides biased size-frequency and species compositions as well as underestimates population densities resulting from avoidance behavior and patchiness. Major factors leading to these problems are visual detection of the net during daytime trawling and during the night due to bioluminescence triggered from other organisms in the water. These nets can generate noise or vibrations that leads to early escape responses by organisms. The net opening and shape as well as the net size results in size-dependency of caught organisms. Speed, mode of fishing (vertically, horizontally, descending, ascending) and diel vertical migration or schooling behavior impact on the probability of certain organism to be caught (Wormuth and Roper, 1983). Not only the net itself influences the catch probability of cephalopods, but also the individual activity level of the organisms at the time of trawling and diel changes in behavior such as schooling (Wormuth and Roper, 1983). The same has been observed in fish. A study investigating the minimum capture probability of several fish species found that only 3 to 40% of the fish present in the towed water body were caught, depending on species, net size and towing speed (Barkley, 1972). Fish trawls have also been suggested to invoke avoidance reactions in mesopelagic fish, behaviors similar to those initiated by approaching predators (Kaartvedt et al., 2012). As a result, when comparing acoustics with net trawls abundance estimates, acoustics always appear to yield higher estimates (Kaartvedt et al., 2012; Kloser et al., 2009; Koslow et al., 1997; Pakhomov and Yamamura, 2010). A comparison between the results of mid-water net hauls for squid and the diets of squid predators feeding in the same area indicated that the average size of squid in net catches was

biased towards small species and was below the minimum size of individuals caught by predators (sperm whales) (Clarke, 1983). Patchiness is another difficulty in sampling cephalopods. Many species do not occur in shoals and inhabit a vast area. To a certain degree, patchiness can be compensated for by sampling a larger water volume, however, this becomes progressively more expensive and is not always suitable for research vessels, limited in available ship time and sampling gear. Additionally, a single net type is most effective for assessing only a part of the cephalopod assemblage (Hoving et al., 2014) and different nets exhibit different detection rates for different cephalopod species (Judkins et al., 2017).

Not only are species with strong swimming capabilities difficult to capture with nets, but also fragile and delicate animals, such as gelatinous zooplankton. The underestimation of gelatinous zooplankton in marine ecosystems is a result of the dominance of towed net sampling for quantitative estimations and geographical distributions of gelatinous zooplankton. As with cephalopods, gelatinous zooplankton is patchily distributed and can show escape responses (Hamner et al., 1975). Many taxa are fragile and delicate, which leads to their destruction when captured with nets. As a result, relative abundance estimates of gelatinous zooplankton captured with nets are often incorrect. Even if collected successfully, taxa may dissolve in preservatives like formalin and ethanol (Pugh, 1989). It was not until the invention of *in-situ* observation methods - where organisms can be studied in their natural environment via submersibles and cameras - that the importance and diversity of gelatinous zooplankton became more obvious (Hamner et al., 1975; Pugh, 1989).

Visual observations

Visual observations are suitable to assess small-scale distribution or behavior of species. The first use of automated cameras for photographing squid was described in 1957, capturing images of *Sthenoteuthis pteropus* at 1000 m in the Atlantic Ocean (Baker, 1957). The invention of manned and unmanned submersibles, remotely operated vehicles (ROVs) or camera systems towed or lowered from a ship has proven useful in the detection and description of cephalopods (Bush et al., 2009; Hoving et al., 2012; Hoving and Vecchione, 2012; Robison, 2004, 2000; Robison et al., 2003; Seibel et al., 2005; Vecchione and Young, 1991). For instance, in 1994 the mating behavior of deep-water octopuses was filmed from the deep-diving

submersible *Alvin* (Lutz and Voight, 1994), the same submersible that also observed the first egg brooding in *Graneledone* and *Benthoctopus* at 2600 m (Voight and Grehan, 2000). Most striking are the observations of elusive and rather large squids such as the giant squid *Architeuthis dux* first observed alive at 900 m in the North Pacific in 2005 (Kubodera and Mori, 2005) or *Taningia danae* at 240 and 940 m in the North Pacific in 2007 (Kubodera et al., 2007), showing attacking and bioluminescence behaviors. Both species have been suggested to be rather sluggish and inactive squid due to the ammonia solution within their flesh, with this solution enabling neutral buoyancy whilst making the body musculature flabby and soft to touch in captured animals (Hanlon and Messenger, 2018; Nixon and Young, 2003; Norman, 2000). However, visual observations proved both squids to be muscular, active predators with extreme maneuverability and swimming capabilities. Visual observations also made it possible to investigate species of which no known adult specimen has ever been captured, such as *Magnapinna* sp. (Guerra et al., 2002; Vecchione et al., 2001). This renders visual observations extremely important to investigate the behavior and natural morphology of deep-sea species that cannot be obtained from net catches. The same holds true for gelatinous zooplankton. Some taxa are exclusively observable by ROVs or submersibles and completely missed by other censuses. Some submersibles and ROVs are able to collect living specimens with suction devices or other tools to perform experiments (Hoving et al., 2012; Hunt, 1996; Jacoby et al., 2009; Robison et al., 2003). However, visual observation devices share the same characteristics that cause avoidance behavior compared to nets. An approaching submersible can cause bioluminescence and makes noise and vibrations, potentially scaring away cephalopods and fishes close by. Additionally, they are mostly operated with bright lights that might attract, scare or even damage species.

The use of environmental DNA to assess biodiversity

The limitations of the traditional means for biodiversity monitoring mentioned above have raised the need for alternative approaches. With the development of next-generation sequencing technologies which are capable of rapidly sequencing hundreds of samples at relatively low costs, new methods have emerged taking advantage of this new capability. One of these is environmental DNA (eDNA) analysis. Environmental DNA analysis is the extraction of DNA from environmental samples such as soil, sediment, water or air without first isolating the organism. Every organism interacting with the environment releases DNA into its surrounding environment (Figure 6). This DNA can stem from excreted cells or tissue such as mucus, urine (Valiere and Taberlet, 2000) or feces (Deagle et al., 2010; Pompanon et al., 2012; Valentini et al., 2009) or from dead organisms shedding genetic material. It includes intracellular (from living cells) and extracellular (from dead cells resulting in the destruction of cell structures) DNA (Nielsen et al., 2007). Environmental DNA samples are typically analyzed by amplification using polymerase-chain-reaction (PCR). Amplification is done either by a single-species approach using a specialized primer or a multi-species approach with a universal primer, known as metabarcoding with subsequent DNA sequencing (Figure 7). In principle, metabarcoding works similarly to DNA barcoding of DNA extracts. Both techniques rely on the fact that short standardized DNA regions can be amplified by PCR, sequenced and finally being used as barcodes to identify and discriminate taxa.

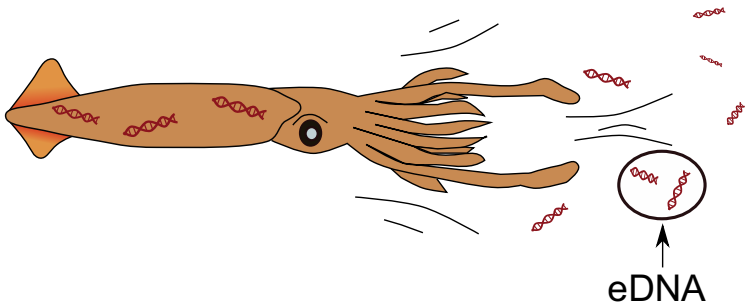

Figure 6| Environmental DNA is released by every organism interacting with its environment. Sources of eDNA are e.g., excreted cells or tissue such as urine, feces, skin and dead individuals.
Illustration by Alice Nauendorf.

Environmental DNA analysis is first mentioned in 1987 in an extraction protocol for eDNA found in sediments (Ogram et al., 1987) (Figure 8). Until the beginning of the 2000s, eDNA was predominantly used in microbiology (Giovannoni et al., 1990; Handelsman et al., 1998). In 2003, the first DNA metabarcoding study based on macroorganisms indicated that eDNA can be used to identify megafauna (mammoth, bison and horse) and ancient plant DNA from permafrost (Willerslev et al., 2003). Since then, eDNA analysis has become a powerful molecular tool to non-invasively survey species richness (Andruszkiewicz et al., 2017; Port et al., 2016; Sigsgaard et al., 2017b; Stat et al., 2019; Thomsen et al., 2016), species distribution (Doi et al., 2017; Guardiola et al., 2016) and population genetics (Sigsgaard et al., 2020, 2017a). It has also been applied successfully to detect rare or invasive species (Dejean et al., 2012; Jerde et al., 2011; Mächler et al., 2014; Tréguier et al., 2014) and has been tested to assess species biomass estimates (Doi et al., 2017; Lacoursière-Roussel et al., 2016; Takahara et al., 2012).

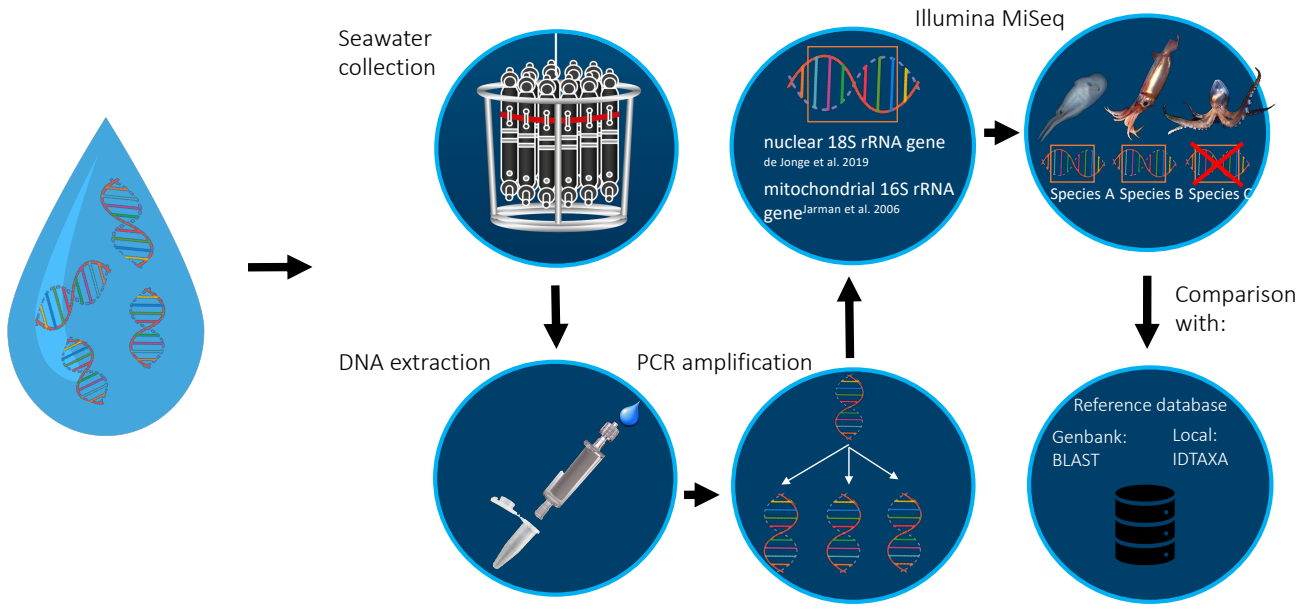

Figure 7| Workflow of eDNA metabarcoding from sample collection to species assignment.
In the field, seawater is collected with a CTD rosette, filtered and stored frozen at -80°C until further analysis in the laboratory. In the laboratory, the DNA is extracted directly from the filter and amplified by PCR. All PCR products are pooled into a library with unique barcode tags for each sample and sequencing on an Illumina MiSeq. On the computer, the eDNA data is cleaned and assigned to taxa.

In the marine environment, eDNA of macroorganisms was first analyzed to monitor the expansion of echinoderm populations in Tasmanian waters (Deagle et al., 2003), followed by the monitoring of benthic metazoans (Chariton et al., 2010; Fonseca et al., 2010; Guardiola et al., 2016, 2015; Leray and Knowlton, 2015), fish (Kelly et al., 2014; Miya et al., 2015; Thomsen et al., 2012) and marine mammals (Foote et al., 2012). Since then, eDNA analysis has become an increasingly used tool in biological monitoring of marine macroorganisms inhabiting various habitats. In 2017, an eDNA metabarcoding study targeting macroorganisms in seawater was able to detect 287 taxa across the tree of life by applying several sets of universal primers and also isolated haplotype diversity from a specific fish genus (Stat et al., 2017). The newly designed universal fish primer by Miya et al. (Miya et al., 2015) was successfully tested in aquaria with known species diversity. The fish primer detected 93.3% of all species present (123 genera, 59 families) and was also applied in natural coral reefs detecting 232 fish species (152 genera, 70 families). Another study on eDNA from seawater was able to detect the same or more fish species compared to traditional sampling methods, including rare species (Thomsen et al., 2012). A study conducted around the New Caledonian archipelago showed the efficiency of eDNA metabarcoding by detecting 44% more shark species than traditional underwater visual censuses and baited video (Boussarie et al., 2018).

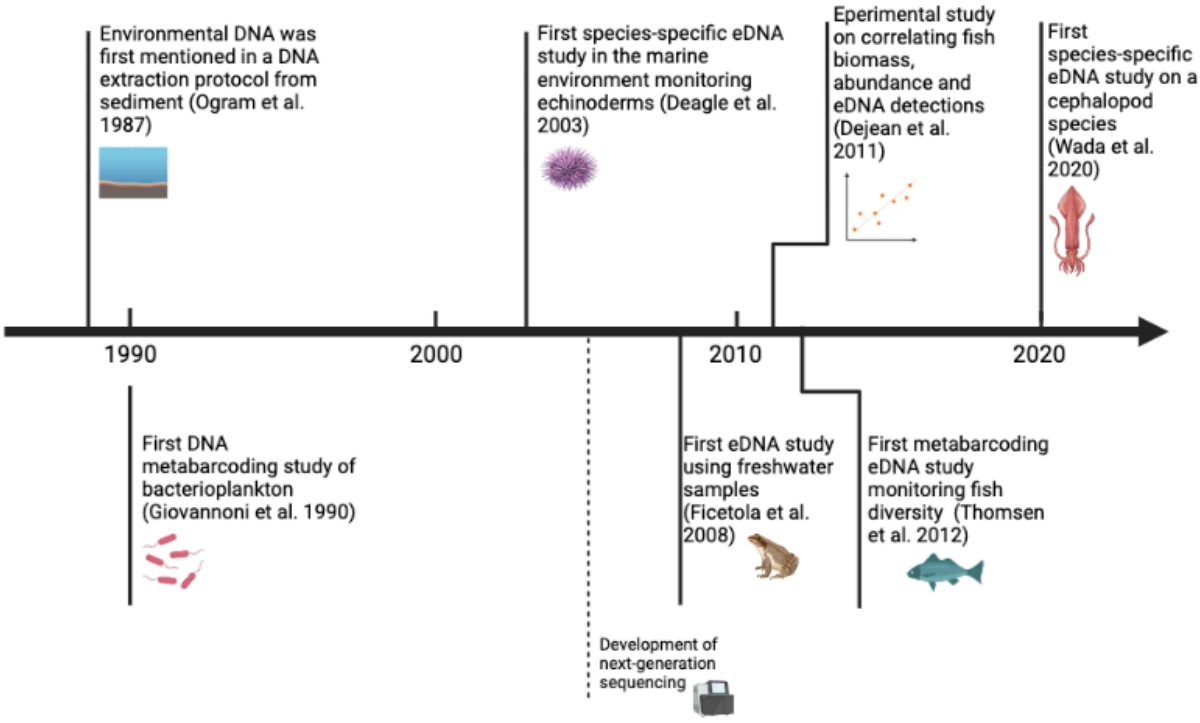

Figure 8| History of environmental DNA. Timeline from the first mention of eDNA in a DNA extraction protocol to its first application on marine macroorganisms and following studies.

Environmental DNA analysis is particularly promising for the assessment of gelatinous zooplankton diversity, as those organisms are often under-sampled and destroyed by nets. In the Maizuru Bay, Kyoto, eDNA concentration of the Japanese sea nettle (*Chrysaora pacifica*) correlated positively with the number of individuals observed by eye, reflecting the temporal fluctuation pattern of this species (Minamoto et al., 2017). In another species-specific eDNA approach, eDNA of four cubozoan species was successfully detected in waters off Magnetic Island, Queensland. However, this study showed a poor correlation between eDNA concentration and abundance of jellyfish, likely due to the detection of polyps located within the substratum. This renders eDNA analysis a valuable tool to detect both the medusae and likely the polyps of cubozoans (Bolte et al., 2021).

Application of eDNA in the deep sea

Most eDNA studies investigating pelagic marine diversity have been conducted in surface waters, and eDNA studies focusing on the deep sea are rare. In the mesopelagic, Govindarajan et al. suggested eDNA analysis to be more effective than net trawls in detecting marine metazoan communities by comparing the amount of filtered seawater per method (net trawls vs. eDNA) (Govindarajan et al., 2021). In the Labrador Sea at depths between 500 to 3000 m, eDNA sampling provided comparable results in the detection of fish diversity when compared to assessments based on conventional trawls. Their study also indicated that seawater samples from depths below 1400 m had significantly lower DNA concentrations than samples from the surface or mid-depth (McClenaghan et al., 2020). A study from Southwest Greenland analyzed fish eDNA in seawater samples between 188 to 918 m depth in comparison to trawling. The eDNA reads correlated with biomass and abundance data obtained by trawling and the most abundant taxa in the eDNA reads were also the most abundant in the trawls (Thomsen et al., 2016). Next to seawater, marine sediments are another source of eDNA in the oceans. Metabarcoding of deep-sea sediment for eukaryotes between 100 and 2250 m resulted in the detection of 1629 molecular operational taxonomic units (MOTUs) with Metazoa being one of the most dominant groups (Guardiola et al., 2015). However, a worldwide analysis of sediment in bathyal and abyssal depths revealed large gaps in reference databases, characterized by large numbers of unassigned MOTUs (Sinniger et al., 2016). These findings emphasize that eDNA analysis augments traditional methods, but especially in less investigated areas with a lack of

extant taxonomic knowledge and appropriate reference databases such as the deep sea, eDNA analysis needs to be integrated with traditional approaches. For cephalopods, eDNA metabarcoding has only been applied in studies that did not specifically look for cephalopods, but focused on larger taxonomic groups from coastal areas such as eukaryotes (Stat et al., 2017), metazoans (Günther et al., 2018) and invertebrates (Borrell et al., 2017). In a species-specific approach, eDNA analysis has been used to detect the giant squid *Architeuthis* in Japanese waters (Wada et al., 2020). To our knowledge, this thesis is the first dedicated effort to use eDNA metabarcoding to specifically target cephalopods in the deep sea.

Technical properties of environmental DNA

Environmental DNA is mostly degraded and fragmented, therefore, eDNA studies typically target short DNA barcodes (<100 -150 bp and seldom longer than ~250 bp) (Taberlet et al., 2012). However, eDNA stemming from recently shed skin cells or saliva might contain complete cells or long DNA fragments and could be used for eDNA analysis (Deiner et al., 2017). The DNA barcodes used are typically located on mitochondrial, chloroplast or ribosomal RNA genes. The choice of barcode for eDNA studies is of fundamental importance, because it can greatly impact the results. DNA barcodes used for eDNA studies need to be represented in a reference database in which every DNA sequence was retrieved from barcoding of a voucher specimen that has been identified morphologically by a taxonomist. Although there are several initiatives with the goal to sequence e.g., all of the world's fish (www.fishbol.org), mammal (www.mammaliabol.org) and bird species (www.barcodingbirds.org), there still is a massive gap in sequences for a variety of organisms. On average, only 20.76% of each phylum analyzed in Kvist (Kvist, 2013) are covered by DNA barcodes and for molluscs the number is even lower (8.34%). Therefore, it is vital to establish a study-specific local database for the organism group of interest covering the chosen marker genes. Most barcodes represented in reference databases belong to the mitochondrial cytochrome c oxidase subunit I gene (COI). Mitochondrial DNA is typically present in much larger quantities in the cell than nuclear DNA and therefore suggested to be more abundant and easier to quantify. COI is maternally inherited and a variety of universal primer sequences have been published which are effective across a wide range of eukaryotes (Folmer et al., 1994). Although COI is widely used and one of the best characterized part of the eukaryotic genome, it has been shown to be sub-optimal for

metabarcoding (Deagle et al., 2014). Due to the maternal inheritance of mitochondrial genes, interspecific hybridization and endosymbiont infections can transfer mitochondrial genes outside of an individuals' evolutionary group leading to misidentifications (Dasmahapatra and Mallet, 2006). To delineate species by molecular data, the rate of evolution in a marker gene needs to be high enough to differentiate closely related species, but low enough to not differ within a species. Mitochondrial DNA evolves 5-10% faster than nuclear DNA (~2% per million years in bilaterian metazoans) making it useful for species and genus level identification (Ballard and Kreitman, 1995; Brower, 1994; Brown et al., 1979). Yet, molluscs have been reported to exhibit elevated rates of evolution in COI (Folmer et al., 1994; Yamazaki et al., 1997) resulting in problems when using COI to correctly identify taxa due to paraphyletic lineages, unequal rates of inter- and intra-specific variation, poor taxonomy or underlying genetics (Meyer and Paulay, 2005; Strugnell and Lindgren, 2007). Cephalopods show inconsistent rates of evolution for COI with a wide variability of rates reported. For example, COI amino acid sequences vary more within Octopodiformes (mean divergence = 5.9%) and less within Decapodiformes (mean divergence = 2.3%) (Carlini and Graves, 1999). Also, the sequencing of the entire COI locus (~1200 bp) from two morphologically distinct giant squid, *Architeuthis,* did not reveal any sequence differences (Strugnell and Lindgren, 2007). Although COI has been shown to perform well within some families or genera such as Gonatidae (Lindgren et al., 2005) or *Illex (Carlini et al., 2006)*, it does not appear to behave similarly in all cephalopods, stressing the need for further investigations into the usefulness of this marker and the evolutionary rates of COI. Mitochondrial genomes of molluscs have shown to have unusual gene arrangements, duplications and deletions of genes (Boore, 1999; Boore and Brown, 1995, 1994) and some bivalves are heteroplasmic, that is, they contain two copies of the mitochondria inherited from each parent (Hoeh et al., 1996). In cephalopods, two copies of COI have been found in at least two species of Oegopsida (*Watasenia scintillans* and *Todarodes pacificus*), but not in the octopus *Octopus vulgaris (Yokobori et al., 2004)*. Entire mitochondrial genome sequences are missing for the majority of cephalopod species and therefore, the effects of multiple copies of COI are unknown. Another difficulty in using mitochondrial sequences for barcoding studies is mitochondrial DNA sequences that translocate to the nucleus (*Numts*) (Benesh et al., 2006; Bensasson et al., 2000; Williams and Knowlton, 2001). Once arrived in the nucleus, *Numts* lose their original function, can acquire mutations and can be misinterpreted as "real" mitochondrial sequences.

The elevated and inconsistent rates of evolution in COI, multiple copies of COI and *Numts* are problematic for barcoding as well as metabarcoding. However, for barcoding (only one species present in a sample), protocols can be optimized that fail to amplify initially, while for metabarcoding (several species in a sample) failed amplification of certain taxa can be masked by the successful amplification of others. For example, COI metabarcoding of a mixture of known arthropod species resulted in failed detections between 24% and 36% (Yu et al., 2012). This is in concordance with another metabarcoding study on arthropods that used generic COI primers, and recovered only between 43 and 64% of species that were added to the DNA mixture (Clarke et al., 2014). Alternatives to COI are primers targeting ribosomal RNA (rRNA). Ribosomal RNA allows to design highly conserved primers that provide very wide taxonomic coverage, but often with lower taxonomic resolution. Mitochondrial rRNA genes have higher taxonomic resolution comparable to COI, but also allow for the design of more conserved primers (Deagle et al., 2014). However, the difficulty with rRNA primers are length variations in rRNA coding regions. Those can result in differences in sequence recovery depending on the taxon.

There is not one perfect metabarcoding primer, hence, the choice of the primer is study-specific. Best practice is to apply several primers targeting different genes to increase the likelihood to amplify all DNA of interest in a given sample. Environmental DNA metabarcoding has proven to be a powerful method in describing communities and biodiversity, but it also has the potential to detect interactions and functional ecology over large spatial scales.

Considerations and pitfalls

Despite its potential to revolutionize biodiversity assessments and other fields in biology, the precision and accuracy of eDNA metabarcoding is constrained by several factors during the whole workflow, from the field, within the laboratory and during the bioinformatic analysis (Deiner et al., 2017; Thomsen and Willerslev, 2015). In the field, the study design can greatly impact the outcome of eDNA studies, as the statistical power and analytical interpretation will be influenced. This includes for example the sampling effort, such as the volume of water filtered, spatial sampling strategies, replication (biological and technical) and controls. In the laboratory, the used DNA extraction protocol can greatly influence the accuracy of the following PCR by more or less reducing inhibitors present in the sample (Djurhuus et al., 2017; Hinlo et

al., 2017). Several controls are needed to account for possible contaminations, false-positives or false-negatives (Ficetola et al., 2016) and PCR primers need to be chosen carefully (Freeland, 2017). When analyzing sequencing data, one challenge is to recognize artefactual sequences that can be mistaken for rare species (Flynn et al., 2015; Kunin et al., 2010). Sequence errors can occur at various steps, as a result of replication errors during PCR and during sequencing. A variety of programs have been developed to identify and remove both PCR and sequencing errors (Coissac et al., 2012). The appropriate parameters of the bioinformatic steps can highly influence the results (Taberlet et al., 2012). For example, Flynn et al. (2015) compared commonly used clustering methods on a series of mock communities and on natural communities of zooplankton. The estimated operational taxonomic units (OTU) varied by two and three orders of magnitude for mock communities and natural communities, respectively. To overcome these problems related to the construction of OTUs, new methods have been developed that use models and algorithms to remove spurious sequences and assign all unique sequences identified as biological variants (Callahan et al., 2016).

Deep-sea model regions to study diversity in a changing ocean

Cabo Verde

Cabo Verde is comprised of a group of ten volcanic islands and eight islets situated in the North Atlantic Ocean, 450 – 600 km off the west-coast of Africa. The archipelago sits upon the Cape Verde Rise, having its highest point at 2200 m below sea level and the deepest at over 3000 m depth (Ramalho, 2011). This results in a quick descend of the continental slope of Cabo Verde, and the occurrence of deep-sea habitats close to the islands. Traditionally, Cabo Verde was placed within a group of volcanic islands located in the North Atlantic known as Macaronesia (Azores, Madeira, Canaries, Salvages and the North East coast of Africa, from Morocco to Senegal), but recent studies suggest Cabo Verde to be its own, separated ecoregion (Freitas et al., 2019). Due to Cabo Verdes' oceanic isolation, the archipelago harbors a high level of endemism within benthic invertebrates (Briggs, 1970; McDowall, 1968). For fish, 315 species are reported to occur off Cabo Verde including manta ray (*Mobula* sp.) and whale sharks (*Cetorhinus maximus*) as well as 20 endemic species (Reiner, 1996; Wirtz et al., 2013). Cabo

Verde's fish diversity is comparable to the Canary Islands (330 coastal species, (Brito et al., 2002)) and higher compared to Madeira (226 coastal species, (Wirtz et al., 2008)) and the Azores (190 coastal species,(Porteiro et al., 2010; Wirtz et al., 2013)). The endemism of fish species for Cabo Verde is much higher than for the Azores (one endemic species), Madeira (no endemic species) and Canary Islands (no endemic species). This can be explained by the distance to the African mainland and currents. The North West African Upwelling (NWAU) brings cold water to the surface, acting as an effective biogeographical barrier between Cabo Verde and the African mainland (Wirtz, 2012, 2009), while the Canary Current separates Cabo Verde from the Canary Islands in the North (Freitas et al., 2019). More than 17 cetacean species have been reported in waters around Cabo Verde (Reiner et al., 1996). The archipelago serves as a breeding area for humpback whales (*Megaptera novaeangliae*) (Jann et al., 2003) and also sperm whales occur here (Moore et al., 2003). Sperm whales and other animals feeding on cephalopods profit from the occurrence of 58 cephalopod species detected around Cabo Verde (Clarke, 2006; Voss et al., 1998). One of the species, *Octopus vulgaris*, is suggested to be a potential new endemism (Sampaio et al., 2018). *Octopus vulgaris* off Cabo Verde has a reduced size and is excluded from subtidal areas. The behavioral and morphological alterations of this octopus species may result from developmental plasticity or from genetic differentiation. This needs to be confirmed in the future with genetic analysis. Several seamounts are located in the vicinity of the main islands, which support diverse fish communities (Hanel et al., 2010; Monteiro et al., 2008). Submesoscale and mescoscale eddies form at the productive coast of the African mainland throughout the year, propagating westwards and transporting nutrient-rich waters (Schütte et al., 2015). Those eddies can become hotspots of biological activity (Godø et al., 2012; McGillicuddy Dennis J. et al., 2007; Menkes et al., 2002), that eventually may lead to the development of oxygen minimum zones (OMZs) (Karstensen et al., 2015). Cabo Verde is a hotspot for marine biodiversity (Freitas et al., 2019) and due to its steep continental slopes a perfect study area for deep-sea organisms.

Azores

The Azores are located about 1400 – 2000 km from continental Europe and North America, respectively, rendering it the most remote archipelago in the North Atlantic. It consists of nine volcanic islands, numerous seamounts and sits on the mid-Atlantic ridge at a triple tectonic junction (Afonso et al., 2020). Its location favors temperate climate conditions, combined with the Azores Current that introduces subtropical conditions (Caldeira and Reis, 2017). Combined with high seafloor complexity, the Azores attract a wide variety of oceanic vertebrate megafauna. The archipelago is a hotspot for cetacean diversity with 24 species of toothed and baleen whales occurring regularly in the region, which is one of the highest cetacean biodiversity in the world. This includes resident species (e.g., bottlenose dolphins, Risso's dolphins), species that are present year-round (e.g., sperm whales, pilot whales) and seasonal species (baleen whales, northern bottlenose dolphin) (Silva et al., 2014). This high diversity renders the Azores a model region to study cetaceans. Dedicated observational time-series are taking place in the Azores to e.g., track migration patterns of baleen whales (Visser et al., 2011), and study the behavior of e.g., Risso's dolphins (Hartman et al., 2008; Visser et al., 2011; Visser et al., 2021) and deep-diving beaked whales (Aguilar de Soto et al., 2020). The wide variety of cetacean species is attracted by the availability of abundant prey. Seasonal visitors such as baleen whales and dolphins feed on e.g., krill and baitfish, respectively, in spring and summer (Afonso et al., 2020). Year-round species such as sperm whales feed on deep-sea squid (Clarke et al., 1993) and dolphins on mesopelagic prey (Clarke et al., 1996, 1995). Some cetacean species also use the Azores as a nursery such as sperm whales, common and spotted dolphin (Silva et al., 2014). The Azores are a cephalopod diversity hotspot with 83 confirmed species (Pereira et al., 2016). Contrary to Cabo Verde, the cephalopod diversity of the Azores is well studied. A major contributor was Malcolm R. Clarke, who compiled species lists for this area and investigated the stomachs of predator that feed on cephalopods (Clarke et al., 1993, 1996, 1995; Clarke, 2006; Clarke, 1996). In terms of fish species, 190 coastal species are known including over 60 species of benthic and pelagic sharks and rays (Das and Afonso, 2017; Porteiro et al., 2010; Wirtz et al., 2013). The Azores function as a crossroad linking eastern to western basins as well as the cold productive boreal waters in the North to the tropical and equatorial waters in the South (Afonso et al., 2020). The archipelago can serve as a model region to investigate predator-prey dynamics due to its high diversity and abundance of top predators.

Fram Strait - Arctic Ocean

The diversity of the Arctic Ocean has been mainly shaped by recurrent invasions, habitat fragmentation and impacts of glacial and interglacial periods like e.g., bathymetric changes (Hewitt, 2000; Ronowicz et al., 2015; Weydmann et al., 2018). The Arctic Ocean is divided into five ocean basins (Canada, Makarov, Amundsen, Nansen and Eurasian Basin), which are separated by mid-oceanic ridges. Those ridges limit species dispersal within the Arctic (Bluhm et al., 2011) as well as the inflow of water masses from adjacent oceanic regions (Carmack and Wassmann, 2006). Combined with the glacial history of the Arctic, those dispersal barriers resulted in distinctive marine biota assemblages that simultaneously are still closely related to species found in neighboring oceanic regions (Bucklin et al., 2010). The number of extant species in the Arctic is estimated to be about 8000 (Bluhm et al., 2011), however, several thousand species are estimated to be undescribed (Appeltans et al., 2012; Bluhm et al., 2011). In the Arctic, 242 fish species are known to occur including six endemic species (Mecklenburg et al., 2010). Highest diversities are found in benthic and demersal fishes (87%) versus pelagic fishes (13%) (Bluhm et al., 2011). The distribution of 59% of the 242 species is considered boreal, predominantly boreal or widely distributed, while 41% are considered Arctic, predominantly Arctic or Arctic-boreal. A total of 16 marine mammals are found in the Arctic with nine species that are ice-associated and occur year-round and seven species that are seasonal visitors or just occur occasionally (Huntington and Moore, 2008). Arctic biodiversity is shaped by the seasonality of light and sea-ice coverage. Increased ice-free conditions, ocean warming and increased inflow of Atlantic water into the Arctic (“atlantification”) favor the migration of Atlantic species further north into the Arctic (“borealization”). This has been observed in phytoplankton (Hegseth and Sundfjord, 2008), zooplankton (Weydmann et al., 2014), fish (Mueter and Litzow, 2008) and marine mammals (Moore, 2008). Some of these migrations led to the replacement of long-lived, slow-growing Arctic species with faster-growing temperate species. Baseline studies are needed to further identify potential range expansions and shifts in community composition in the Arctic due to climate change. The Fram Strait, the only deep-basin connection between the Arctic and neighboring Oceans, serves as a transition zone between the northern North Atlantic and the Arctic Ocean, allowing the exchange of Arctic and Atlantic water (Schauer et al., 2004). Therefore, it is a good study area

to investigate atlantification and borealization (Asbjørnsen et al., 2020; Wang et al., 2020). The HAUSGARTEN Observatory LTER established by the Alfred-Wegener Institute, Helmholtz-Centre for Polar and Marine Research (AWI) has been initiated to detect and track environmental changes in the Fram Strait for over 20 years (Soltwedel et al., 2005). However, most research within this initiative focuses on benthic or planktonic-surface communities. This thesis aims to close that gap by adding observations of nekton species of the pelagic realm.

Objectives

This thesis aims to answer three objectives.

The **first objective** is to establish an eDNA pipeline from universal and species-specific primer design and fieldwork to species assignment to assess nekton diversity and distribution. Due to the difficulties that are affiliated with using COI primer for cephalopod diversity assessments stated above, a universal cephalopod primer targeting the nuclear 18S rRNA gene was designed (**Chapter 4**). This universal primer was then applied in seawater samples off the Azores (**Chapter 1**) and Cabo Verde (**Chapter 2**) and in seawater and sediment of the Arctic Ocean (**Chapter 3**). As I was interested in specific species, I designed two species-specific primers. One of them indicated the horizontal and vertical distribution of the elusive squid *Taningia danae* off Cabo Verde (**Chapter 2**). The second primer investigated the migration range of the pyrosome *Pyrosoma atlanticum* in Cabo Verdean waters to understand its role in the carbon cycle (**Chapter 5**).

The **second objective** of this thesis is to assess nekton biodiversity and distribution in hotspots of biodiversity or climate change. Off the Azores, a biodiversity baseline of cephalopods was established using molecular techniques and literature records (**Chapter 1**), while off Cabo Verde, a cephalopod baseline was established by combining eDNA analysis with net catches, video surveys and literature records (**Chapter 2**). The diversity baseline off the Azores was used to reconstruct the cephalopod prey spectra of two cetacean top-predators (**Chapter 1**). The hypothesis that Cabo Verdes' cephalopod diversity is different from other archipelagos in Macaronesia was tested with the cephalopod diversity baseline constructed for Cabo Verde (**Chapter 2**). As a hotspot of climate change, the cephalopod and fish diversity of the Arctic was investigated in seawater and sediment (**Chapter 3**) also using molecular techniques to detect range expansions due to climate change.

The **third objective** is to predict taxa that potentially contribute actively or passively to the vertical transport of carbon. Three different approaches were applied. First, cephalopod taxa that undergo ontogenetic migration or diel vertical migration off the Azores were identified, as

they actively distribute carbon from surface layers to the deep sea (Chapter 1). Second, in a metabarcoding and species-specific approach, two abundant squid species and one pyrosome species occurring off Cabo Verde were discussed that potentially contribute passively to the carbon cycle as foodfalls (Chapter 2 & 5). Third, cephalopod and fish eDNA in seawater and sediment of the Fram Strait in the Arctic Ocean were compared to test whether eDNA of pelagic origin can be detected in sediments to identify potential foodfall species (Chapter 3).

Thesis outline and author contributions

This thesis contains five chapters based on five separate manuscripts of which two are first-author manuscripts, one is a shard first-authorship and two are co-authorships.

CHAPTER 1:

Fleur Visser, **Véronique J. Merten**, Till Bayer, Machiel G. Oudejans, Danielle S.W.. de Jonge, Oscar Puebla, Thorsten B. H. Reusch, Janina Fuss, Henk-Jan T. Hoving (2021) "**Deep-sea predator niche segregation revealed by combined cetacean biologging and eDNA analysis of cephalopod prey**". *Science Advances*: 7 (14) eabf5908. DOI: 10.1126/sciadv.abf5908

This is a shared first-authorship manuscript.

Contribution	Author
Conceived the study	FV, HJTH
Design of the study	FV, HJTH, **VJM**
Sample collection	FV, **VJM**
Investigation process	FV, HJTH, **VJM**, DSWdJ, TB, MGO.
Analysis and processing	FV, HJTH, **VJM**, DSWdJ, TB, MGO
Drafting of manuscript	FV, HJTH, **VJM**, DSWdJ, TB, MGO
Support during eDNA analysis and data collection from samples	TB, TBHR, OP
Sequencing	JF

CHAPTER 2:

Véronique Merten, Till Bayer, Thorsten B. H. Reusch, Oscar Puebla, Janina Fuss, Julia Stefanschitz, Alexandra Lischka, Helena Haus, Philipp Neitzel, Uwe Piatkowski, Stephanie Czudaj, Bernd Christiansen, Anneke Denda and Henk-Jan T. Hoving (2021) **"An Integrative Assessment Combining Deep-Sea Net Sampling, in situ Observations and Environmental DNA Analysis Identifies Cabo Verde as a Cephalopod Biodiversity Hotspot in the Atlantic Ocean"**. *Frontiers in Marine Science 8:760108. DOI: 10.3389/fmars.2021.760108*

Contribution	Author
Conceived the study	HJTH
Design of the study	**VJM**, HJTH, TB, TBHR, OP
Sample collection	**VJM**, AL, UP, SC, AD, HJTH
Lab work	**VJM**, JS, JF
Data contribution	AL, UP, SC, HH, BC, AD, HJTH
Data analysis	**VJM**, HJTH
Drafting of manuscript	**VJM**, HJTH
Critical review of manuscript	TB, TR, OP, JF, JS, AL, HH, UP, SC, BC, AD

CHAPTER 3:

Véronique Merten, Oscar Puebla, Till Bayer, Thorsten B.H. Reusch, Janina Fuss, Julia Stefanschitz, Katja Metfies, Henk-Jan T. Hoving **"Arctic cephalopod and fish distribution and diversity in the Fram Strait uncovered by seawater and sediment eDNA metabarcoding"**

Contribution	Author
Conceived the study	HJTH
Design of the study	**VJM**, HJTH, TB, TBHR, OP
Sample collection	**VJM**, KM, HJTH
Lab work	**VJM**, JS, JF
Lab facilities and samples	KM
Data analysis	**VJM**
Drafting of manuscript	**VJM**, HJTH
Critical review of manuscript	OP, TB, TBHR, KM

CHAPTER 4:

Danielle de Jonge, Véronique J. Merten, Till Bayer, Oscar Puebla, Thorsten B.H. Reusch , Henk-Jan T. Hoving (2021) „A novel metabarcoding primer pair for environmental DNA analysis of Cephalopods (Mollusca) targeting the nuclear 18S rRNA region". *Royal Society Open Science*: 8 (2). DOI: 10.1098/rsos.201388

Contribution	Author
Conceived the study	HJTH, OP, TBHR
Design of the study	VJM, HJTH, TB, TBHR, OP
Collection of samples	VJM, HJTH
Lab work	DSWdJ, VJM, HJTH
Data analysis	DSWdJ, VJM, TB
Drafting of manuscript	DSWdJ
Critical review of manuscript	VJM, HJTH, OP, TBHR, TB

CHAPTER 5:

Vanessa I. Stenvers, Helena Hauss, Karen J. Osborn, Philipp Neitzel, Véronique Merten, Stella Scheer, Bruce H. Robison, Rui Freitas & Henk Jan T. Hoving (2021) "Distribution, associations and role in the biological carbon pump of Pyrosoma atlanticum (Tunicata, Thaliacea) off Cabo Verde, NE Atlantic". *Scientific Reports*: 11, 9231. DOI: 10.1038/s41598-021-88208-5

Contribution	Author
Conceived the study	HJTH, HH
Collection of samples	HJH, HH, KJO, BHR, SS, RF,
Lab work	VIS, PN, KJO, VJM, SS
Data analysis	VIS, KJO, VJM
Drafting of manuscript	VIS, HJTH, HH, KJO, VJM (eDNA part)
Critical review of manuscript	VJM, SS, BHR, RF

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Chapter 1

Deep-sea predator niche segregation revealed by combined cetacean biologging and eDNA analysis of cephalopod prey

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Abstract

Fundamental insight on predator-prey dynamics in the deep sea is hampered by a lack of combined data on hunting behavior and prey spectra. Deep-sea niche segregation may evolve when predators target specific prey communities, but this hypothesis remains untested. We combined environmental DNA (eDNA) metabarcoding with biologging to assess cephalopod community composition in the deep-sea foraging habitat of two top predator cetaceans. Risso's dolphin and Cuvier's beaked whale selectively targeted distinct epi/meso- and bathypelagic foraging zones, holding eDNA of 39 cephalopod taxa, including 22 known prey. Contrary to expectation, extensive taxonomic overlap in prey spectra between foraging zones indicated that predator niche segregation was not driven by prey community composition alone. Instead, intraspecific prey spectrum differences may drive differentiation for hunting fewer, more calorific, mature cephalopods in deeper waters. The novel combination of methods presented here holds great promise to disclose elusive deep-sea predator-prey systems, aiding in their protection.

1.1. Introduction

The pelagic deep sea is the largest and least explored habitat on the planet, harboring a large and unexplored biodiversity (1, 2). Cumulative impacts of climate change and industrial exploitation impose increasing pressure on deep-sea systems, challenging marine ecosystem health and services at a global scale (1, 3). While key to our understanding of food web dynamics, interactions between elusive and sometimes giant deep-sea predators and prey occur outside of the range of human observations and remain virtually unknown, limiting effective conservation (3). Advanced on-animal recorders have revealed extensive use of meso- and bathypelagic waters as foraging grounds by multiple species of cetaceans with diverse, often cephalopod-dominated diets (4–7). As top predators, cetaceans are essential in maintaining marine diversity and ecosystem functioning (8). Their exploitation of the deep sea may have extensive effects on deep-sea prey populations and food webs and constitutes direct ecological linkage between deep and shallow ocean systems. Despite its apparent homogeneity, the pelagic deep sea hosts a myriad of foraging niches (9). As air-breathing marine predators, cetaceans have evolved a suite of specialized morphological and physiological traits, enabling extreme diving and localization and capture of deep-sea prey (10, 11). Optimal foraging theory predicts that these traits and associated behavioral strategies evolve toward maximization of foraging performance (i.e., net energetic gain) (12). In contrast to their terrestrial counterparts, cetacean predators face a trade-off between selective forces arising from the dependency on air at the surface and prey at depth. Their hunting strategy on remote deep-sea prey puts stringent pressure on the need for efficient foraging, balancing oxygen use (i.e., from modulation of dive depth, duration, and movement energetics) with energetic return [calorific intake per dive (13)]. Hence, in line with their specialized adaptations, deep-diving cetaceans may optimize foraging performance by selective targeting of distinct foraging zones that hold specific prey communities.

Despite the high global biomass and pivotal role of cephalopods in oceanic food webs, knowledge on deep-sea cephalopod community composition is still very limited (14). Many cephalopod species have never been observed alive in their habitat or captured as adults [e.g., (14, 15)]. Cetacean cephalopod prey spectra can be assessed using nets, stomach content analysis, or optical methods (16). Two issues associated with physical and optical sampling of cephalopod diversity are avoidance behavior and patchiness, resulting in sampling bias toward less mobile and more abundant specimens (17). Alternative methods are needed for an efficient and complete assessment of regional

cephalopod biodiversity. Environmental DNA (eDNA) metabarcoding enables the detection of species on the basis of genetic material (e.g., mucus and feces) that they release in their environment (18). eDNA has been successfully used to reconstruct the horizontal distribution, diversity and migration of open-ocean nekton (19, 20). Yet, to the best of our knowledge, it has not been used to investigate cephalopod biodiversity in the deep sea.

We investigated the foraging zones and prey spectra in the habitats of two co-occurring, deep-diving cetaceans, Risso's dolphin (*Grampus griseus*) and Cuvier's beaked whale (*Ziphius cavirostris*), representing two distinct deep-sea foraging strategists, targeting epi/meso- and bathypelagic waters, respectively (4, 6). Stomach content analyses of both species, based on relatively few specimens, show diverse, partially overlapping diets dominated by oceanic deep-sea squids (7). Risso's dolphins belong to a group of deep-diving cetaceans foraging at depths between the surface and around 800 m (6). Individuals can actively switch between mesopelagic and near-surface foraging, targeting often dynamic prey patches or scattering layers (6, 21).

Beaked whales (Ziphiidae), along with members of the sperm whale family, are the deepest diving cetaceans, routinely foraging at depths beyond 1000 m. Cuvier's beaked whale holds the current world record for extreme diving, at depths to 2992 m during dives that may exceed 2 hours (4, 22). Prey search is not initiated until several hundreds of meters deep (4) and may continue to the bathyal seafloor (23), but zones of prey capture within this foraging habitat spanning several kilometers have rarely been reported [only in (4)]. Cetacean foraging depth is often inferred from maximum diving depth. Characterization of target prey layers, however, requires assessment of the target foraging zone, which may vary between dives as a function of dynamic variation in the presence of prey [e.g., (21)]. Whereas we expect prey community composition to be a main driver of observed niche segregation in deep-sea predators, methodological challenges associated with sampling cetacean foraging zones in the extreme deep-sea environment have thus far prevented rigorous testing of this hypothesis in the field. Here, we pioneer a combination of methods that enabled matching of prey community composition with deep-sea predator foraging behavior. We combined cephalopod eDNA analysis with biologging of cetacean diving and acoustic behavior and hypothesize that, to efficiently capitalize on nonuniformly distributed deep-sea prey, (i) cetacean predators target discrete foraging zones that (ii) hold specific prey spectra.

1.2. Material and Methods

Foraging habitat

Annual field effort was conducted off Terceira Island (Azores, Portugal), between May and August 2013 to 2019 (Fig. 2). Shore- and vessel-based observations were conducted to record the locations of Cuvier's beaked whale groups and Risso's dolphin foraging dives. Risso's dolphin daytime foraging dive starts are energetic and can be identified from visual observation at the surface. Cuvier's beaked whales forage throughout the 24-hour day whereby foraging dives are alternated with short series of non-foraging dives (Fig. 3) with limited movement between consecutive surfacing locations (i.e., typically <2 km off Terceira Island; Fig. 2). Observations were conducted from shore-based lookouts elevated at 65 m or higher above sea level or from the research vessel and comprised (i) standardized surveys, recording all groups present and their location, and (ii) focal follow observations, tracking one group to record location and behavior following a standardized protocol (48). We recorded all foraging dive starts (Risso's dolphin) and surfacing or dive locations (Cuvier's beaked whale). Location was recorded using a theodolite linked to a computer running visual tracking software, VADAR (shore based) (49), or using a hand-held GPS (vessel based). Area bathymetry and bottom depth was derived from EMODnet bathymetry data (50) and matched to sighting and dive locations using QGIS (51).

Foraging zones and diet

To identify foraging zone depths, dive and acoustic data were collected from individuals instrumented with noninvasive, high-resolution digital acoustic recording tags [Dtag version 3; 240-kHz sound, 200-Hz accelerometer, magnetometer, and pressure sensor (24)]. Dtags were attached to the dorsal area of individuals using suction cups, with a 6- to 8-m-long hand-held pole. Risso's dolphin and Cuvier's beaked whale forage using sound. They detect and track prey by emission of echolocation click series (biosonar) (10). Following the dive start, individuals initiate the search phase, emitting broadband click series at regular intervals [interclick interval (ICI); e.g., ICI mean = 143 ms for Risso's dolphin (52)]. Upon selection and close approach of a suitable prey item, the click train transitions into a discrete, rapid click series at lower amplitude termed "buzz" (mean ICI = 3.6 ms for Risso's dolphin), indicating a prey capture attempt (10, 52). Buzzes therefore form accurate indicators of foraging effort and presence of prey. In combination with the dive profile, buzzes were used to define (i)

foraging dives (all dives deeper than 20 m with one or more buzz) and (ii) foraging zones (range of buzz depths). The timing of the start and end of echolocation click series and foraging buzzes of the tagged animals were obtained manually through customized auditing scripts from the DTAG toolbox (soundtags.st-andrews.ac.uk) using MATLAB 2014b (MathWorks, MA, USA). Tagged whale clicks can be readily distinguished from clicks produced by nearby conspecifics by their fairly consistent angle of arrival on the two tag hydrophones and the existence of artificial low-frequency energy (<15 kHz), which is absent in clicks produced by conspecifics (53).

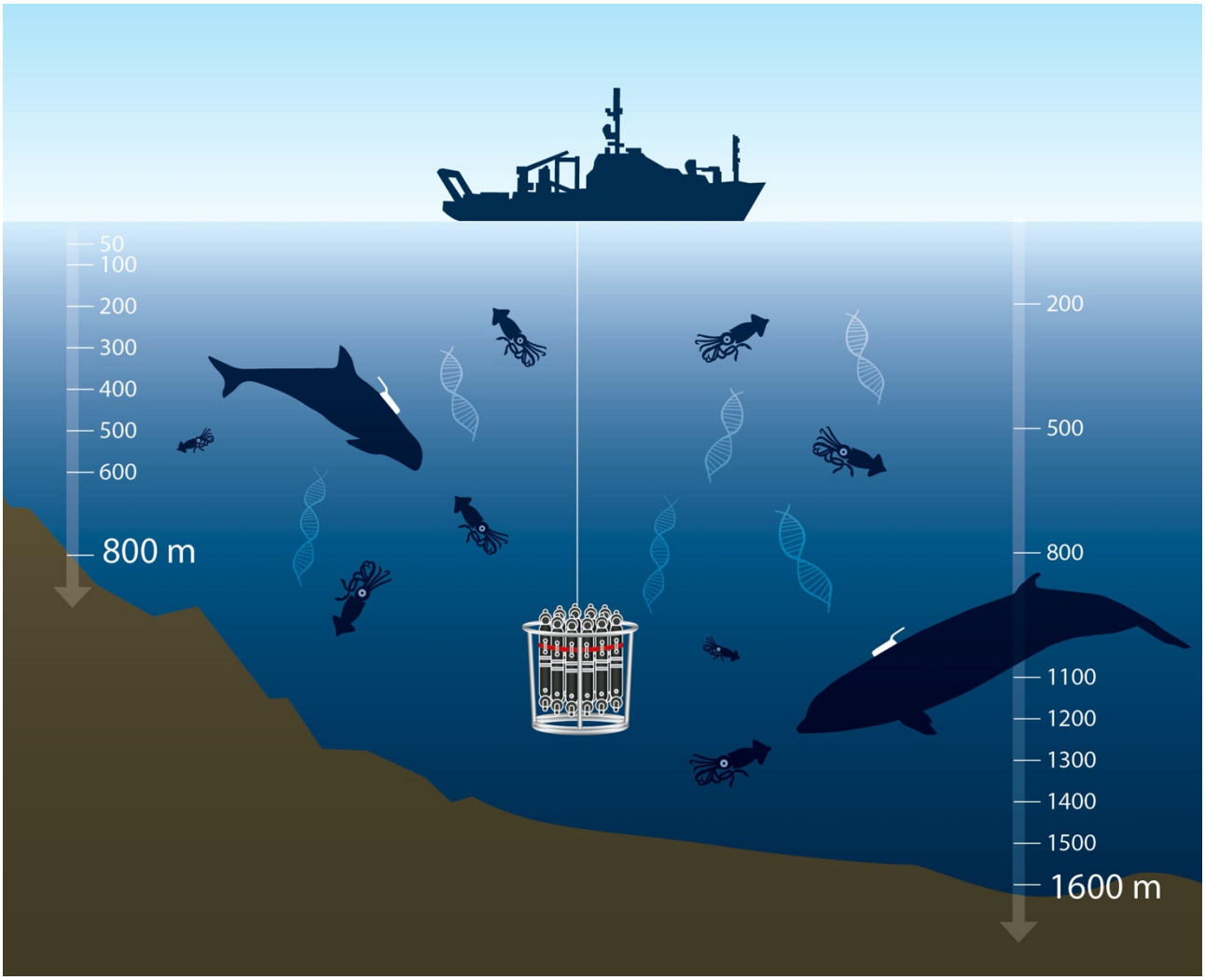

Figure 1| Pelagic sampling of cephalopod eDNA in cetacean foraging zones. Targeted sampling of cephalopod eDNA across the foraging zones of cetacean deep-sea predators, Risso's dolphin (*G. griseus*; left) and Cuvier's beaked whale (*Z. cavirostris*; right). as determined from biologging of their diving and biosonar foraging behavior using noninvasive sound and movement recording tags (24).

The presence of differential dive types in both species was assessed using HMM [package momentuHMM using R (54, 55)]. HMM models classifying foraging dives were run for one to four states, with the two covariates maximum dive depth and SD of within-dive buzz depth (proxy for width of the foraging zone). Parameters improving model fit were retained in the final fitted models. The model with the lowest value of the Akaike information criterion was selected as the best model. For Cuvier's beaked whale, we analyzed the closest distance to the bottom for each foraging dive from the echoes of clicks emitted by the tagged whale, which were recorded on the tag (because of physical properties of Risso's dolphin, returning click-echoes are likely blocked by the melon and rarely recorded on the tag). We used customized scripts from the DTAG toolbox to visualize the echogram, composed of a window of 50 consecutive clicks with all returning echoes up to 0.6 s (mean click train ICI; using MATLAB 2014b). Echoes originating from the seafloor are reverberant and stronger than from objects passing by in the water column (56). The ICI to the next click defines the maximum time window for a returning echo detection and thus closest distance at which the seafloor can be detected, in this case 450 m. The moment of closest proximity to the seafloor was recorded for every dive with clear echo patterns from visual inspection of the echogram. The distance to the seafloor was calculated using the time delay between the produced click and its returning echo [two-way travel time (TWT)] as follows: sound speed in water x TWT/2. This represents maximum closest distance to the seafloor as the angle at which the seafloor is hit by the echolocation beam (and thus distance of travel) will depend on the orientation of the individual with respect to the seafloor.

To test whether Risso's dolphin and Cuvier's beaked whale foraging zones held differential prey spectra, we performed a random forest (RF) classification (57). The RF model was set up to aim to discriminate between sampling records originating from the Risso's dolphin (n = 28; 50 to 600 m) or Cuvier's beaked whale (n = 10; 900 to 1600 m) foraging zone. The RF model was run using 1000 trees, with random selection of 20 predictor variables (taxa) at each node and using a subsample of two-thirds of the dataset. Model selection was performed by running the full model without the variable with the lowest variable importance. Diet data were derived from the literature, extracting all taxa occurring in the diet of Risso's dolphin and Cuvier's beaked whale in comparable regions (North Atlantic Ocean and Mediterranean Sea).

eDNA sample collection, filtration, and extraction

eDNA was sampled centrally in the foraging habitats of Risso's dolphin (n = 4) and Cuvier's beaked whale (n = 2; Fig. 1 and table S3) at maximum bottom depths of 922 and 1600 m, respectively. Sampling was conducted from the RV Pelagia in July 2018, overlapping the annual period of tag data collection. At each station, water was collected using Niskin bottles mounted on a CTD rosette (24 12-liter bottles) at seven or eight specific depths, in biological triplicates, resulting in a total of 144 discrete water collections. Water was immediately transferred from the Niskin bottles to 2-liter sterile single-packed urine bags and stored at 4°C. Water was then filtered through 0.22-µm Sterivex-GP filters (Merck Millipore) using sterile 60-ml syringes. The Sterivex filters were stored in −80°C until further processing. DNA was extracted from the filters using the QIAGEN DNeasy Blood and Tissue *K* it (modified protocol). DNA extracts were quantified using a Qubit fluorometer and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) and stored at −20°C. To identify potential contaminations, filtration negative controls (Milli-Q), DNA extraction negative controls (using elution buffer from the extraction kit) and polymerase chain reaction (PCR) negative controls (PCR water instead of DNA extract) were included. Detailed description of all our procedures, including reduction of contamination risks, is provided in Supplementary Methods.

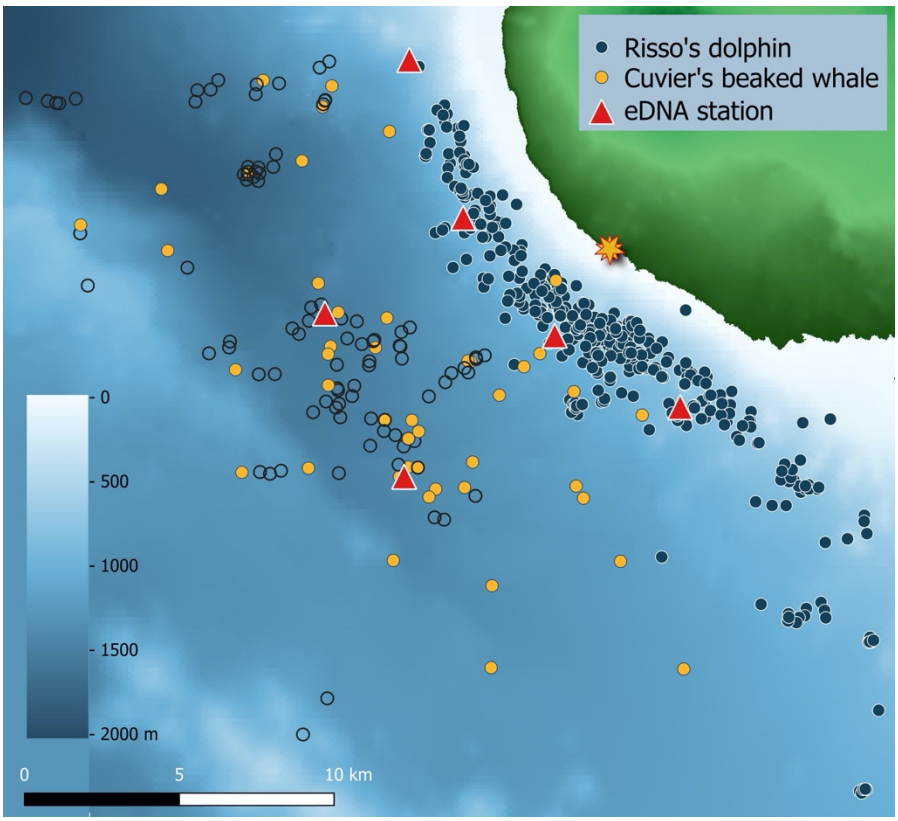

Figure 2| Risso's dolphin and Cuvier's beaked whale foraging habitat and associated eDNA sampling stations. eDNA surface to deep-sea CTD sampling locations (red triangles) placed centrally in the foraging habitats of Risso's dolphin (G. griseus; blue circles, foraging dive locations) and Cuvier's beaked whale (Z. cavirostris; orange circles, group sighting location; open circles, sequential observations of sighted group), off Terceira Island, Azores. Bathymetry derived from (50).

Library preparation and sequencing

Two universal cephalopod primer sets were applied. The first primer set targeted the nuclear 18S rRNA gene yielding an amplicon of 140 to 190 base pairs (bp) (Ceph18S_forward, 5'-CGCGGCGCTACATATTAGAC-3' and Ceph18S_reverse, 5'-GCACTTAACCGACCGTCGAC-3') (58). The second primer set targets the mitochondrial 16S rRNA gene yielding an amplicon of 212 to 244 bp [CephMLS_forward, 5'-TGCGGTATTWTAACTGTACT-3' and CephMLS_reverse, 5'-TTATTCCTTRATCACCC-3' (59)]. For PCR amplification, a two-step PCR protocol was used. The first PCR amplified the cephalopod DNA sequences present in the sample with the universal primer sets mentioned above including a sequencing tail, and the second PCR attached a unique indexing primer combination to every PCR product of each sample to be able to pool the samples. PCRs were carried out in duplicate for every biological replicate (resulting in six replicates per site and depth). On every 96-well plate, one negative control and three positive controls were added in duplicate. The first PCR had a total volume of 20 µl and included 7 µl of PCR-grade water, 4 µl of 5xKAPA HiFi Buffer (Roche), 0.6 µl of 10 mM deoxynucleotide triphosphates (dNTPs; Roche), 1 µl of dimethyl sulfoxide (DMSO), 1 µl each of the 10 µM forward and reverse primers, 0.4 µl of KAPA HiFi polymerase (5 U/µl; Roche), and 5 µl of the DNA extract. The PCR program started with an initial denaturing step at 95°C for 5 min, 35 cycles of 98°C for 20 s, annealing temperature of primer for 15 s (62°C for 18S and 55°C for 16S), 72°C for 1 min, and a final extension step of 72°C for 10 min. Fragment sizes were verified on a 2% agarose gel stained with GelRed (Biotium). The PCR product of the first PCR was diluted 1:100 and used as a template for the second PCR. During the second PCR, a unique indexing primer combination was used for every sample. The second PCR was performed in 10 µl volumes of 1 µl of PCR-grade water, 2 µl of 5xKAPA HiFi Buffer (Roche), 0.3 µl of 10 mM dNTPs (Roche), 0.5 µl of DMSO, 0.2 µl of KAPA HiFi polymerase (5 U/µl; Roche), 0.5 µl of a reverse and forward indexing primer, and 5 µl of template.

The PCR products were pooled to equimolar concentrations according to the DNA concentrations measured using a Qubit fluorometer. This resulted in two libraries, one for each primer set. The fragment size of the libraries was validated on a 2% agarose gel and stained with GelRed, the correct bands were cut out and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research) following the manufacturer's protocol. The library pool was quantified with the Qubit dsDNA HS Assay Kit (Molecular Probes, Life Technologies). Insert size distribution was determined with the 4200 Tapestation D5000 ScreenTape (Agilent). The working solution was diluted to 2 nM, and loading solution was prepared according to protocol. The library pools were loaded with 8 pM and 20% PhiX

spike-in to increase diversity. Sequencing was done on the Illumina MiSeq with the MiSeq Reagent Kit v3 (600 cycles).

Cephalopod reference database

To complement public databases for the targeted mitochondrial 16S rRNA and nuclear 18S rRNA gene, additional cephalopod reference tissue samples, collected during WH383 on RV Walther Herwig III, were barcoded. For the nuclear 18S rRNA gene, the sequences barcoded in (58) were used. For Sanger sequencing of the mitochondrial 16S rRNA gene, the same DNA extracts were used as for the 18S rRNA gene, resulting in 33 successful cephalopod voucher sequences, including 32 different species and 15 different families. Tissue samples had been stored in 70% ethanol at −20°C. DNA was extracted using QIAGEN DNeasy Blood and Tissue following the manufacturer's protocol. PCRs were performed with the CephMLS primer sets used for metabarcoding. Forward and reverse strand sequencing of the PCR products was performed using the Sanger Sequencing Kit (Applied Biosystems). Primers and low-quality ends were trimmed from the sequences, checked manually, edited, and assembled using CodonCode Aligner (version 3.7.1).

These sequences were added to the public sequence databases used for training IDTAXA that were based on the MIDORI (16S) and SILVA (18S) databases (60), which were updated with sequences retrieved from the National Center for Biotechnology Information (NCBI) GenBank database in June 2020. Briefly, the taxonomic information of sequences contained in the MIDORI (version 20180221 unique) and SILVA (version SILVA_138_SSURef_NR99) databases was updated to reflect the taxonomy assigned by NCBI as of June 2020. Cephalopod sequences were extracted from the databases and used as input for the "eukref_gbretrieve.py" program from the EukRef project (https://unieuk.org/) to recursively query GenBank until no new sequences were retrieved. This resulted in a total of 2116 and 169 sequences representing 144 and 81 genera for 16S and 18S, respectively. These cephalopod sequences were then merged with the full MIDORI or SILVA databases by removing duplicates, and these databases used to generate two IDTAXA training sets for 16S and 18S.

Bioinformatic analysis

After sequencing, the sequences were demultiplexed and sorted by sample without indexing primer. The primer sequences were removed using cutadapt (version 1.18). Untrimmed sequences were

discarded, and the maximum accepted error rate was set to 0.1. Allowed errors are mismatches, insertions, or deletions. The pipeline used for data analysis is summarized in Supplementary Methods. The sequencing analysis was conducted with the Divisive Amplicon Denoising Algorithm with an implemented quality-aware model of Illumina amplicon errors (DADA2, version 1.15.0), an R package that corrects for amplicon errors without constructing operational taxonomic units, in RStudio version 1.1.463 (54, 61). Forward and reverse reads were truncated after a quality score ≤2 and merged with at least 80 bp overlap. Only merged sequences ranging from 150 to 300 bp for CephMLS and 80 to 215 bp for Ceph18S were retained (table S7). The taxonomic assignment of the environmental samples and all controls against the training set was performed by IDTAXA with the R package DECIPHER version 2.6.0. IDTAXA was used because it combines features of phylogenetic, distance-based, and machine learning classification methods, which is especially suitable for incomplete training sets and has been shown to have higher accuracy than popular classifiers (62). The confidence threshold for accepting a classification was set to 60%, providing a conservative classification with relatively low mis- and overclassifications. In addition, the BLAST classifier was applied, which assigns a sequence based on its nearest neighbor in a training set.

Ethical statement

Fieldwork was conducted under scientific permits issued by the Direcção Regional dos Assuntos do Mar, Secretaria Regional do Mar, Ciência e Tecnologia (Regional Directorate for Science and Technology). Access and Benefit Sharing (ABS) Regulation: Portugal is party to the Nagoya Protocol, but does not regulate access to genetic resources. The ABS regulations of the Autonomous Region of the Azores were followed by obtaining the required declaration of conformity, establishing informed consent for the collection and export of biological material from the Direção Regional da Ciência e Tecnologia. Cabo Verde (where some reference tissue samples were collected) has not ratified the Nagoya protocol. To fulfill the national ABS regulations of Cabo Verde, we obtained the required permit for the publication of results based on samples collected in Cabo Verde waters from the Direcção Nacional do Ambiente (National Directorate for the Environment of Cabo Verde).

1.3. Results

Deep-sea foraging niche segregation

Risso's dolphins and Cuvier's beaked whales targeted distinct foraging zones that were spatially segregated in both horizontal and vertical space (Figs. 1 to 4). Risso's dolphin foraging habitat off Terceira Island (Azores) was situated along a narrow zone over the island slope, at a mean (SD) distance of 3.1 (1.6) km from shore (range, 0.7 to 13.0 km; *n* = 134 groups, 455 foraging dive observations) and mean bottom depth of 811 (203) m (range, 81 to 1261 m). Cuvier's beaked whale foraging habitat was located offshore [mean (SD) = 8.8 (3.3) km; range, 4.1 to 21.9 km; *n* = 47 groups, 148 observations], over deep waters of the bathyal seafloor [bottom depth, 1411 (186) m; Fig. 2 and fig. S1]. Risso's dolphin mesopelagic (MESO) and shallow (SH) foraging dive types [mean maximum (SD) depth, 508 (52) and 204 (81) m, respectively], as recorded from eight noninvasive, dive and acoustic recording tags [Dtags ((24)], characterized two foraging zones (Figs. 3 and 4 and tables S1 and S2). Foraging effort during dives was determined from echolocation vocalizations (buzzes; n = 1188 in 145 dives). Depth distribution of buzzes indicated that individuals performed prey capture attempts between depths of 12 and 623 m. Mesopelagic dives targeted prey across a wide zone (66 to 623 m; within-dive foraging depth SD = 126 m) with main effort in the mesopelagic, between 450 and 570 m. Shallow dives targeted a relatively narrow near-surface zone with main effort between 130 and 250 m (within-dive foraging depth SD = 60 m; Figs. 3 and 4).

Cuvier's beaked whale foraging zone was deeper, between 911 and 1782 m [*n* = 8 tag records and n = 60 dives, 2068 buzzes; mean diving depth (SD) = 1420 (255) m; Figs. 3 and 4 and table S1]. Individuals performed three foraging dive types, based on maximum dive depth and width of the within-dive foraging zone (table S2). Dive type I, deep layer–restricted (DLR; *n* = 12), were relatively shallow dives [mean diving depth (SD) = 1042 (66) m], with layer-restricted foraging [within-dive foraging depth SD (SD) = 37 (11) m]. Foraging occurred in a narrow-depth zone around the meso-bathypelagic boundary (850 to 1050 m; Figs. 3 and 4). Deep layer-restricted dives were typically pelagic, with 70% of dives remaining at least 450 m above the seafloor. In contrast, dive types II and III showed foraging across a wide depth zone [within-dive foraging depth SD (SD): type II, 113 (42) and type III, 203 (37) m], into the bathypelagic [mean diving depth (SD): type II, 1299 (148) and type III, 1609 (109) m].

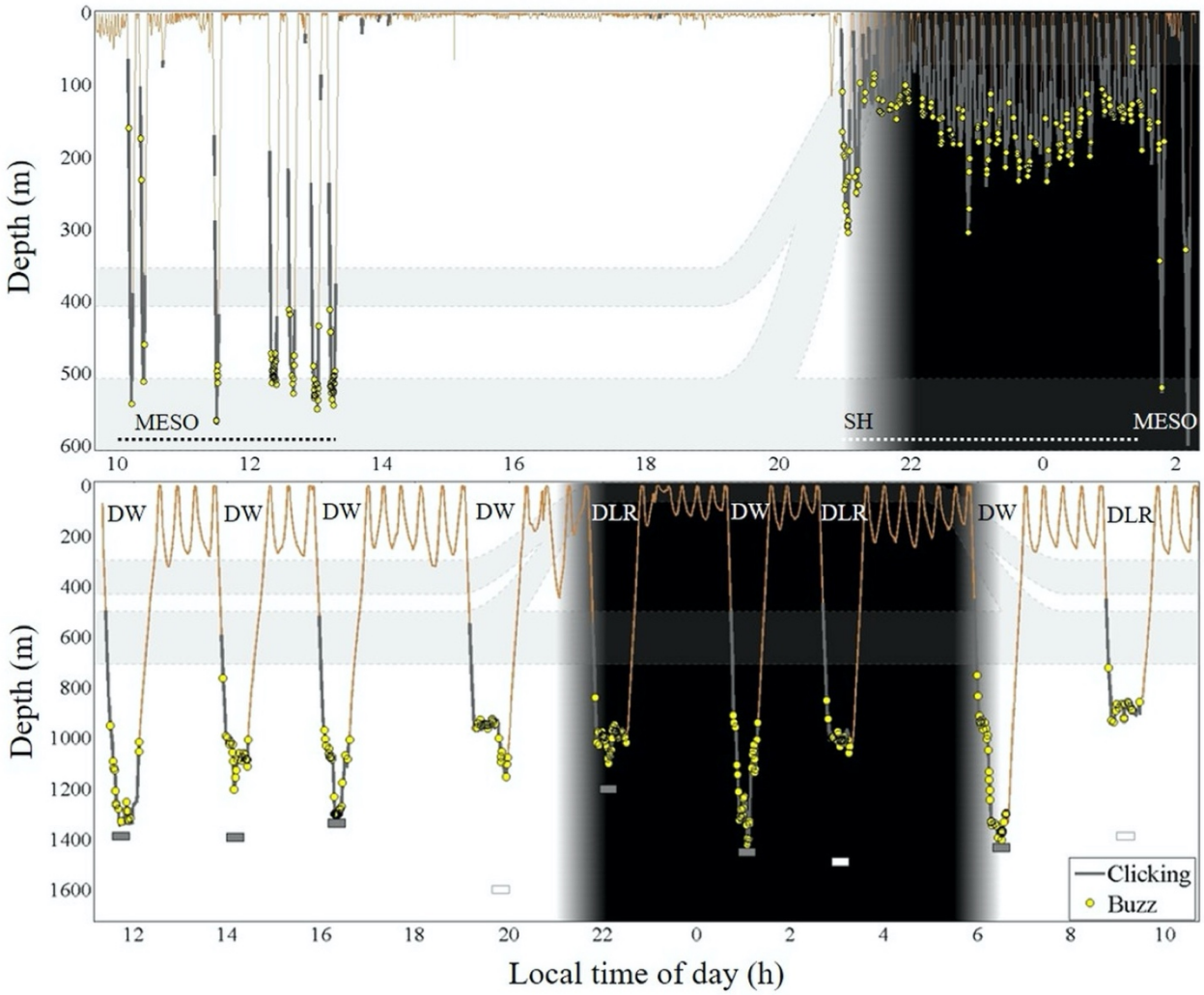

Figure 3| Risso's dolphin and Cuvier's beaked whale foraging behavior off Terceira Island, Azores. Example 17-hour and 24-hour dive profile (orange line) and associated foraging vocalizations of Risso's dolphin (*G. griseus*; gg15_229a; top) and Cuvier's beaked whale (*Z. cavirostris*; zc18_185a; bottom). Echolocation signals (clicking and buzzes) emitted by the tagged individual define foraging effort. Clicking (gray thicker outline) indicates prey search. Buzzes (yellow circles) are emitted at close approach of a target, indicating a prey capture attempt. Both species perform different foraging dive types: Risso's dolphin, mesopelagic (MESO) and shallow (SH); Cuvier's beaked whale, deep-wide (DW) and deep layer–restricted (DLR). Gray rectangles, bottom depth at nearest distance from foraging whale; white rectangle, bottom not detected, minimum depth of nearest bottom; gray bands, schematic representation of depth of the deep scattering layers at the Azores (25).

Type II represented an intermediate strategy, comparable to type III but over shallower bottom depths (bottom depth restricts both maximum dive depth and potential width of the foraging zone). These types were therefore merged into one dive category: deep-wide (DW; n = 48). Deep-wide dives reached the seafloor (38%), near-bottom waters within 200 m above the seafloor [31%; mean (SD) = 132 (47) m] or remained pelagic [31%; mean (SD) = 317 (72) m]. Deep-wide foraging occurred in pelagic, near-bottom, and bottom habitats, with main effort between 800 and 1700 m (Figs. 3 and

4). In all dives to the bottom, individuals performed foraging buzzes and thus prey capture attempts, up to and after reaching the seafloor.

Cephalopod diversity and zonation identified from eDNA

To sample prey spectra of the two deep-sea predators, we performed CTD (conductivity, temperature, depth) casts at the locations of known foraging habitat of Risso's dolphin (n = 4) and Cuvier's beaked whale (n = 2; Figs. 1 and 2). eDNA sampling was conducted at 50- to 300-m intervals between the surface and the mesopelagic or bathyal seafloor at six stations (45 sampling records; 50 to 1600 m; table S3). This enabled the reconstruction of cephalopod community composition spanning the water column (Figs. 4 and 5). Analysis of the resulting nuclear 18S ribosomal RNA (rRNA) and mitochondrial 16S rRNA gene sequences revealed 39 cephalopod taxa,

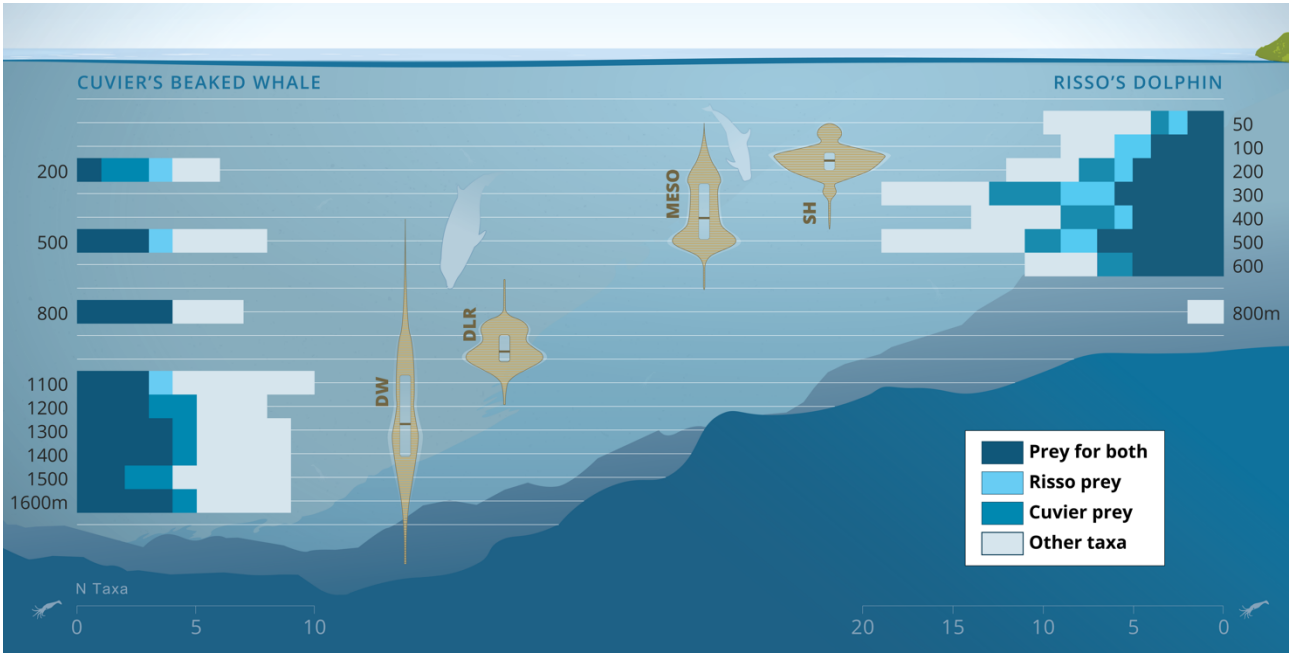

Figure 4| Deep-sea foraging niche differentiation in cetacean top predators. Risso's dolphin (G. griseus) and Cuvier's beaked whale (Z. cavirostris) depth distributions of prey capture attempts (buzzes; violin plots) show discrete foraging zones for the two cetacean predators. Both predators perform two foraging dive types targeting different zones: mesopelagic (MESO) and shallow (SH) for Risso's dolphin and deep-wide (DW) and deep layer-restricted (DLR) into the lower meso- and bathypelagic for Cuvier's beaked whale. Foraging zones of both predators match with the presence of diverse cephalopod prey communities (bar plots; color indicates the number of taxa that are prey for both or either predator).

representing 17 families. Most taxa (79%) could be identified to genus (n = 8) or species level (n = 23; Fig. 5 and table S4). The most widely detected taxa were *Enoploteuthis leptura* (60% of sampling records), *Liocranchia reinhardti* (38%), *Pterygioteuthis* sp. (36%), *Abralia redfieldi* (33%), and *Histioteuthis reversa* (33%). All remaining taxa were detected in less than 25% of sampling records. Additional cephalopod taxa that could not be identified to family level (Teuthida and Cephalopoda) were present in 64 and 4% of sampling records, respectively (Fig. 5).

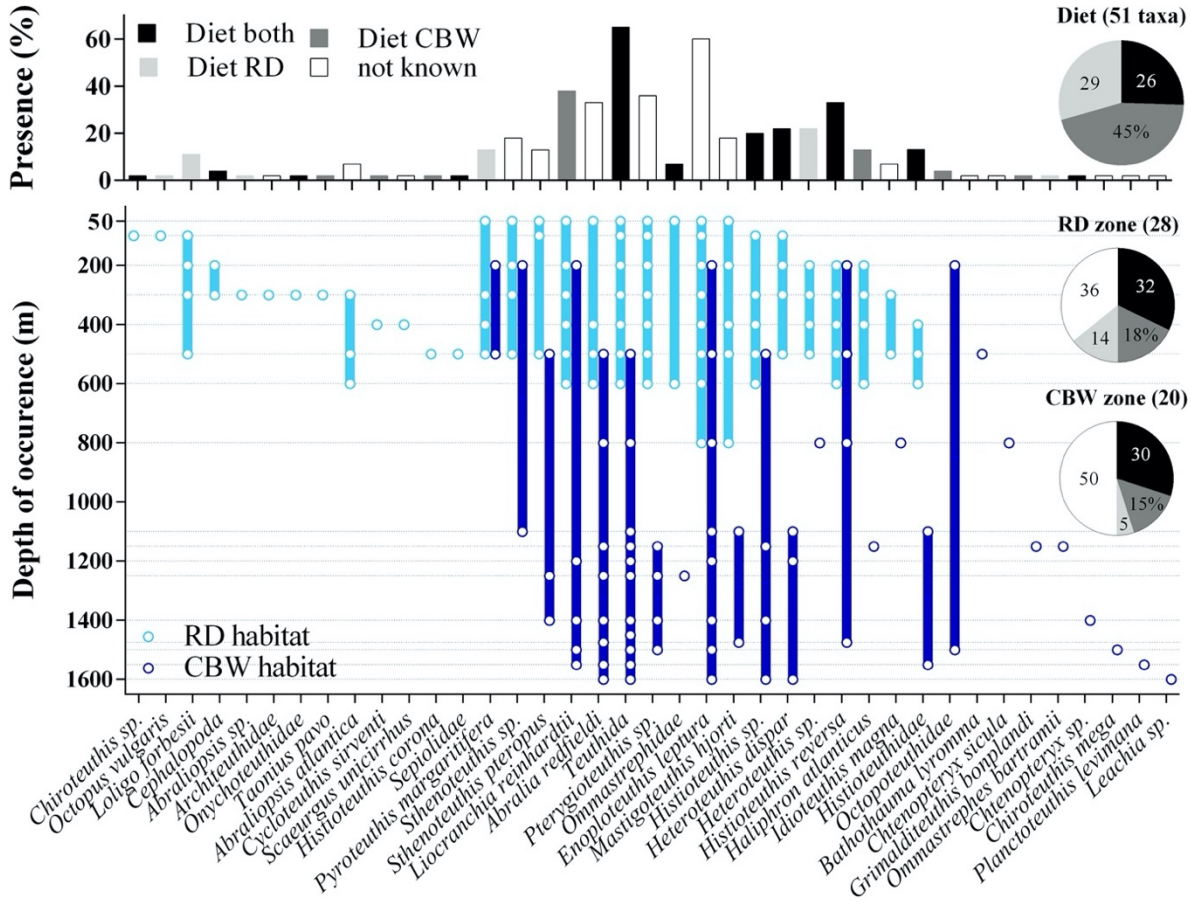

Figure 5| Cephalopod species community and depth distribution from eDNA reveal cetacean deep-sea prey spectra. (Top) Frequency of presence (percentage of sampling records) of the 39 cephalopod taxa identified from eDNA. The color indicates occurrence in the diet of both predators (black), one of the predators (dark/light gray), or not reported in the diet (white; table S5). (Bottom) Cephalopod taxon detection depth and range of occurrence (open circles, filled bar) in Risso's dolphin (*G. griseus*, RD; light blue) and Cuvier's beaked whale (*Z. cavirostris*, CBW; dark blue) habitat, with respective bottom depths of ~800 and ~1600 m. Dotted lines represent sampling depths (Risso's dolphin habitat, 50 to 800 m; Cuvier's beaked whale habitat, 200, 500, 800, and 1100 to 1600 m). Pie graphs show the percentage of cephalopod species reported in the diet of both predators, that are prey of both or either predator (top) and the percentage of taxa recorded in the foraging zones that are prey for both or either predator. Numbers indicate percentage of total taxa.

Cephalopod diversity in the Risso's dolphin zone increased with depth from the surface to 300 to 500 m (9 to 19 taxa; Figs. 4 and 5). This pattern matches the depth of the deep scattering layers (DSLs) at the Azores, which occur at depths between ~300 and 700 m during the day, partially migrating to surface waters at dusk (25). Cuvier's beaked whale habitat also contained a diverse cephalopod community in the mesopelagic (6 to 8 taxa), which diversified further in the bathypelagic and through to the bottom (8 to 10 taxa). Highest diversity was recorded around 1100 m (Figs. 4 and 5). Taxa showed strong variation in their spatial distribution, ranging from a single depth horizon, to a confined depth range spanning several hundreds of meters, to most of the water column (Fig. 5). Thirteen taxa (37%, at family or lower taxonomic level) occurred in both foraging zones. Fifteen (43%) and seven taxa (20%) were restricted to Risso's dolphin and Cuvier's beaked whale foraging zone, respectively (Fig. 5 and fig. S2). Sampling records from the two foraging zones were all classified as part of the same community, i.e., this spatial variation did not translate to significant differences in cephalopod species composition between foraging zones. Moreover, taxonomic overlap between the two habitats was not restricted to the same depth zone, with overlapping community composition between epi/mesopelagic (50 to 600 m) and lower meso/bathypelagic (800 to 1600 m) waters in Risso's dolphin and Cuvier's beaked whale habitat, respectively (Fig. 5). Sampling effort was larger in Risso's dolphin habitat than in Cuvier's beaked whale habitat (four versus two casts), and it is possible that more taxa would have been detected with additional casts in the latter. However, additional sampling would result only in few additional detections (mean of two new detections of species with lower eDNA density presence per cast after second cast; fig. S3 and table S4). Total cephalopod diversity in the two foraging habitats was in the same range (14 to 21 versus 18 to 20 taxa per cast, 30 and 26 taxa in total, for Risso's dolphin and Cuvier's beaked whale habitat, respectively).

Deep-sea predator-prey dynamics

Whereas the predators displayed strict niche segregation, prey spectra in both foraging zones could provide ample foraging opportunity for either predator (Figs. 4 and 5). Known prey species of both predators were present from near-surface to the deep sea, with overlapping prey spectra recorded across the foraging zones. Presence of suitable prey in the foraging zones was confirmed by consistent, ample recordings of prey capture attempts during foraging dives. On average, Cuvier's beaked whale performed 30 and 34 prey capture attempts per hour of foraging effort in deep layer-restricted and deep-wide dives. Risso's dolphin rate of prey capture attempt was higher, with a mean

of 41 and 51 buzzes per hour in shallow and mesopelagic dives. The literature review showed that both cetacean species have diverse diets (31 and 36 cephalopod taxa reported from the North Atlantic and Mediterranean Sea for Risso's dolphin and Cuvier's beaked whale, respectively; tables S5 and S6) that vary between geographic locations, in terms of detected prey species and their importance in the diet. However, most cephalopod families are consistently preyed upon, and diet shows a considerable degree of taxonomic overlap between areas (table S5). Both habitats in our study area held a diverse prey community for the cetacean predators, harboring 83% (10 of 12) and 47% (8 of 17) of prey families recorded in Risso's dolphin and Cuvier's beaked whale diet, respectively (Fig. 5 and table S4). In total, 46% of taxa recorded by eDNA in the cetacean foraging zones represented known prey [13 of 28 for Risso's dolphin (46%) and 9 of 20 for Cuvier's beaked whale

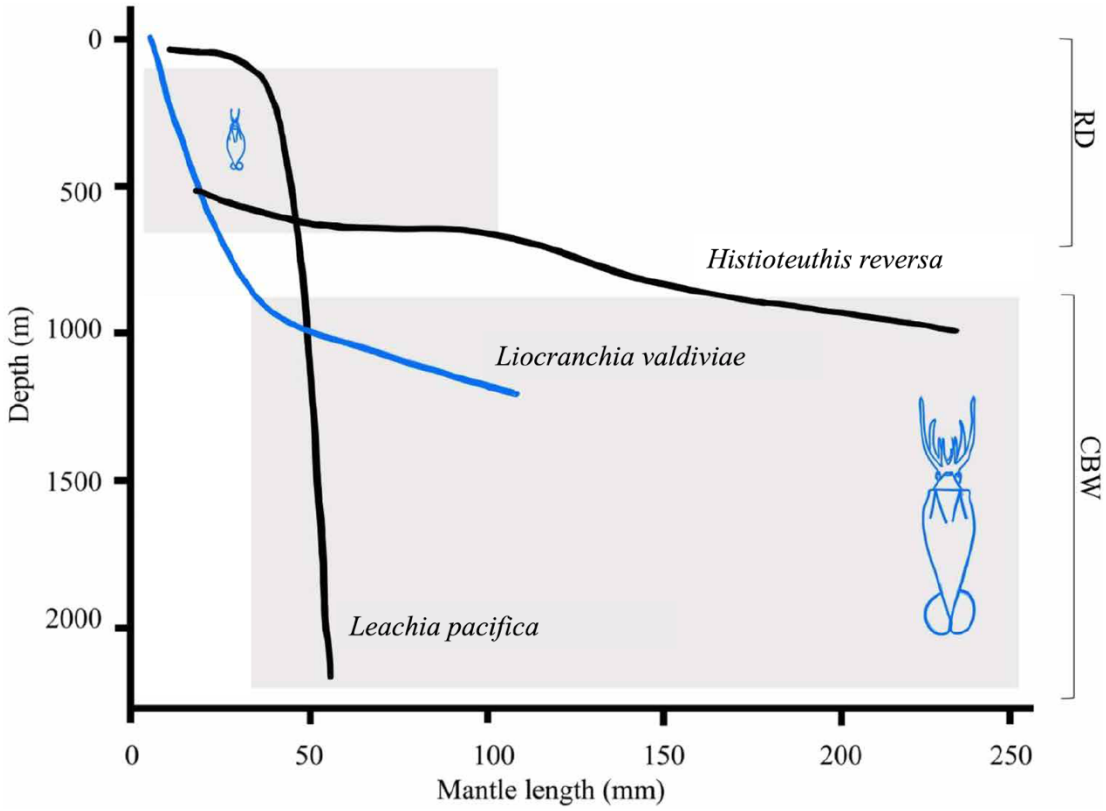

Figure 6| Deeper waters offer larger, more mature cephalopod prey through vertical ontogenetic migration. Literature-derived data of ontogenetic migration from epi- and mesopelagic waters to lower meso- and bathypelagic waters for three cephalopod genera predated by both Risso's dolphin (*G. griseus*, RD) and Cuvier's beaked whale (*Z. cavirostris*, CBW). All three genera were detected in the predator foraging zones off Terceira Island, Azores. Data derived from the Mediterranean Sea (*H. reversa*) and Pacific Ocean (Hawaii; *Leachia pacifica* and *Liocranchia valdiviae*). Juveniles occur shallow, or in the upper 1000 m, and migrate deeper when maturing (36, 63). Inset: Size comparison of juvenile (26 mm) versus adult (240 mm) cranchiid *L. valdiviae*, occurring in the epi-and mesopelagic versus meso-and bathypelagic zone, respectively [data from the Pacific (64)]. The sister species *L. reinhardti* was detected off the Azores and is a known prey for Cuvier's beaked whales. Drawings of *L. reinhardti* adapted from (64). Gray boxes indicate range of prey sizes present in the respective foraging zones.

(45%)]. The most commonly detected families—Enoploteuthidae, Histioteuthidae, Pyroteuthidae, and Cranchiidae—include main dietary components of Risso's dolphin (Histioteuthidae) and Cuvier's beaked whale (Histioteuthidae and Cranchiidae; tables S5 and S6). Accordingly, eDNA of main prey species was detected at high frequency [e.g., *H. reversa* (33% of sampling records) and *Heteroteuthis* sp./*Heteroteuthis dispar* (22%/22%) for Risso's dolphin and Cranchiidae (two species; 40%) and *Histioteuthis* sp./*H. reversa* (20%/33%) for Cuvier's beaked whale; Fig. 5 and table S4). Thus, combined overlap in diet (Risso's dolphin, 13 of 31; Cuvier's beaked whale, 13 of 36 shared known prey taxa; Fig. 5) and prey spectra (Risso's dolphin prey in Cuvier's beaked whale habitat, 35% of taxa; Cuvier's beaked whale prey in Risso's dolphin habitat, 50% of taxa) resulted in the presence of potentially suitable prey for either predator across the discrete foraging zones.

1.4. Discussion

Whereas terrestrial foraging niches are often delineated by structural components, such as the different parts of a tree, habitat structuring in the open ocean is governed by gradients in environmental and oceanographic conditions, including light, pressure, and nutrient and oxygen availability (26). We demonstrate that the resulting zonation and prey distribution can offer specialized foraging niches for mammalian deep-sea predators. We present the first reconstruction of cephalopod communities in the three dimensions of the pelagic environment, using eDNA analysis. Matched with high-resolution data on cetacean foraging behavior, this enabled examination of the relation between the fine-scale distribution of cephalopod prey and top predator foraging zones. Confirming our first hypothesis, the two co-occurring cetaceans exploited entirely discrete deep-sea foraging niches. Their target zones held diverse, overlapping cephalopod species communities, largely composed of known preferred prey. Contrary to expectation, cephalopod community composition alone did not fully explain the strict niche segregation observed between the two deep-sea predators. Instead, cephalopod life history patterns, and the observation of lower prey capture rates by the predator foraging at the largest depths, support an alternative hypothesis. Through the process of ontogenetic migration, performed by several of the most frequently detected cephalopods in the foraging zones, deeper waters may contain larger, more calorific individuals of the same prey (27). Hence cetacean top predators may forage on similar species, but differentiate by targeting individuals of different size and maturity.

Cetacean deep-sea foraging niche segregation

For every hunt, mammalian deep divers need to access a remote foraging zone from the surface. Optimal foraging theory predicts that their foraging strategy should balance the cost of travel in such a way that net energetic return from prey patches is maximized (12). Hence, deep-diving cetaceans have evolved specialized energy- and oxygen-conserving locomotion strategies, modulating speed, fluking patterns, dive duration, and depth as a function of target prey (11). Risso's dolphins foraged in the top 600 m and relatively close to shore, targeting layers and patches that can show dynamic patterns in time and space, such as the DSL (6, 21). Although their foraging zone also held suitable prey species for Cuvier's beaked whale (50% of recorded taxa), they did not capitalize on the dense DSL resources. Instead, Cuvier's beaked whale targeted prey at greater depths, between 800 m deep in the pelagic and the bathyal seafloor. Dives either targeted relatively narrow, pelagic prey layers (layer-restricted foraging) or covered a wide foraging zone, across the pelagic, benthic boundary layer (up to 200 m from the seafloor) and bottom habitat, with individuals foraging directly at the seafloor. The extreme diving strategy of Cuvier's beaked whale may help avoid competition with other mammalian predators. The highly social Risso's dolphin can benefit from long-term stable associations and large numbers for competition and social defense (28, 29). In contrast, Cuvier's beaked whale is a cryptic flight strategist that occurs in small, likely ephemeral groups with limited capability for interspecific competition or defense (30). Whereas the shallower foraging zone of Risso's dolphin is also accessible to many potential cetacean competitors and other marine predators, only few large predators are capable of targeting larger prey at beaked whale foraging depths (30). Extreme breath-hold dives, however, require a high calorific intake to render deep foraging effort energetically rewarding (12).

Deep-sea predator-prey dynamics

Energetic return from prey can be modulated from the number, volume, catchability, and calorific content of individuals targeted. In our study, Cuvier's beaked whale, on average, targeted 7 to 21 fewer prey per hour of foraging effort than Risso's dolphin. The combination of a larger body size and higher energetic requirements through more extreme diving predicts that Cuvier's beaked whale targets larger or more calorific prey (31). Comparative data on calorific content of deep-sea cephalopods of different size and maturity are limited. However, five of seven shared prey families plus another four families in the diet of Cuvier's beaked whale are known to migrate ontogenetically,

including important prey such as *H. reversa* and cranchiid squids (Fig. 6) (32, 33). Many squids spend the paralarval and juvenile phase in surface waters to profit from increased primary productivity (14, 34). As part of ontogenetic migration, larger and more mature individuals descend to deeper layers for reproduction and better protection against predators (35). For example, mature females of *H. reversa* have not been captured above 800 m (36), indicating that sexual maturation of this species takes place beyond Risso's dolphin but inside Cuvier's beaked whale hunting zone. Segregation of foraging niches by targeting different ontogenetic stages of deep-sea cephalopods would allow Risso's dolphin and Cuvier's beaked whale to feed on different life stages and sizes of the same abundant species in the region, while reducing interspecific competition (Fig. 6). Cephalopods have only one reproductive episode before death (semelparity), leading to a relatively high gonadal investment (14, 16) and mature individuals of enhanced energetic value and volume (i.e., carrying ripe eggs). Moreover, mating and brooding squids can be compromised in their escape responses (35, 37). Whereas Risso's dolphin foraging was predominantly pelagic, Cuvier's beaked whale also targeted benthic habitat, suggesting beneficial and possibly enhanced foraging opportunity at and near the bathyal seafloor. Hence, given extensive overlap in diet (and prey spectra in the two predator's foraging zones), this indicates that enhanced energetic demands from extreme deep dives may be balanced by a prey community offering high-calorific prey, that may be easier to catch.

eDNA elucidates deep-sea cephalopod community

eDNA proved to be an efficient and potent technique to establish diversity and distribution of cephalopods in the deep sea, in particular using a vertically stratified approach. We detected 21 of the 83 cephalopod species that have been reported in waters around the Azores to date, plus an additional two new species for the region (*Chiroteuthis mega* and *Cycloteuthis sirventi*) (38), as well as giant squid (*Architeuthis*; second eDNA detection worldwide (39)). Moreover, cephalopod distribution patterns were biologically meaningful. Risso's dolphin prey veined squid (*Loligo forbesii*), for example, was only detected over the island slope at relatively shallow depths (100 to 500 m), matching its known habitat, as well as depth of catches from local fisheries (33, 40). As a confirmed prey species of the Azorean population of Risso's dolphins (41), its absence offshore may help explain the species' preference for mesopelagic foraging over slope versus bathyal waters. Strictly deep-sea species such as *Planctoteuthis levimana* and *Chtenopteryx* sp. were only recorded at large depth

(1600 m) (33). These data show that cephalopod eDNA is not a homogeneous mixture as a result of currents, upwelling, or biological vectors such as whale defecation (42).

Trophic coupling in changing oceans

The predator-prey systems revealed here play a key ecological role in deep-sea food webs. Yet, they have thus far remained largely undocumented because of the challenging deep-sea environment and the elusive nature of both foraging whales and cephalopods. As top predators, cetaceans capture many and large cephalopods and shape the population size and structure of their prey (8). The coexisting predators have differentiated into entirely discrete foraging niches. Their foraging zones, however, are linked by common occurrence of shared cephalopod prey species, creating interdependent food web dynamics between the two systems through the processes of carbon and nutrient transport and emergent facilitation (43). The cephalopods in our study area likely perform considerable migration between depth zones and transport biomass into deeper waters through ontogenetic migration. In combination with the deposition of carcasses after terminal reproduction (44), this implies considerable fluxes of nutrients between depth zones driven by cephalopod movements (45) and predator consumption and defecation (7, 42). Combined data on deep-sea prey spectra available in cetacean target foraging zones also represent critical knowledge aiding in the understanding of marine top predator foraging performance and how this may change under disturbance settings. Absence of knowledge on prey communities has been identified as a limiting factor in the understanding of population-level effects of predator behavioral responses causing impeded foraging, particularly for beaked whales, which are highly sensitive to disturbance from anthropogenic noise (46, 47).

Unravelling the specifics and magnitude of predation coupled with prey distributions and population composition is pivotal for an integrative understanding of the food webs and carbon budgets of deep-sea waters, which cannot directly benefit from nutrient input through primary production. The combination of methodologies pioneered here can be transferred to other predator-prey systems, thus creating major opportunity for the advancement of our knowledge of open-ocean and deep-sea food web processes.

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Author contributions

F.V. and H.J.T.H. conceived the study. F.V., H.J.T.H., and V.J.M. designed the study. F.V., H.J.T.H., V.J.M., D.S.W.d.J., T.B., and M.G.O. conducted the investigation process, performed formal analysis and processing, and drafted the manuscript. T.B., T.B.H.R., and O.P. provided critical support during eDNA analysis and data collection from samples. J.F. performed the sequencing. All authors critically revised the manuscript. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials or are available from the PANGAEA repository at https://doi.pangaea.de/10.1594/PANGAEA.926840. Additional data related to this paper may be requested from the authors.

1.5. References Chapter 1

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Chapter 2

An integrative assessment combining deep-sea net sampling, *in situ* observations and environmental DNA analysis identifies Cabo Verde as a cephalopod biodiversity hotspot in the Atlantic Ocean

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Abstract

The deep sea is among the largest, most biologically diverse, yet least-explored ecosystems on Earth. Baseline information on deep-sea biodiversity is crucial for understanding ecosystem functioning and for detecting community changes. Here, we established a baseline of cephalopod community composition and distribution off Cabo Verde, an archipelago in the eastern tropical Atlantic. This baseline served to test the hypothesis that Cabo Verde is biogeographically separated from other Macaronesian archipelagos and allowed the identification of cephalopod species which may play a role in the Macaronesian carbon cycle and oceanic food web. To investigate cephalopod community composition, this study used 746 individual cephalopods obtained by nets (0 - 1000 m) and 52 cephalopod encounters during video surveys with either towed camera (0 - 2500 m) or manned submersible (0 - 375 m). Additionally, environmental DNA (eDNA) metabarcoding on 105 seawater samples (50 - 2500 m), using an 18S rRNA universal cephalopod primer pair, and a species-specific primer pair for *Taningia danae* resulted in the detection of 32 cephalopod taxa. When combined, the three methods detected a total of 87 taxa, including 47 distinct species. Each method contributed between 7 and 54 % of taxa that were not detected by the other methods, indicating that multiple methodological approaches are needed for optimal deep-sea cephalopod biodiversity assessments. This study documents the occurrences of six species and three genera for the first time in waters surrounding Cabo Verde. Video surveys and eDNA analysis detected *Taningia danae* recurrently (100 - 2500 m). Environmental DNA metabarcoding proved to be a powerful tool for cephalopod biodiversity monitoring and complementary to traditional sampling methods. When also including literature records, Cabo Verde hosts at least 102 cephalopod taxa including 30 families and 64 benthic and pelagic species. The total number and species composition of Cabo Verde cephalopods is similar to the Canary Islands and Azores, two known cephalopod biodiversity hotspots, but the Cabo Verde octopus fauna seems to differ. Due to a range of life history characteristics, we hypothesize that the squids *Taningia danae* (Octopoteuthidae) and *Sthenoteuthis pteropus* (Ommastrephidae) are important in the carbon cycle of Macaronesia. As a cephalopod biodiversity hotspot Cabo Verde could function as a model region to investigate cephalopod biology and ecology in a rapidly changing Atlantic Ocean.

2.1. Introduction

The deep sea (ocean depths below 200 m) is home to one of the most diverse communities on earth with the highest faunal biomass and greatest number of individual organisms (Ramirez-Llodra et al., 2010; Robison, 2004). The pelagic zone of the deep sea comprises 73% of the deep-sea water volume, yet despite this vast size, remains the least explored habitat with less than 0.0001% of its volume having been sampled (Herring, 2002; Ramirez-Llodra et al., 2010). Climate change and anthropogenic impacts are influencing the deep sea on a global scale (Breitburg et al., 2018; Keeling et al., 2010; Levin and Le Bris, 2015; Schmidtko et al., 2017) and altered environmental gradients in temperature and oxygen may cause changes in deep-sea species diversity and distribution (Robison, 2009). To preserve essential deep-sea ecosystem services both within and beyond exclusive economic zones, and to accurately evaluate the impacts of ocean changes, baseline information on deep-sea ecosystem functioning and biodiversity is needed (Robison, 2009; Thurber et al., 2014). This is particularly true for deep-sea cephalopods, abundant marine invertebrates that are known to respond rapidly to environmental change and play a pivotal role in the food web.

Cephalopods are marine molluscs that connect lower and higher trophic levels as both predators and prey (M.R Clarke, 1996; Piatkowski et al., 2001). They occur from coastal areas to the deep sea and are typically predators, feeding on fish, crustaceans, other cephalopods and some species, also on gelatinous zooplankton (Hoving et al., 2014; Hoving and Haddock, 2017; Merten et al., 2017; Villanueva et al., 2017). A range of predators e.g., cetaceans, tuna, swordfish and sharks, hunt for cephalopods as their primary prey (Clarke, 1996a,b,c; Smale and Clarke, 1996). Most cephalopod species are semelparous, that is, after a single reproductive event, the individuals die and, depending on the species, either sink to the seafloor or float to the surface (Boyle and Rodhouse, 2005; Hoving et al., 2015; Nesis et al., 1998). Hence, post-spawning mortality events may result in localized pulses of carcasses, creating organically enriched patches contributing to regional carbon fluxes and providing scavengers with food (Hoving et al., 2017; Martin and Christiansen, 1997; Stockton and Delaca, 1982). The biology, ecology and physiology of only 8% (~60 species) of the 800 extant cephalopod species known today have been investigated in detail (Jereb and Roper, 2010), with these studies being biased towards commercially important or coastal species. Much less is known about

the 45% of species comprising non-commercially-exploited, open-ocean or deep-sea squids and octopuses (Sweeney and Roper, 1998).

In the Atlantic Ocean, 43 cephalopod families are known with 34 of these occurring exclusively in the open ocean (Rosa et al., 2008). The highest cephalopod species diversity in the northern hemisphere is found in the western North Atlantic (74 species). Lowest cephalopod diversities are found in polar regions (14 species in the Arctic Ocean, 22 species in the Antarctic Ocean) (Rosa et al., 2008). Known Atlantic hotspots near oceanic islands are the Canary Islands (83 cephalopod species, Escánez et al., 2020) and the Azores (83 cephalopod species, Pereira et al., 2016; Visser, Merten et al., 2021). Another potential hotspot of cephalopod biodiversity is the Cabo Verde archipelago in the eastern tropical Atlantic, but a comprehensive overview of cephalopod diversity is lacking for this region. The topography of Cabo Verde induces local upwellings and high biological productivity (Doty and Oguri, 1956; Gove et al., 2016). The islands accommodate a unusually high number of endemic species and an eclectic mix of temperate, subtropical and tropical taxa (Duda and Rolán, 2005; Freitas et al., 2019; Sampaio et al., 2018). Due to the islands' steep slopes, deep-sea ecosystems occur within the exclusive economic zone of the archipelago of Cabo Verde. Historically, the archipelago has been included with the Azores, Madeira, Selvagens and the Canary Islands in the Macaronesian biogeographic region (Ávila, 2005; Borges et al., 2010; Cordeiro and Ávila, 2015; Freitas et al., 2019; Wirtz et al., 2013). However, the coastal and benthic composition of marine biota of Cabo Verde differ significantly from other Macaronesian archipelagos (Freitas et al., 2019), with the Cabo Verde front potentially functioning as a biogeographical barrier preventing the dispersal of marine organisms from and to other island groups in the North (Freitas et al., 2019). The North-West African Upwelling may form another biogeographical barrier between Cabo Verde and the African mainland limiting marine species dispersal by upwelling cold water to the surface (Freitas et al., 2019; Terashima et al., 2007; Türkay, 1982; Wirtz, 2012). To test the hypothesis that Cabo Verdes' cephalopod fauna is distinct from other Macaronesian archipelagos, this study provides an overview of Cabo Verde cephalopod community composition combining novel data from seven expeditions, complemented by literature records. The paucity of knowledge about deep-sea cephalopod biodiversity and distribution is in part due to the difficulties associated with sampling these organisms. Net sampling surveys are biased by gear characteristics such as mesh size or net mouth opening as well as deployment parameters (e.g., towing speed and depth) (Heino et al., 2011). Video surveys and in situ observations with remotely operated vehicles (ROVs) and towed camera systems

have successfully documented certain cephalopod species throughout the water column and can also document a range of behaviors and ecological interactions (Bush et al., 2009; Hoving et al., 2017, 2019a; Robison et al., 2017; Vecchione, 2019). However, the light associated with underwater video surveys and the noise of hydraulic systems on ROVs may result in selective and species-specific documentation. As a general rule, net sampling and ROV surveys are expected to result in avoidance behavior and sampling bias in favor of species with reduced swimming capacity and less sensitive sensory capabilities (Clarke, 1977; Wormuth and Roper, 1983).

A cost-effective, non-invasive tool to study community compositions is the metabarcoding of environmental DNA i.e., the genetic material that organisms leave in the water. Environmental DNA analysis is a rapidly advancing field and becoming widely used in: biomonitoring and conservation, establishing local biodiversity patterns, temporal dynamics in migration and detection of range expansions, as well as documenting rare and invasive species occurrences (Cowart et al., 2018; Everett and Park, 2018; Jeunen et al., 2019; Laroche et al., 2020; Sigsgaard et al., 2017b; Taberlet et al., 2012; Yamamoto et al., 2017). For cephalopods, eDNA analysis has thus far been used in two field studies. The first study designed a species-specific eDNA primer and was able to detect the giant squid *Architeuthis dux* in Japanese waters (Wada et al., 2020). The second study used universal cephalopod primers to reconstruct cephalopod community composition in the foraging zones of deep hunting predators off the Azores, detecting 39 cephalopod taxa from 17 families from depths between 50 and 1600 m (Visser, Merten et al., 2021). The latter study showed that not all taxa can be detected with the general 18S primer being used. Therefore, a species-specific primer for specific elusive squid should additionally be developed. Here, we developed such a primer for *Taningia danae* (Octopoteuthidae), a large species that was encountered frequently during video surveys around Cabo Verde, but which was not detected with nets or the same universal 18S cephalopod primer used here. Using a combination of optical, physical and genetic sampling as well as data from the literature, the aim of this study was to set a baseline for cephalopod community composition and vertical distribution off Cabo Verde. The community baseline could then be used to compare cephalopod community compositions from Cabo Verde with those of the Canary Islands and Azores to test the hypothesis that Cabo Verde's cephalopod communities are distinct from Macaronesia. Additionally, such a diversity assessment will allow more accurate predictions to be made as to which cephalopod species may contribute substantially to the regional carbon cycle.

2.2. Material and Methods

Cephalopod surveys

Sampling

Net catches of cephalopods were obtained during the cruises POS320/2 (March 2005), MSM49 (November/December 2015) and WH383 (March/April 2015) off Cabo Verde at a total of 18 stations at depths between 0 and 1000 m (Figure 1). Cephalopods were caught during POS320/2 with either an Isaacs-Kidd midwater trawl (IKMT) with a 6 m^2 net opening, 4 mm mesh size equipped with a flowmeter, a Hydro-Bios Multinet Maxi with a 0.5 m^2 net opening and 500 µm mesh size between the surface and 250 m water depth, or an 80 feet bottom trawl (Kraus, 2005). Net sampling during MSM49 was conducted with two types of multiple opening/closing nets (MOCNESS) and an IKMT. The smaller MOCNESS had a net opening of 1 m^2 (three nets with a mesh size of 2 mm and six nets with a mesh size of 335 µm) and the larger MOCNESS a net opening of 10 m^2 (five nets, mesh size: 1.5 mm) and were deployed between the surface to 1000 m. The IKMT had a net opening of 7 m^2 and ended in a cod end of 500 µm mesh size. It was deployed to a maximum depth of 500 m (Christiansen et al., 2016). During WH383 a pelagic trawl ('Aalnetz', Engel Netze, Bremerhaven, Germany) with a mouth opening of 16 x 30 m, length of 150 m including a multiple net opening-closing device, 260 meshes by 180 cm stretched mesh size at the front, a cod end 20 mm stretched mesh-opening and a 1.8 mm inlet sewn into last 1 m of cod end (Harrison,1967) was used with a multisampler (Construction Services AS, Bergen, Norway; (Engås et al., 1997)) allowing depth-stratified sampling (Fock, 2015; Fock and Czudaj, 2019). During WH383, three strata (mean vertical extension of ca. 40 m) were trawled mostly during night, though once during daytime, at depths between 30 and 700 m in horizontal tows for 30 minutes per stratum with a mean speed of three knots (2.8-3.3 kn). During this cruise, night trawls took place at 10:00 pm local time, and the day-time trawl at 12:00 pm local time. Onboard, cephalopods were identified morphologically to the lowest taxonomic level possible (species, genus or family), with whole specimens preserved in formalin as voucher. In addition, tissue samples from some specimens were collected and preserved in ethanol for barcoding and the genetic reference database used for eDNA metabarcoding.

In situ observations

Pelagic video transects with the Pelagic In-Situ Observation System (PELAGIOS, (Hoving et al., 2019a)) were conducted during the cruises MSM49 (Christiansen et al., 2016) (transects between 30 and 1000 m, total hours of observation > 80h), MSM61 (Fiedler et al., 2020) (transects between 80 and 1200 m, total hours of observations > 32h), POS520 (Hoving et al., 2018, p. 520) (transects between 30 and 2500 m, total hours of observations 27h), POS532 (Hoving et al., 2019b) (transects between

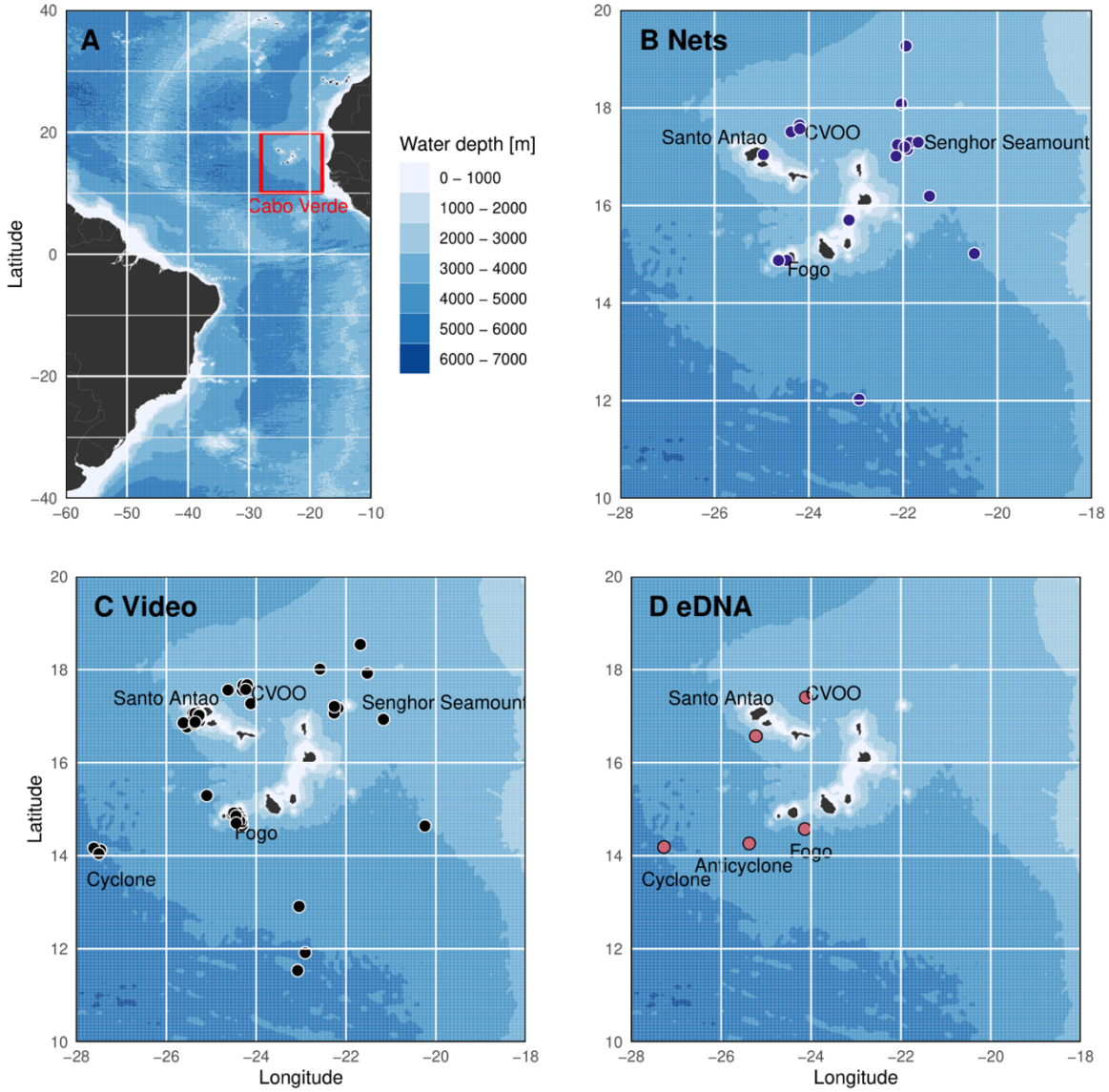

Figure 1| Sampling sites in the exclusive economic zone of Cabo Verde in the eastern tropical Atlantic. (**A**) Cabo Verde in the eastern tropical Atlantic. (**B**) Blue rectangles show the 18 net stations collecting cephalopods during the cruises POS320/2, MSM49 and WH383 between 2015 and 2018. (**C**) Black triangles visualize the 40 video deployments conducted during the cruises MSM49, MSM61, POS520, POS532 and M119 between 2015 and 2019. (**D**) Red circles depict the five eDNA sampling stations collected during POS532 in 2019.

30 and 990 m, total hours of observations 19h) and M119 (Brandt, 2016) (transects between 50 and 700 m, total hours of observations > 20h) between 2015 and 2019 (Figure 1). PELAGIOS is a battery powered, high-definition camera system that is towed horizontally via a single-wired conductive sea-cable at 0.5 m s^{-1}. Approximately 0.45 m^3 of the water column in front of the camera is illuminated with an LED array. The attached depth sensor and/or a sensor for conductivity, temperature and depth (CTD) with oxygen sensor allows for hydrographic measurements and depth monitoring during transects. Pelagic video transects lasted between 11-33 minutes per depth, towing the camera horizontally at specified depths. A deep-sea telemetry system allows for transmission of a low-resolution preview of the recorded video. Part of the data of the PELAGIOS transects collected during MSM49 are presented in (Hoving et al., 2019a) with a focus on the methodology and using video from one station (Senghor NW) as an example for data analysis. The PELAGIOS cephalopod data from MSM49 was also analyzed with focus on gelatinous fauna in (Hoving et al., 2020), but with no detailed taxonomic information on cephalopods presented, which is provided here. During the cruises POS520 and POS532 the manned submersible JAGO (GEOMAR, Helmholtz Centre for Ocean Research Kiel, (Hissmann and Schauer, 2017)) was used for 30 deployments, each of approximately four hours duration, between the surface and 375 m water depth. During the dives, video was recorded by a high-resolution camera (see details in (Stenvers et al., 2021)). The videos taken during the PELAGIOS and JAGO dives were annotated manually using the Video Annotation and Reference System (VARS) developed at the Monterey Bay Aquarium Research Institute (Schlining and Jacobsen, 2006), which allows annotation with the congruent collection of video frames.

eDNA collection

A total of 105 environmental DNA samples were taken during POS532 in February 2019 from five stations (Hoving et al., 2019b) (Figure 1). The stations off the islands Santo Antão and Fogo were close to the coast (maximum sampled depth 2500 m), CVOO (Cabo Verde Ocean Observatory) was an open ocean station (maximum sampled depth 2200 m) and the stations Cyclone and Anticyclone were located within eddies that had formed in the wake of Fogo and had propagated southwards (maximum sampled depths 2200 and 600 m, respectively) (Supplementary Figure 1). All stations described in this study were located inside the exclusive economic zone of Cabo Verde. Per sampled depth, three biological replicates, each of two liters of seawater were collected from three different 10-liter Niskin bottles mounted on a CTD rosette. For filtration, 0.22 µm pore size Sterivex-GP filter (Merck Millipore) were directly connected to the Niskin bottle with sterile tubing and two liters of

seawater filtered per filter. Each of the replicates was filtered separately and included individually in the following analysis. The filters were closed with sterile plastic caps and stored at -80°C until further processing in the laboratory.

DNA extraction

The DNA extraction protocol followed Visser, Merten et al. (2021). Briefly, DNA was extracted from the filters using the DNeasy Blood and Tissue Kit (Qiagen) with modified volumes. The DNA extractions were processed in sterile conditions under a clean bench to minimize contamination. After cleaning the outside of every filter with bleach, 720 µl of ATL-buffer and 80 µl of Proteinase K were added directly into the filter and incubated at 56°C for 2 hours with agitation. After incubation, 600 µl of the buffer mix from the filter was transferred to a 2.0 ml Eppendorf tube and mixed with 600 µl AL-buffer and 600 µl 99% high grade ethanol. After this step, the normal DNeasy Blood and Tissue protocol was followed. DNA was eluted in 2x30 µl AE-buffer from the kit.

Rigorous laboratory measures were followed to reduce contamination. Only single-use consumables and single-capped PCR-tubes were used. DNA extractions, pre- and post-PCR were physically separated and sampling devices as well as laboratory equipment and surfaces cleaned with 50% bleach or RNAse AWAY (Carl Roth) containing bleach. To control for possible contaminations, negative controls were included at the filtration (MilliQ instead of seawater), DNA extraction (a blank filter was extracted and treated the same way as eDNA filter samples) and PCR (PCR-grade water instead of DNA sample) stages. As indicated below, positive controls were also included at the PCR stage.

Amplification and sequencing of cephalopod eDNA

A universal cephalopod primer targeting the nuclear 18S rRNA gene (Ceph18S_forward: CGCGGCGCTACATATTAGAC, Ceph18S_reverse: GCACTTAACCGACCGTCGAC, amplicon length = 140 – 190 bp (de Jonge et al., 2021)) was used. The mitochondrial 16S rRNA locus (Jarman et al., 2006) was amplified as well following Visser, Merten et al. (2021). However, the positive controls on this sequencing run failed, so this data was excluded from further analysis.

For PCR amplification of the 18S rRNA gene, a 1-Step PCR protocol was used with the Illumina linker, Illumina index, Illumina adapter and a spacer for greater variability directly added to the primer

sequence resulting in primer lengths of between 86 and 97 bp. All samples were amplified in duplicate resulting in six PCR products per sampling depth and site (three biological replicates x two PCR replicates) with individual Illumina tags for every replicate. Three positive controls (tissue derived DNA from *Filippovia knippovitchi* and *Rossia palpebrosa*, two cephalopod species that do not occur in the eastern Atlantic and one mock community with ~10 ng/µl of each positive control species) and one negative control (PCR-grade water instead of DNA extract in the PCR reaction) were run in duplicate with individual taqs, and included on every PCR run.

The PCR reaction had a total volume of 25 µl and included 10 µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 9 µl PCR-grade H_2O, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM) and 5 µl DNA extract. A touchdown PCR program was applied starting with an initial denaturation step at 95°C for 5 min, 8 cycles of 94°C for 30 sec, 70°C for 30 sec (decreasing this temperature by 1°C after every cycle) and 72°C for 1 min. Subsequently, 32 additional cycles were run with 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min. The program was terminated by a final extension step of 72°C for 5 min. DNA fragment sizes were verified on a 1.5% agarose gel stained with GelRed (Biotum). The PCR products were measured using a Qubit fluorometer and then pooled in equimolar concentrations, resulting in one library. The fragment size of the library was validated on a 2% agarose gel stained with GelRed (Biotum). Subsequently, the correct band was cut out (band size between 300 – 380 bp) and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research) following the manufacturer's protocol. The library pool was quantified with the Qubit dsDNA HS Assay Kit (Molecular Probes Life Technologies). The insert size distribution was determined with the Tapestation4200: D5000 ScreenTape (Agilent). The working solution was diluted to 2 nM and the loading solution was prepared according to the Agilent protocol. The library pools were loaded with 8 pM. To increase diversity, 20 % PhiX was spiked in the library. Sequencing was done on an Illumina MiSeq with the MiSeq Reagent Kit v3, 600 cycles (PE), 2x 300bp (Illumina) at the Institute of Clinical Molecular Biology (IKMB), Kiel, Germany.

Reference database

As a reference for the obtained eDNA data, the 18S database described in Visser, Merten et al. (2021) was used. Briefly, cephalopod sequences from the SILVA 18S database were used to recursively search the NCBI Genbank database (accessed in June 2020) using a BLAST-based script from the EukRef project (https://unieuk.org/) until no further cephalopod sequences were found. This resulted

in 169 sequences from 119 species. These cephalopod sequences were combined with all other eukaryotic 18S sequences from the SILVA database to prevent spurious assignments of non-cephalopod amplicons. Additionally, 18S rRNA sequences from 16 tissue extracts of voucher specimens collected during two research cruises (MSM49, WH383) in the eastern tropical Atlantic and North Atlantic were sequenced and added to this database (GenBank accession numbers OK663489: OK663503). DNA was extracted from tissue samples using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturers protocol. DNA concentrations were measured with the NanoDrop Spectrophotometer (Thermo Fisher) and amplified with the same primer used for eDNA metabarcoding, Ceph18S. For PCR amplification, we used the same parameters as for the touchdown PCR program outlined above. All PCR products were Sanger sequenced on a 3730xl DNA analyzer (Applied Biosystems) at the Institute of Clinical Molecular Biology (IKMB) in Kiel, Germany. Forward and reverse strand sequencing of the PCR products was performed using the Sanger Sequencing Kit (Applied Biosystems). Primers and low-quality ends were trimmed from the sequences, checked manually, edited, and assembled using CodonCode Aligner (version 3.7.1). Information on the construction of the reference database can be found in Visser, Merten et al. (2021) and resulted in a total of 34,811 sequences of which 169 originate from cephalopods representing 107 species for the 18S rRNA gene.

Bioinformatic analysis

The raw sequences from the eDNA metabarcoding were demultiplexed by the sequencing center (IKMB) and forward and reverse PCR primer sequences were removed with *cutadapt* version 1.18 (Martin, 2011). The subsequent bioinformatic analysis was conducted with the R package DADA2 version 1.15.0 (Callahan et al., 2016, p. 2) in RStudio version 1.1.463 (R Core Team, 2018; RStudio Team, 2020). Paired reads were merged and trimmed at a quality score ≤ 2. The maximum expected error rate that was accepted was set to three for forward reads and five for reverse reads. Expected errors (EE) are calculated from EE = sum(10^(-Q/10)) with Q being the quality score. Potential chimeras were removed and an amplicon sequence variant table created. Taxonomic assignment of unique reads was performed using IDTAXA with the R package DECIPHER version 2.6.0 (Murali et al., 2018) using the databases described above as training set. Taxonomic assignments occurring in the negative controls and blanks were removed from the corresponding plates or batch of samples, respectively. After taxonomic assignments by IDTAXA, sequences that were assigned to only genus

or family level were rechecked with applying the naïve Bayesian classifier method used by BLASTn in GenBank (Altschul et al., 1990). A hit by BLASTn was accepted to species level with a percent identity of at least 99% and no closer hit than 90% to another species.

Species-specific *Taningia danae* primer design

The universal Ceph18S primer targeting the 18S rRNA gene used in this study could not discriminate reliably to species level within the family Octopoteuthidae (de Jonge et al., 2021). To obtain this information, a novel species-specific primer targeting the cytochrome oxidase I (COI) region of the mitochondrial gene of *Taningia danae* and amplifying a 250 bp segment was designed using Primer3 (Untergasser et al., 2012) (Taningia_COI_forward: TCATGCAGGTCCCTCTGTTG, Taningia_COI_reverse: AGGTGTTGGTATAAGATGGGGT, amplicon length = ~250 bp). Sequences for designing the primer were obtained from the GenBank database (NCBI, Accession: AY393902.1, Accession: MG591434.1, Accession: EU735402.1). The specificity of the primer was checked by carrying out an in-silico PCR with ecoPCR by OBITools (Ficetola et al., 2010). The in-silico results showed amplification of *T. danae,* but also 15 other species, mostly belonging to Gonatidae, that do not occur in Cabo Verdean waters. Subsequently, the specificity of the primer was tested on DNA samples derived from tissue from seven cephalopod species (*Bathypolypus* sp., *Megaleledone* sp., *Gonatus* sp., *Rossia* sp., *Brachioteuthis* sp., *Pareledone felix*) and *T. danae* and sequenced using the Sanger Sequencing Kit (Applied Biosystems). The DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's protocol and diluted in 100 µl TE-Buffer.

Analysis of eDNA samples with *Taningia danae* primer

A subset of eDNA seawater samples from four stations (Santo Antão, Fogo, CVOO, Cyclone) were re-analyzed with the species-specific *T. danae* primer developed for this study, along with the above-mentioned universal cephalopod primer. All biological replicates were pooled and analyzed together with a positive (DNA extract of *T. danae* as a template) and a negative (PCR-grade water instead of DNA template) control, amplified via PCR in triplicate to increase the detection probability, which resulted in a total of 16 samples. Each PCR mixture contained 10 µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 7 µl PCR-grade water, 0.5 µl dsDNase (ArcticZymes Technologies), 0.5 µl DTT Inactivation Buffer (ArcticZymes Technologies), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM). The PCR-Mix was incubated for 20 min at 37°C to reduce contaminating DNA,

followed by 20 min at 60°C for DNAse deactivation. After incubation, 5 µl DNA template was added to the PCR-mix and run with the following program: Initial denaturation step for 10 minutes at 95°C, 30 cycles with 94°C for 30 seconds, an annealing temperature of 60°C for 30 seconds and 72°C for 1 minute, followed by a final elongation step of 72°C for 5 minutes. All PCR products were examined on a 1.5% Agarose gel stained with GelRed (Biotum). Only samples showing a band on the gel were sequenced using the Sanger Sequencing Kit (Applied Biosystems) at IKMT. Sequences were processed with CodonCode Aligner v. 3.7.1.2. The forward and reverse primer sequences were cut off and forward and reverse reads assembled. All sequences were run against the NCBI database (22/01/2021) using BLASTn (Altschul et al., 1990). Only hits with a percent identity > 96% and e-value below 1e-80 were accepted as positive detections for *Taningia danae* (Supplemental Table 1).

Statistical analysis

A species accumulation curve (SAC) for the number of sampled sites was calculated only for eDNA using *specaccum* from the R package *vegan* (Oksanen et al., 2019). The method *random* was used calculating the mean SAC and its standard deviation from 100 random permutations of the data (Gotelli and Colwell, 2001). The Jaccard index, a measure of community dissimilarity, was calculated with the function *vegdist* (Oksanen et al., 2019) to measure similarity between the Canary Islands, Cabo Verde and Azores for all cephalopod taxa and for taxa belonging to the order Octopoda based on presence/absence data.

Biodiversity analysis

To compare cephalopod taxa occurring in the waters around Cabo Verde, the Canary Islands and the Azores, an extensive literature research was conducted. For the Cabo Verde islands, the species list generated in this study incorporated observations from three studies describing cephalopods in Cabo Verdean waters (Clarke, 2006; Collins et al., 2001; Voss et al., 1998) and our data. For the Azores, the species list from Visser, Merten et al. (2021) was used as it included all currently available data from both the literature and eDNA. For the Canary Islands, a recent review on cephalopod diversity off the Canary Islands was used, which included species from 48 published documents (Escánez et al., 2020) following the authors' exclusion of species that were only hypothesized to occur off the Canary Islands due to their wide geographical distribution, but have not as yet been positively detected there.

2.3. Results

Community composition and distribution of cephalopods off Cabo Verde

Net catches

Trawling in the Cabo Verde exclusive economic zone (EEZ) during three research cruises in 2005 (POS320) and 2015 (MSM49 and WH383) resulted in the collection of 746 specimens belonging to 69 taxa (Supplementary Table 1, observations that could only be identified to the level Cephalopoda (*n*=1) or Teuthida (*n*=9) were excluded from further analysis due to low taxonomic resolution, all included taxa were identified to either species, genus or family level). Of the 69 taxa, 41 could be identified to species level, with these belonging to 20 families. The most frequently captured taxon (in terms of number of individuals collected) was *Liocranchia reinhardtii* (Cranchiidae, 25%, *n*=184, Supplementary Table 2), which occurred over the whole sampled depth range from the surface to 1000 m (Figure 2). The next most frequently captured species' were *Pterygioteuthis gemmata* (Pyroteuthidae, 12%, *n*=93) occurring between 0 and 1000 m and *Abraliopsis atlantica* (Enoploteuthidae, 9%, *n*=65), which was captured from 0 to 1000 m (Figure 2, Supplementary Table 2). All other taxa represented less than 6% (*n*<41) in terms of frequency. Twenty-two taxa were only observed once, e.g., *Ornithoteuthis antillarum*, *Ommastrephes bartramii* and *Taonius pavo.* The most frequently encountered family in the catches was the Cranchiidae with 43% (*n*=318), to which the most abundant species collected, *Liocranchia reinhardtii* belonged. Pyroteuthidae made up 17.2% (*n*=128) of the total captured cephalopods followed by Enoploteuthidae with 16.8% (*n*=125). All other families made up less than 4% (*n*<30). The Cranchiidae was also the family with the highest species diversity collected (16 taxa, 11 could be identified to species level) followed by Enoploteuthidae (eight taxa, five could be identified to species level). All other families were represented by fewer than seven taxa. Families represented by only one taxon were Argonautidae, Bathyteuthidae, Lepidoteuthidae, Lycoteuthidae, Pholidoteuthidae, Sepiidae, Sepiolidae and Vampyroteuthidae (Table 1). *Taningia danae* was not captured.

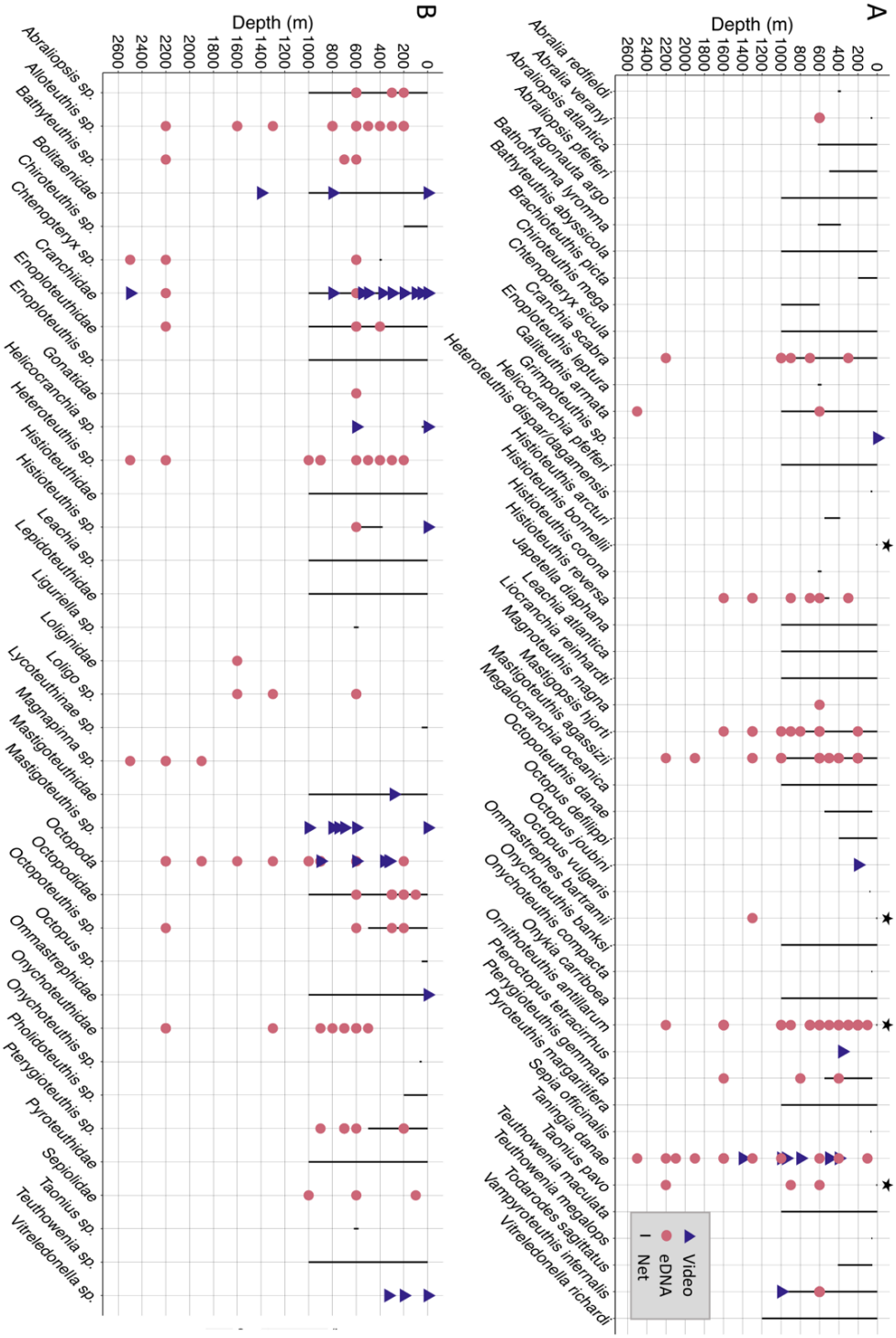

Figure 2| Depth distribution of taxa detected with eDNA, net catches and video surveys conducted off Cabo Verde in the eastern tropical Atlantic. Red dots depict eDNA detections, blue triangles video observations and black bars the depth distribution found in net catches with opening/closing nets. Black stars are cephalopods that fell out of the nets, so that the corresponding depth can not be assigned. Some taxa were observed in videos during down- or upcasts, in this case, the maximum depth of the down- or upcast was used. The taxa Cephalopoda and Teuthida were excluded from this graph. Many net tows were conducted with open nets from 1000 m to the surface, making it impossible to define at which specific depth the cephalopod individual was captured. Those tows are defined by the solid bars spanning from 0 to 1000 m. (A) Depth distribution of all species caught by nets, observed by video surveys and detected with eDNA metabarcoding off Cabo Verde. (B) Depth distribution of all genera, families and one order detected with nets, video surveys and eDNA metabarcoding off Cabo Verde.

Pelagic video surveys

During five research cruises between 2015 and 2019, 52 cephalopod individuals were observed (excluding observations that could only be identified to Teuthida, *n*=162 or Cephalopoda, *n* = 2, all taxa included were identified to species, genus or family level) with either PELAGIOS or JAGO (Supplementary Table 3). Ninety percent (*n*=47) of individuals were observed with PELAGIOS and 10% with JAGO (*n*=5). Most individuals could not be identified to species or genus level, because the video footage did not reveal sufficient taxonomic characteristics.

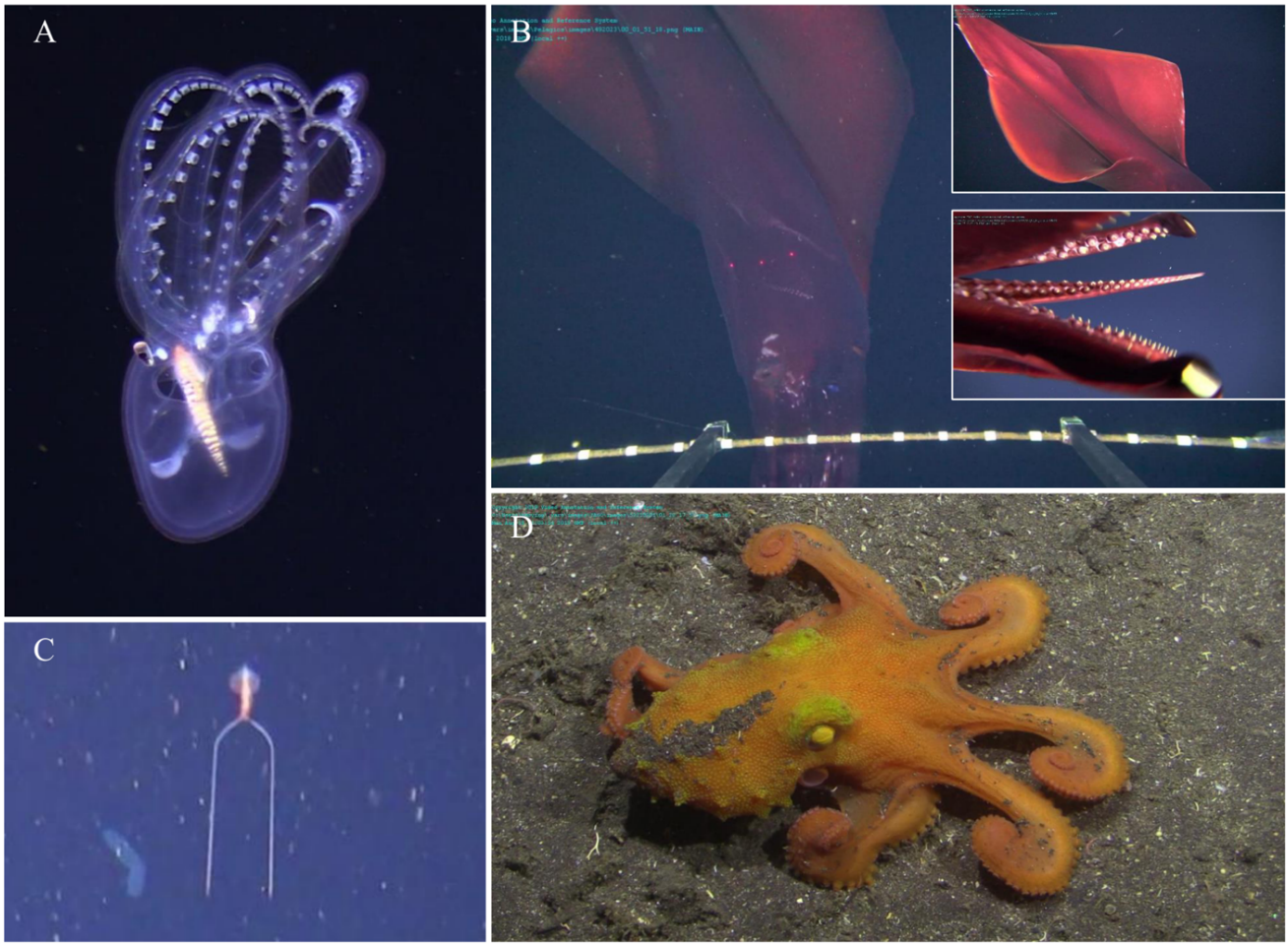

Figure 3| Pictures of cephalopods detected with JAGO and PELAGIOS off Cabo Verde in the eastern tropical Atlantic. (A) The glass octopus *Vitreledonella* sp. between 300 – 330 m photographed with the manned submersible JAGO during the cruise POS532. **(B)** The octopoteuthid *Taningia danae* approaching the towed underwater camera-system PELAGIOS at 600 m during the cruise MSM49. The small pictures are also *T. danae* during the cruise M119 (lower right) and POS520 (upper right) with unknown depth. **(C)** *Mastigoteuthis* sp. detected with PELAGIOS during the cruise POS520 at 285 m. **(D)** The Atlantic fourhorn octopus *Pteroctopus tetracirrhus* at 365 m detected with JAGO during POS532.

In total, 14 taxa were identified as belonging to one order (Octopoda), four families (Cranchiidae, Mastigoteuthidae, Ommastrephidae, Bolitaenidae), five genera (*Mastigoteuthis*, *Helicocranchia, Histioteuthis, Grimpoteuthis,* and *Vitreledonella*) and four species (*Taningia danae, Octopus joubini, Pteroctopus tetracirrhus* and *Vampyroteuthis infernalis*). Most cephalopods observed in situ belonged to the family Cranchiidae with 32% of individuals (*n*=17) and occurred between 50 and 2500 m (Figure 2). Bolitaenidae was the second most observed family, with 6% of individuals (*n*=3), occurring below 800 m to depths of up to 1400 m. The most frequently observed cephalopod which could be identified to species level was *T. danae* with seven observations (12%) at depths of between 400 and 1400 m (Figure 2). The observations of *T. danae* differed from the other cephalopod observations since *T. danae* seemed to be attracted to the lights of the towed camera system (Figure 3). Individuals came very close to the camera and distinct taxonomic features were visible including the large photophores on the arms (II), the purple body color and hooks on the arms. Individuals approached the camera system, some even charged it and then swam away. We observed some individuals "flashing" their arm photophores by opening and closing the covering tissue over the photophores. Octopoda (*n*=5) were detected between 323 and 900 m, including the benthic Atlantic pygmy octopus *Octopus joubini* at 200 m and *Pteroctopus tetracirrhus* at 365 m (Figure 2). Both these species were detected with JAGO.

Environmental DNA metabarcoding

A total of 14,130,249 sequences were obtained after sequencing with a mean (± standard deviation [SD]) of 53,523 (± 48,799) reads per sample. The number of reads in the negative controls ranged between 11 and 3,693 excluding the contamination of the positive controls, which amounted to 37% of the contamination in the negative controls. After the DADA2 analysis, 2,897,366 sequences remained that were classified into 908 unique amplicon sequence variants (ASVs) with a mean (±SD) of 196 (± 2233) reads per ASV (range 1 – 96,089 reads). A conservative approach was applied for data cleaning and all taxa present in the negative controls deleted from the corresponding samples. In detail, all taxa present in a negative control (filtration negative control, DNA extraction negative control and/or PCR negative control) were removed from the samples that were either processed in the same filtration and/or DNA extraction step or run on the same PCR plate as the negative control. After deleting rare ASVs with a frequency below 10 reads per sample and excluding contaminants from the negative controls as well as removing of the positive controls, 78 ASVs remained (Table 2). The remaining ASVs ranged between 11 and 4,035 reads with a mean (± SD) of 102 (± 294) reads.

The number of reads per sample ranged between 132 – 59,494 with a mean (± SD) of 15,069 (± 11,337).

Environmental DNA analysis of seawater samples from POS532 in 2019 resulted in the detection of 32 taxa (Figure 2). Thirty-eight percent of the detections could be identified to species level (*n*=12), 31% to genus (*n*=10), 22% to family (*n*=7), 6% to order (*n*=2) and 3% to class (*n*=1). ASVs that were only assigned to the taxa Cephalopoda or Teuthida and not further were excluded from the following analyses due to their low taxonomic resolution which resulted in the detection of 30 taxa in total with eDNA. All included taxa were assigned to species, genus or family level. Species or genera that were found with eDNA, but are not known to occur in the eastern tropical Atlantic Ocean were reduced to the next lower taxonomic level known from the Atlantic. This approach was applied to *Doryteuthis pealeii* (western Atlantic species), which was assigned to Loliginidae, *Heteroteuthis hawaiiensis* (Pacific species) which was assigned to *Heteroteuthis* sp. and *Pterygioteuthis microlampas* (Pacific species) which was assigned to *Pterygioteuthis* sp. *Pterygioteuthis microlampas* and *H. hawaiiensis* are morphologically very similar to their sister species *P. gemmata* and *H. dispar,* respectively, both of which occur in the Atlantic (Judkins et al., 2016; Lindgren, 2010). All four species were underrepresented in the database here used (just one reference sequence included) and were therefore potentially difficult to differentiate by the used marker gene.

Ornithoteuthis antillarum (Ommastrephidae) was the most frequently detected taxon (47% detections) with eDNA analysis, followed by Octopoda (34%, not including octopus taxa identified to genus or species level), *Alloteuthis* sp. (34%, Loliginidae), *Mastigoteuthis agassizii* (31%, Mastigoteuthidae) and *Heteroteuthis* sp. (31%, Heteroteuthidae) (Supplementary Figure 2). All other taxa were found in less than 30% of the samples. Considering the frequency of detections (detections/total samples analyzed * 100%) the most commonly detected families with eDNA were Mastigoteuthidae (14%), Ommastrephidae (11%) and Loliginidae (10%). The depth distribution of the eDNA data of all taxa detected was consistent with the known depth distribution patterns of the cephalopods that were detected (Figure 1).

Exceptions were the occurrence of shallow and midwater species (*Chtenopteryx* sp., Loliginidae, *Loligo* sp., *Alloteuthis* sp. and *O. antillarum)* in deep water (below 1000 m). Most of the taxa (69%, *n*=22) were detected throughout the water column from shallow to deep depths, whereas 25% of the detected taxa only occurred in the upper 1000 m, including *Abralia veranyi, Abraliopsis* sp., *Histioteuthis* sp., *Magnoteuthis magna*, Octopodidae, *Pterygioteuthis* sp., Sepiolidae and *Vampyroteuthis infernalis.* Six percent of the taxa were only detected below 1000 m (*Ommastrephes bartramii* at 1300 m and *Magnapinna* sp. between 1900 and 2500 m).

The number of taxa per surveyed depth ranged between one and 26. The highest number of taxa was detected at depths of 600 (26 taxa) and 2200 m (15 taxa, Supplementary Figure 3). Twenty-nine taxa were detected in the upper 1000 m and 23 taxa below 1000 m. When considering species only, the highest number of species (*n*=10 species) was found at 600 m, followed by 5 species at 900, 1000, 1300, 1600 and 2200 m (Supplementary Figure 3). However, these results should be interpreted with caution since the sampling effort at different depths was imbalanced, ranging from one to four sampled stations at each depth. On the other hand, the distribution of cephalopod eDNA off Santo Antão and Fogo, two of the stations with the most sampled depths, and with sampled depths between the two stations being identical, showed a similar trend in the upper 1000 m with the highest number of species at 600 m (17 taxa). Below 1000 m, highest numbers of species were detected at 1600 m with nine species (Supplementary Figure 4).

The *Taningia danae* primer successfully amplified *T. danae* tissue samples to species level (> 98% sequence match in Genbank). All matches ranged between 96 and 99% identity and the next species hit was below 90% identity (Supplementary Table 4). The six test cephalopod species (*Bathypolypus* sp., *Megaleledone* sp., *Gonatus* sp., *Rossia* sp*., Brachioteuthis* sp*., Pareledone felix)* were not amplified by the species-specific *T. danae* primer. *Taningia danae* eDNA was detected off Fogo, Santo Antão and the open ocean station CVOO between 100 and 2500 m depth (Supplementary Table 4). Only the negative control and two eDNA samples (Fogo at 400 m and Santo Antão at 200 m) yielded no amplification.

Cephalopod diversity off Cabo Verde: eDNA, nets, video and literature combined

Nets, video surveys and eDNA were able to detect 69, 14 and 30 cephalopod taxa off Cabo Verde, respectively. Three taxa (Cranchiidae, *Histioteuthis* sp. and *Vampyroteuthis infernalis*) were identified by all three methods, 15 taxa by eDNA and nets only, four taxa by nets and video only and one taxon by eDNA and video only (Figure 4A, excluding Cephalopoda and Teuthida). However, it is important to point out that the different methods were used in different areas, seasons and years, with the exception of data collected during specific cruises (e.g., PELAGIOS observations and MOCNESS net catches during MSM49; eDNA, PELAGIOS and JAGO during POS532). Therefore, a direct comparison between the three methods across the full data set described here is not possible. The video surveys were able to detect six taxa that were not detected by any other census used in this study, whereas eDNA was able to detect eleven additional taxa and nets 47 additional taxa. The taxonomic resolution was highest in net catches with 61% of detected taxa being identified to species level, 21% to genus and 15% to family. In comparison, eDNA was able to detect 38% to species, 31% to genus and 22 % to family level (Figure 4 B & C). The SAC for the eDNA approach showed a flattening of the curve and a decrease in standard deviation after sampling five stations, however, the curve did not reach the asymptote (Figure 5). Video surveys identified 27% to species, 40% to genus and 27% to family level, but from the video data fewer taxa were determined than the other censuses (Figure 4 B & C). Net catches seemed to be the most efficient sampling technique with 69 identified cephalopod taxa compared to 30 and 14 taxa for eDNA and video, respectively. However, the net data included three cruises, while the eDNA data is only originating from one cruise. When comparing the eDNA data to the three net sampling cruises individually (Figure 6), the predominance of nets over eDNA is no longer so obvious. The overlap in detected species between eDNA and nets for all cruises ranged between 9 to 20%, while eDNA contributed 20 – 43% of additional species

The three censuses combined detected three new genera and six new species records for Cabo Verde waters including *Teuthowenia megalops, Todarodes sagittatus, Onychoteuthis compacta, Mastigopsis hjorti, Magnoteuthis magna*, *Mastigoteuthis agassizii, Loligo* sp., *Alloteuthis* sp. and *Magnapinna* sp. These taxa are known from the Atlantic Ocean, but had not been recorded around Cabo Verde prior to the current study (Jereb and Roper, 2010).

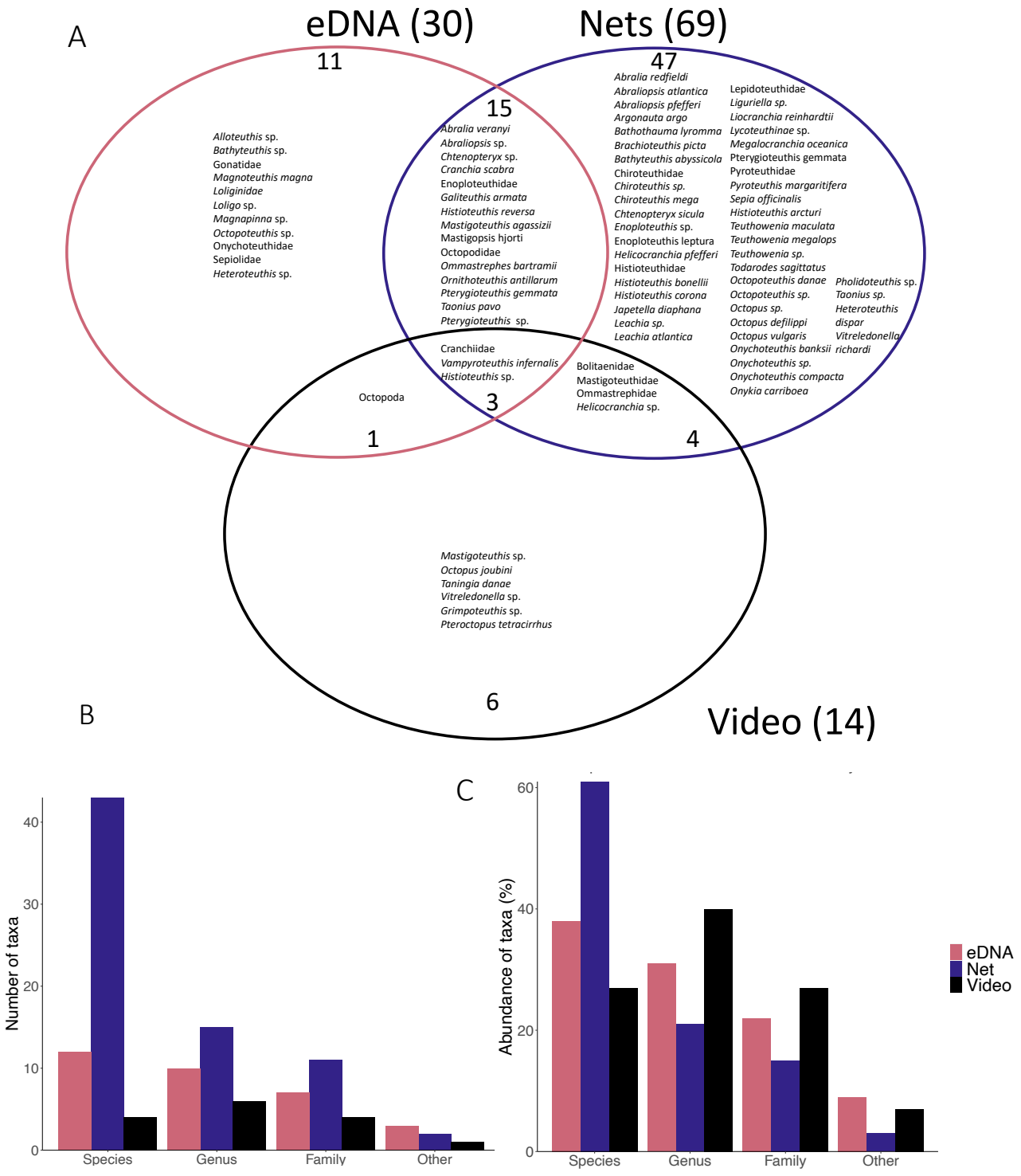

Figure 4| Comparison between taxa found with eDNA, net catches and video surveys off Cabo Verde. **(A)** Venn diagram showing the taxa detected with eDNA (30 in total), net catches (69 in total) and video surveys (14 in total) and their overlap (excluding Cephalopda and Teuthida). **(B)** Number of taxa that could be identified to species, genus, family or any other taxonomic level of the eDNA approach, net catches and video surveys. **(C)** Percentage of taxa that could be identified to species, genus, family or any other taxonomic level with the eDNA approach, net catches and video surveys.

Combining the results of all censuses applied here and the literature review (Clarke, 2006; Collins et al., 2001; Voss et al., 1998) resulted in the detection of 102 taxa of which 64 were identified to species level (Table 1).

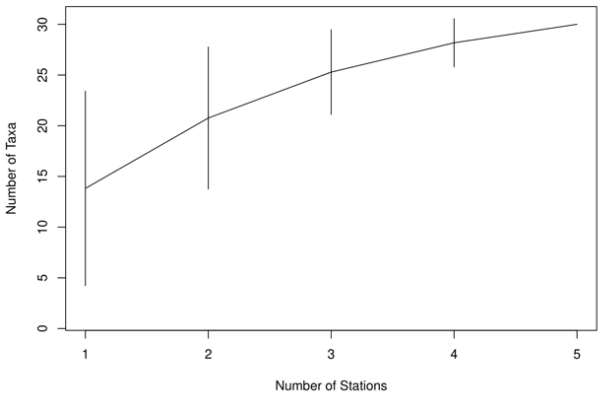

Figure 5| Species accumulation curve derived from the numbers of taxa for a certain number of sampled sites for stations sampled for eDNA metabarcoding. Error bars represent the standard deviation.

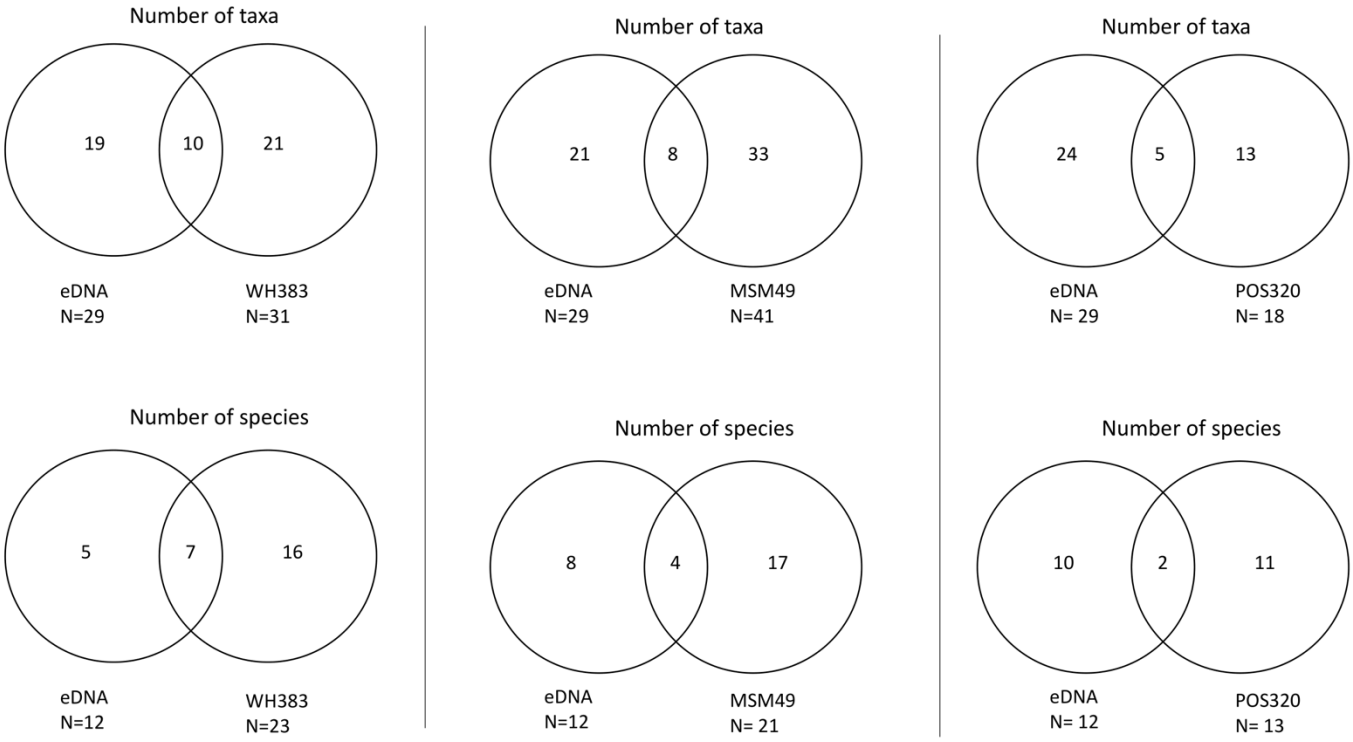

Figure 6| Comparison of eDNA data with each of the three cruises (WH383, MSM49, POS320) collecting cephalopods with net trawls. The upper Venn diagrams show the number of taxa per method and cruise. The lower Venn diagrams show the number of species per method and cruise.

Table 1| Species list of detected cephalopod species off Cabo Verde in the eastern tropical Atlantic compiled by the eDNA approach, net trawls and video surveys from this study combined with literature data. The taxa highlighted in bold are new species records for this area, but known taxa from the broader Atlantic, while the taxa highlighted in grey are dubious detections that have just been detected at the coast of Africa or other islands prior to this study.

Family	Taxon	Taxonomic level	Census	Literature	Migration
Argonautidae	*Argonauta argo*	Species	Net	(Clarke, 2006)	
Vitreledonellidae	*Vitreledonella richardi*	Species	Net	(Clarke, 2006)	Evidence for ontogenetic migration
	Vitreledonella sp.	Genus	Video		
Bathyteuthidae	*Bathyteuthis* sp.	Genus	eDNA		
	Bathyteuthis abyssicola	Species	Net	(Clarke, 2006)	Ontogenetic and diel vertical migration
Bolitaenidae	Bolitaenidae	Family	Net, video		
	Japetella diaphana	Species	Net	(Clarke, 2006)	Ontogenetic migration
Brachioteuthidae	*Brachioteuthis picta*	Species	Net		
	Brachioteuthis riisei	Species		(Clarke, 2006)	
Chiroteuthidae	Chiroteuthidae	Family	Net, Video		
	Chiroteuthis sp.	Genus	Net		
	Chiroteuthis mega	Species	Net		No evidence for ontogenetic migration, some evidence for diel vertical migration
	Joubiniteuthis portieri	Species		(Clarke, 2006)	Diel vertical migration
	Planctoteuthis danae	Species		(Clarke, 2006)	Evidence for ontogenetic migration
Chtenopterygidae	*Chtenopteryx* sp.	Genus	Net, eDNA	(Clarke, 2006)	
	Chtenopteryx sicula	Species	Net	(Clarke, 2006)	Ontogenetic and vertical diel migration
Cirroteuthidae	*Cirrothauma magna*	Species		(Collins et al., 2001)	
Cranchiidae	Cranchiidae	Family	Net, Video, eDNA		
	Helicocranchia sp.	Genus	Net, Video		
	Leachia sp.	Genus	Net		
	Liguriella sp.	Genus	Net		
	Taonius sp.	Genus	Net		
	Teuthowenia sp.	Genus	Net		
	Bathothauma lyromma	Species	Net	(Clarke, 2006; Voss et al., 1998)	
	Cranchia scabra	Species	Net, eDNA	(Clarke, 2006)	Ontogenetic migration

	Galiteuthis armata	Species	eDNA, Net	(Clarke, 2006)	Ontogenetic migration
	Helicocranchia pfefferi	Species	Net	(Clarke, 2006)	Ontogenetic migration
	Leachia atlantica	Species	Net		Ontogenetic migration, potentially diel vertical migration
	Liocranchia reinhardti	Species	Net	(Clarke, 2006)	Ontogentic migration
	Megalocranchia oceanica	Species	Net		Ontogenetic and diel vertical migration
	Taonius pavo	Species	Net, eDNA		Ontogenetic migration
	Teuthowenia maculata	Species	Net		Ontogenetic migration
	Teuthowenia megalops	Species	Net		Ontogenetic migration and evidence for diel vertical migration
	Taonius belone	Species		(Clarke, 2006)	Ontogenetic and diel vertical migration
	Leachia cyclura	Species		(Clarke, 2006)	Ontogenetic migration
	Megalocranchia oceanica	Species		(Clarke, 2006)	Ontogenetic migration
	Sandalops melancholicus	Species		(Clarke, 2006)	Ontogenetic and diel vertical migration
Cycloteuthidae	*Cycloteuthis sirventi*	Species		(Clarke, 2006)	Ontogenetic migration
Enoploteuthidae	Enoploteuthidae	Family	Net, eDNA		
	Abraliopsis sp.	Genus	Net, eDNA		
	Enoploteuthis sp.	Genus	Net		
	Abralia redfieldi	Species	Net		
	Abralia veranyi	Species	Net, eDNA		Diel vertical migration
	Abraliopsis atlantica	Species	Net		Diel vertical migration
	Abraliopsis pfefferi	Species	Net	(Clarke, 2006)	Diel vertical migration
	Enoploteuthis leptura	Species	Net	(Clarke, 2006)	Diel vertical migration
	Abraliopsis affinis	Species		(Clarke, 2006)	Diel vertical migration
	Ancistrocheirus lesueurii	Species		(Clarke, 2006)	Diel vertical migration
Gonatidae	Gonatidae	Family	eDNA		Ontogenetic and diel vertical migration
Grimalditeuthidae	*Grimalditeuthis bonplandi*	Species		(Clarke, 2006)	
Histioteuthidae	Histioteuthidae	Family	Net		Ontogenetic migration
	Histioteuthis sp.	Genus	eDNA, video, Net		
	Histioteuthis bonnellii	Species	Net		
	Histioteuthis corona	Species	Net	(Clarke, 2006)	
	Histioteuthis reversa	Species	Net, eDNA	(Clarke, 2006)	
	Histioteuthis arcturi	Species	Net	(Clarke, 2006)	Ontogenetic and diel vertical migration
	Histioteuthis meleagroteuthis	Species		(Clarke, 2006)	Diel vertical migration

Lepidoteuthidae	Lepidoteuthidae	Family	Net		Evidence for ontogenetic migration
Loliginidae	Loliginidae	Family	eDNA		
	Loligo sp.	Genus	eDNA		
	Alloteuthis sp.	Genus	eDNA		
Lycoteuthidae	*Lycoteuthinae* sp.	Genus	Net		
Magnapinnidae	*Magnapinna* sp.	Genus	eDNA		
Mastigoteuthidae	Mastigoteuthidae	Family	Net, video		
	Mastigoteuthis sp.	Genus	Video		
	Mastigopsis hjorti	Species	Net, eDNA	(Clarke, 2006)	
	Mastigoteuthis magna	Species	eDNA		
	Mastigoteuthis agassizii	Species	Net, eDNA		
	Mastigoteuthis schmidti	Species		(Clarke, 2006)	
Neoteuthidae	*Neoteuthis theilei*	Species		(Clarke, 2006)	
Octopoda	Octopoda	Order	Video, eDNA		Ontogenetic migration
Octopodidae	Octopodidae	Family	Net, eDNA		
	Octopus sp.	Genus	Net		
	Octopus defilippi	Species	Net	(Voss et al., 1998)	
	Octopus joubini	Species	Video		
	Octopus vulgaris	Species	Net	(Voss et al., 1998)	
	Octopus burryi	Species		(Voss et al., 1998)	
	Pteroctopus tetracirrhus	Species	Video	(Voss et al., 1998)	
Octopoteuthidae	*Octopoteuthis* sp.	Genus	Net, eDNA		
	Octopoteuthis danae	Species	Net		
	Taningia danae	Species	Video, eDNA	(Voss et al., 1998)	
Ommastrephidae	Ommastrephidae	Family	Net, Video		Ontogenetic and diel vertical migration
	Ommastrephes bartramii	Species	Net, eDNA		
	Ornithoteuthis antillarum	Species	Net, eDNA		Ontogenetic and diel vertical migration
	Todarodes sagittatus	Species	Net		Diel vertical migration
	Sthenoteuthis pteropus	Species		(Merten et al., 2017)	Ontogenetic and diel vertical migration
Onychoteuthidae	Onychoteuthidae	Family	eDNA		Ontogenetic and diel vertical migration
	Onychoteuthis sp.	Genus	net		
	Onychoteuthis banksi	Species	Net	(Clarke, 2006)	
	Onychoteuthis compacta	Species	Net		
	Onykia carriboea	Species	Net	(Clarke, 2006)	Diel vertical migration
Pholidoteuthidae	*Pholidoteuthis* sp.	Genus	Net		
Pyroteuthidae	Pyroteuthidae	Family	Net		
	Pterygioteuthis sp.	Genus	Net, eDNA	(Clarke, 2006)	
	Pterygioteuthis gemmata	Species	net, eDNA		

	Pyroteuthis margaritifera	Species	Net	(Clarke, 2006)	Diel vertical migration
Sepiidae	*Sepia officinalis*	Species	Net		Diel vertical migration
Sepiolidae	Sepiolidae	Family	eDNA		
	Heteroteuthis sp.	Genus	eDNA		
	Heteroteuthis dispar/dagamensis	Species	Net		
Tremoctopodidae	*Tremoctopus violaceus*	Species		(Clarke, 2006)	
Vampyroteuthidae	*Vampyroteuthis infernalis*	Species	Net, Video, eDNA	(Clarke, 2006)	

Table 2| Overview of the bioinformatic steps after DADA2 analysis. The number of amplicon sequence variants (ASVs) is shown that is retrieved after (1) DADA2 analysis, (2) assignments to the reference database with IDTAXA, (3) removing of all taxa assignments that have less than 10 reads per sample, (4) removing of all samples that belong to negative controls, (5) removing of all samples that belong to positive controls, and (6) removing of taxa assignments from the dataset that were found in the corresponding negative controls. The mean, standard deviation and range is given for the number of reads per sample and the number of reads per ASV.

	Number of ASVs	Bioinformatic step	Number of reads per sample (mean ± standard deviation)	Range of reads per sample	Number of reads per ASV (mean ±standard deviation)	Range of reads per ASV
1	908	After DADA2 analysis	11,186 (± 15,775)	1-100,406	196 (± 2233)	1-96,089
2	567	Taxa assignment				
3	563	ASV with reads below 10 removed	21,390 (± 18,761)	31-100,406	550 (± 3805)	11-96,089
4	333	Negative controls removed				
5	305	Positive controls removed				
6	78	Contamination removed	15,069 (± 11,337)	132-59,494	102 (± 294)	11-4,035

Cephalopod composition off Cabo Verde in comparison with the Azores and Canary Islands

For the Canary Islands, Cabo Verde and Azores 83, 64 and 83 cephalopod species have been reported to date, belonging to 31, 30 and 36 families, respectively (Figure 7). For all three archipelagos, the Cranchiidae was the family with the highest number of species (21 for Cabo Verde and eleven for the Canary Islands and Azores). The second most speciose family for Cabo Verde and the Azores was the Enoploteuthidae (ten and eight species, respectively). For the Canary Islands, Octopodidae, Histioteuthidae and Enoploteuthidae were the second most diverse families with six species each. Thirty-eight species were found to occur in the EEZ of all three archipelagos. The Canary Islands overlapped with five and 17 species with Cabo Verde and the Azores, respectively, whereas Cabo Verde and the Azores shared seven cephalopod species (Figure 7). Cephalopods that only occurred off one island group included 23 species off the Canary Islands, 21 species off the Azores and 14 species off Cabo Verde. The overlapping octopus community composition between Canary Islands, Azores and Cabo Verde consisted of four pelagic species, in addition to *Octopus vulgaris* (Figure 8) which is benthic. Four octopus species only occurred off Cabo Verde, whereas the Azores harbored six and the Canary Islands five species that do not occur at the other archipelagos.

The Jaccard index measures dissimilarity in species composition between the three archipelagos and in this study this index ranged between 0.50 and 0.60, indicating that all islands shared between 40-50% of cephalopod species (Supplementary Table 5). When reducing the analysis to the Order Octopoda, the Jaccard index ranged between 0.5 and 0.75, with Azores and Canary Islands sharing 50% of octopus species, in contrast to Cabo Verde which only shared 25-27% of octopus species between the other two archipelagos (Supplementary Table 6).

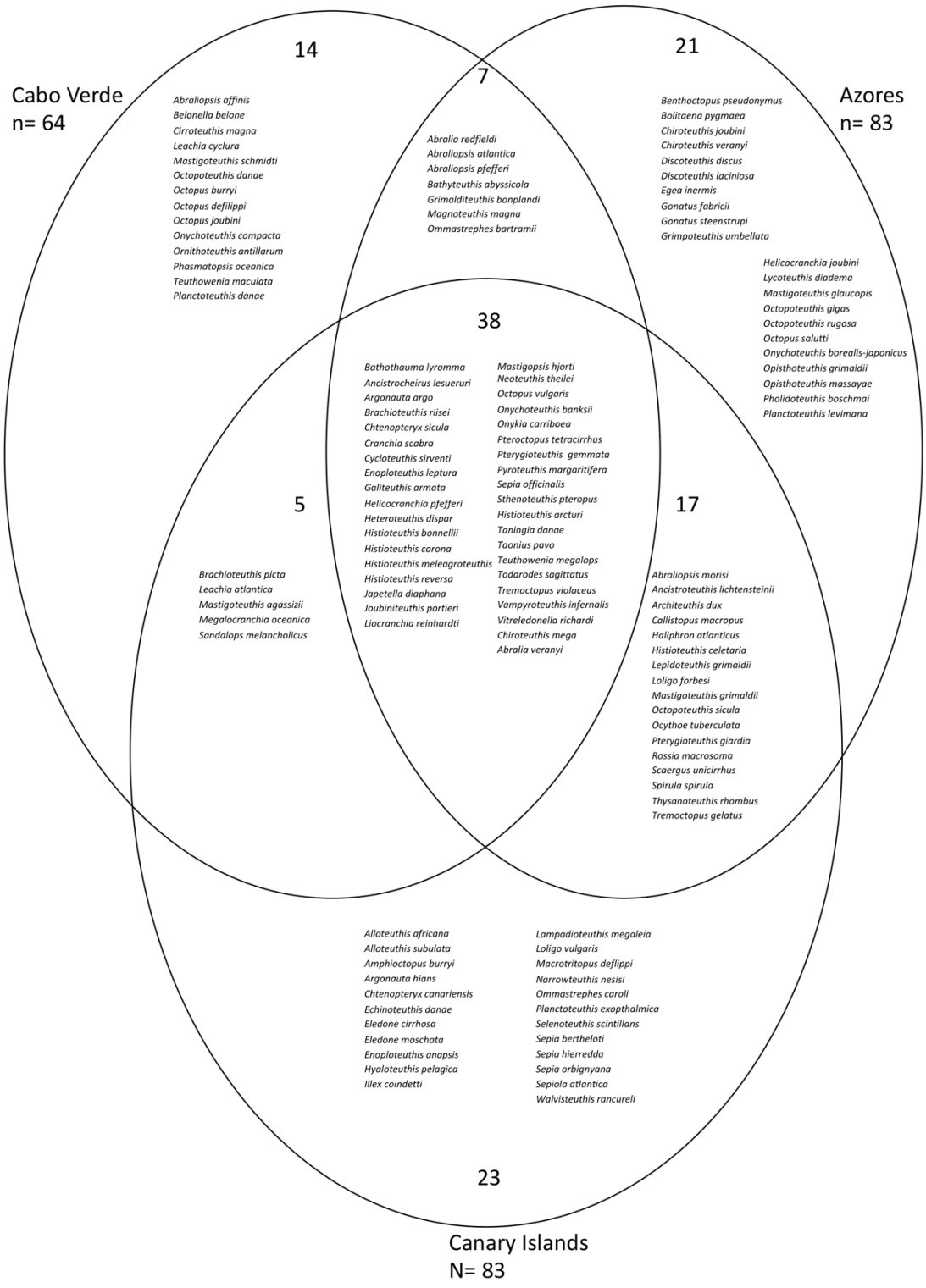

Figure 7| Biodiversity of cephalopod species off Cabo Verde, Canary Islands and the Azores in the eastern Atlantic Ocean from the literature ((Visser, Merten et al., 2021) for Azores; (Escánez et al., 2020) for the Canary Islands) and this study. Overlapping spheres show overlapping species for the corresponding geographical regions. The number of species shared between different geographical regions or being unique are shown in black inside the circles. Only taxa that were identified to species level are included.

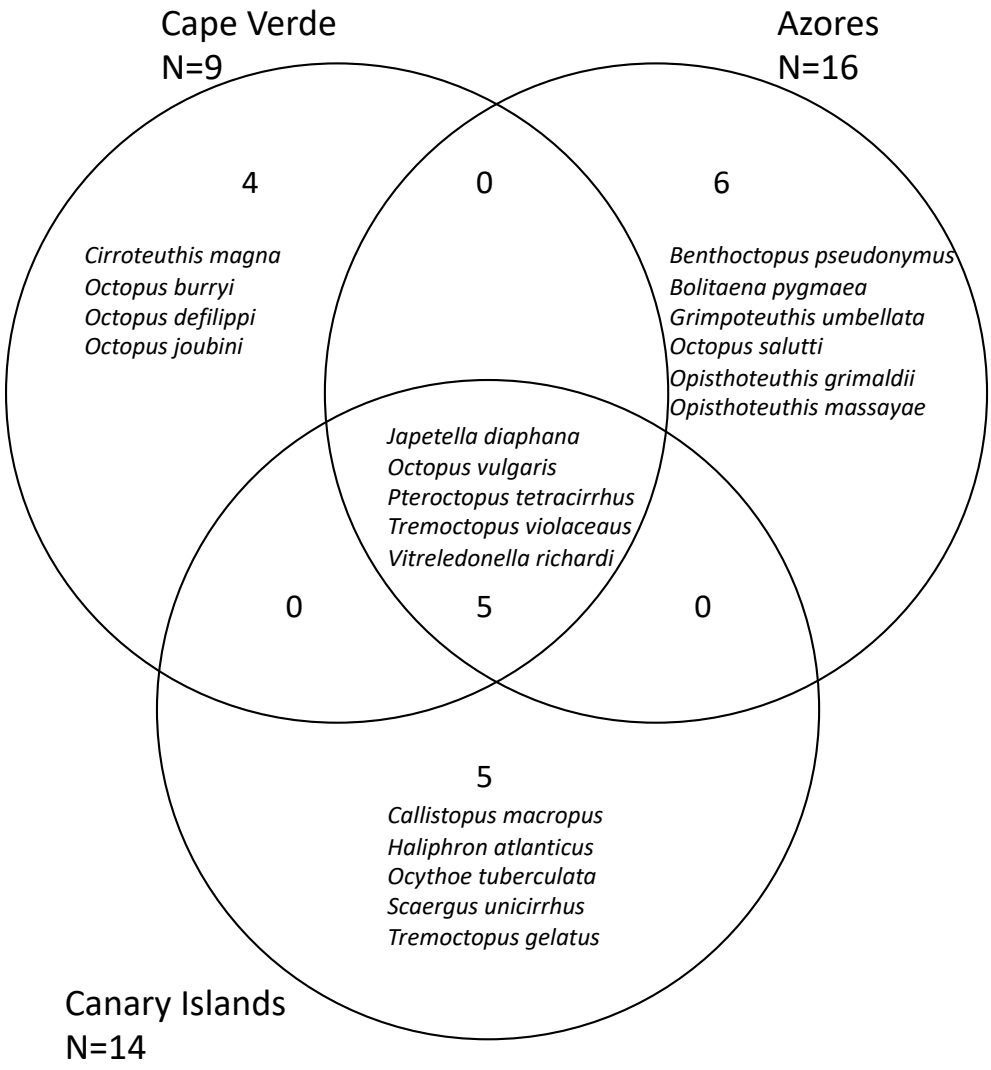

Figure 8| Biodiversity of octopus species off Cabo Verde, Canary Islands and the Azores in the eastern Atlantic Ocean. The number of taxa shared between different geographical regions or only occurring in one geographical region are shown in black inside the circles.

2.4. Discussion

Cabo Verde as a biodiversity hotspot for cephalopods

Combining the data from this study with records from the literature indicates that Cabo Verde is home to at least 102 cephalopod taxa of which 64 were identified to species level from 30 families. Nine of these taxa are rare and were detected in this region for the first time by this study. This includes three genera and six species detected with eDNA that are known from the Atlantic Ocean, but had not been recorded around Cabo Verde before (Jereb and Roper, 2010). With 64 confirmed species, Cabo Verde can be considered a biodiversity hotspot for cephalopods comparable to the Canary Islands (Escánez et al., 2020) and Azores (see literature in Visser, Merten et al., 2021). This species richness is also comparable to those reported from other northern hemisphere open ocean systems identified as areas with high cephalopod species diversities such as the western North Atlantic (74 species), the North Sargasso Sea (68 species), the North African Subtropical Sea (72 species) and the Mediterranean outflow (70 species) (Rosa et al., 2008). The high species richness observed off Cabo Verde may be explained by the sampling sites being in vicinity of several seamounts (e.g., Senghor Seamount) and islands (Cabo Verde), topographical features that are known to enhance species diversity, creating various ecological niches for different species to thrive and evolve (Worm et al., 2003). Additionally, Cabo Verde is located at intermediate latitudes where temperate, subtropical and tropical species ranges overlap, leading to high diversities across trophic levels for predator and prey species (Worm et al., 2003). Many of the here detected cephalopod species belong to deep-sea families (Hoving et al., 2014) such as Cranchiidae, Histioteuthidae, Octopoteuthidae, Mastigoteuthida and Chiroteuthida. This indicates, that the identification of Cabo Verde as being a cephalopod diversity hotspot may be driven particularly by diversity in the deep sea. However, cephalopod diversity in shallow and coastal waters needs to be explored in more detail for a complete understanding of what is driving overall regional cephalopod community composition.

Two unexpected genera were detected with eDNA: *Loligo* and *Alloteuthis*. These genera are coastal and not known from Cabo Verde. They have not been detected by the intense demersal fisheries active around the islands prior to this study. The detections are unlikely to be false positives since they were absent from the corresponding negative controls. Another possibility may be the transport of eDNA by predators. Apex predators such as sperm whales, blue sharks and swordfish are known

to feed on *Loligo* off the Azores and migrate to Cabo Verde (Clarke et al., 1996, 1995; Clarke, 1956), potentially releasing faeces, and therefore eDNA, in Cabo Verdean waters. However, it can also not be ruled out that *Loligo* and *Alloteuthis* are undetected species in Cabo Verdean waters. *Loligo* and *Alloteuthis* are both distributed from tropical to temperate and subpolar coastal waters and occur along the northern and central west coast of Africa (Jereb and Roper, 2010). This indicates that species' distributions may be broader than anticipated and eDNA detections of unknown and unexpected species must be considered carefully. In this context it is worth noting that in the South Atlantic Ocean the bathypelagic and benthopelagic squid *Gonatopsis octopedatus* has been dispersed more than 15 000 km from its region of original species distribution in the North Pacific, potentially by deep-sea currents (Arkhipkin et al., 2010).

This study was able to detect rare and elusive species with eDNA. One example is the bigfin squid *Magnapinna* sp. This squid is a bathypelagic species that is primarily known from underwater observations > 1800 m in the Pacific (Osterhage et al., 2020), Indian and Atlantic Ocean (Vecchione et al., 2001). Only a handful of damaged or juvenile specimens have been captured in the Pacific Ocean (Vecchione and Young, 1998). No observations or records have been made to date from the Cabo Verde region, although individuals have been captured 500 miles south of Cabo Verde from depths of between 998-1962 m in 2007 (Piatkowski, personal communication). This squid species has distinctive and relatively long, slim arms reaching lengths of over 1.5 m, compared to a mantle length of ~ 15 cm (Osterhage et al., 2020). *Magnapinna* sp. is suggested to be globally distributed in bathyal and abyssal depths (Vecchione et al., 2001). This genus was solely detected with eDNA in this study and repeatedly in deep waters (1900 – 2500 m) off Cabo Verde, which is consistent with its known depth distribution in the Atlantic (Vecchione et al., 2001).

Vertical distribution of cephalopod species off Cabo Verde

The depth ranges stated in this study are a combination of general presence/absence of taxa. Since the cephalopod data presented here from the net catches is imbalanced with regard to day/night sampling, we do not differentiate between day and night distributions. In the Atlantic open ocean, cephalopod species diversity is known to be highest in the upper 200 to 1000 m and decreases with depth (Rosa et al., 2008) resulting in a bell-shaped diversity pattern with respect to depth. The same trend has been observed in pelagic fishes and invertebrates (Cartes et al., 2011; D'Onghia et al., 2004; Farré et al., 2016; Gaertner et al., 2013; Haedrich et al., 1980; Moranta et al., 1998; Papiol et al.,

2012; Smith and Brown, 2002). High mesopelagic diversities coincide with enhanced primary productivity and optimal oxygen concentrations in overlying water layers (Levin et al., 2001) resulting in sinking of organic matter and high food supply at intermediate depths. Additionally, in this depth zone, predation pressure from top predators is intermediate and balanced by high or moderate production by low trophic levels providing prey for cephalopods and therefore facilitating their occurrence and coexistence (Gage and Tyler, 1991; Roxburgh et al., 2004). Cephalopod taxa community composition around the Cabo Verde islands of Santo Antão and Fogo, the two stations for which eDNA sampling was balanced with respect to depth, followed the same above-mentioned bell-shaped diversity pattern, with the highest number of species detected in the upper 1000 m, peaking at 600 m. This peak in cephalopod community composition correlates with species and biomass aggregations of myctophid fishes at 400 to 700 m in the central equatorial Atlantic (Kinzer and Schulz, 1985), which are, together with other fish species, primary prey of several cephalopods (Hoving et al., 2014; Merten et al., 2017; Villanueva et al., 2017). Overall cephalopod community composition decreased below 1000 m in comparison to the upper 1000 m, however, two peaks in cephalopod community composition were present at 1600 and 2200 m. The distribution of cephalopod species occurring in bathyal and abyssal depths is less well known than the distribution of epi- and mesopelagic species, partially due to difficulties in sampling the deep sea. Therefore, the species community composition is likely underestimated in these depths. The water layer immediately above the seafloor, the benthic boundary layer (BBL), is expected to support increased biomasses and species diversity (Brandt et al., 2007), which may explain the here observed peak in cephalopod species community composition at 2200 m depths. A previous study on eDNA of cephalopods in the hunting zones of Cuvier's beaked whales (*Ziphius cavirostris*) showed that some predators specifically hunt in the BBL (Visser, Merten et al., 2021). Additionally, the high abundances of e.g., fish and crustaceans reported in the BBL (Dauvin and Vallet, 2006) suggests that BBL assemblages are of great importance for deep-sea food webs and nutrient cycling.

Due to the repeated observations of *Taningia danae* during video transects, but their absence in nets, we were particularly interested in the vertical distribution of this large squid, and hence developed and applied a species-specific primer on the eDNA samples. This species is recognized as one of the largest mesopelagic squid in tropical and subtropical oceans worldwide (Clarke, 1966) growing up to 150 kg and 1.7 m mantle length. Knowledge of this species is based on small juvenile individuals caught with mid-water nets (Roper and Vecchione, 1993), records generated from sperm whale

stomach content analysis (Clarke, 1967; Okutani and Satake, 1978), specimens from fishing nets (González et al., 2003; Quetglas et al., 2006) and in situ observations with midwater camera systems (Gomes-Pereira and Tojeira, 2014; Hoving et al., 2019a; Kubodera et al., 2007). The presence of hundreds of beaks of *T. danae* in sperm whale stomachs suggests that this species is a very abundant deep-sea squid (Clarke, 1996). However, adults are rarely caught with nets, potentially due to their occurrence at considerable depths and their strong swimming capabilities. The relatively frequent detections of *T. danae* during the video transects may be the result of *T. danae* being attracted to the light of the camera system (Hoving et al., 2019a) as seen in other studies applying lured camera systems in midwater (Gomes-Pereira and Tojeira, 2014; Kubodera et al., 2007). Juvenile and young *T. danae* undergo ontogenetic migration from the surface to 200 – 300 m water depth (Roper and Vecchione, 1993) and larger specimens perform short distance diel vertical migration from depths of 600 – 900 m during the day to shallower depths of 240 – 500 m during the night (Kubodera et al., 2007). Considering the hunting grounds of sperm whales and deep-sea sharks, adult *T. danae* were thought to occur below 1000 m (Clarke and Merrett, 1972). This deep occurrence is supported by the eDNA and video data of this study. The video surveys observed *T. danae* between 400 and 1400 m, whereas eDNA detected *T. danae* between 100 and 2500 m, with the majority of detections at 1600 m. The fact that *T. danae* was detected frequently in the eDNA samples highlights the potential importance and abundance of this elusive top-predator in deep-sea ecosystems, and shows the ability of eDNA to detect species that are rarely captured in nets.

Comparing Cabo Verde cephalopod biodiversity with other Macaronesian islands

The three archipelagos Cabo Verde, the Canary Islands and Azores investigated in this study share overall cephalopod community composition. However, the data indicates a difference in octopus community composition between Cabo Verde and the Azores as well as Canary Islands, suggesting a separation of Cabo Verde when focusing specifically on benthic octopuses. These findings are in line with studies proposing a biogeographical separation of Cabo Verde from Macaronesia based on the dispersal of benthic and coastal metazoan species (Freitas et al., 2019). Biogeographical separation and endemism are tightly linked to the larval dispersal ability of organisms as dispersal directly influences gene flow within and among populations. Populations may become either genetically more similar when subject to frequent mixing or they may become more isolated when mixing and

dispersal is reduced or absent. There are strong inter-specific differences in cephalopod dispersal abilities. Cephalopod species that hatch planktonic paralarvae tend to be smaller and reach broader distributional ranges than species with larger, benthic hatchlings (Villanueva et al., 2016). Additionally, species with offshore larval distribution have been shown to have larger mean geographic ranges than species with inshore larval distribution (Macpherson and Raventos, 2006), a trend also observed in fish (Macpherson and Raventos, 2006), highlighting the importance of oceanographic currents as powerful dispersal mechanisms for pelagic species (Bower et al., 1999; Downey-Breedt et al., 2016; Martins et al., 2014; Roberts and van den Berg, 2005; Rowell et al., 1985; Saito and Kubodera, 1993).

Due to their muscular anatomy, strong swimming capabilities and highly mobile life style, some squid species are able to actively migrate hundreds of kilometers. For instance, the ommastrephid squid *Sthenoteuthis pteropus* spawns in the eastern equatorial Atlantic (Zuyev et al., 2002) and its paralarvae are quickly dispersed in the equatorial zone. Adult females are known to migrate 2500 km from Cabo Verde to Madeira and back (Zuev and Nikolsky, 1993; Zuyev et al., 2002). Other oceanic squids occurring off the Cabo Verde islands also occur off the Azores and Canary Islands, such as *Taningia danae* and *Todarodes sagittatus*. Due to their mobility and ability to cope with different oceanographic conditions, many squid species are able to sustain broad horizontal and vertical distribution patterns (Childress and Seibel, 1998; Doubleday et al., 2016; Hoving and Robison, 2012; Rodhouse et al., 2014; Rosa and Seibel, 2010; Zuyev et al., 2002). For species with limited swimming capacities, pelagic egg masses may facilitate dispersal (Roberts et al., 2011; Young and Harman, 1985).

The majority of octopus species have a short planktonic larval stage followed by a benthic adult lifestyle, and as such have a limited dispersal potential due to e.g., larger hatchling sizes, a characteristic linked with limited dispersal (Villanueva et al., 2016) and association with certain substrates. For example, the benthic octopus *Pareledone turqueti* is unable to disperse between sites that are separated by ocean depths > 1000 m, which presents a major physical barrier for the species (Allcock et al., 1997). This limited dispersal ability and therefore reduced gene flow in octopuses is also applicable to the common octopus *Octopus vulgaris* (Sampaio et al., 2018). The common octopus is the only benthic octopus occurring at all three archipelagos, the Canary Islands, Azores and Cabo Verde. However, *O. vulgaris* populations off Cabo Verde potentially show a developing endemism of this species (Sampaio et al., 2018). The other octopus species from the current study to occur across the three archipelagos were all pelagic octopuses that brood their eggs in midwater or have pelagic

paralarvae (*Japetella diaphana*, *Pteroctopus tetracirrhus*, *Vitreledonella richardi* and *Tremoctopus violaceus*). Therefore, with increased dispersal abilities potentially comparable with those of oceanic squids. The oceanographic features of Cabo Verde form natural barriers to the north of the islands and towards the west coast of Africa, whereas the Canary Islands and Azores are connected by the Azores Current and the Madeira current, forming a seaway, with several shallow seamounts functioning as stepping stones for marine organisms (Freitas et al., 2019). As a result, benthic or benthopelagic organisms with reduced mobility, in contrast with the high mobility of squids, may become separated from species of the other archipelagos, leading to a separation of Cabo Verde from Macaronesia. This is indicated in octopus species composition in this study. To fully understand the biogeography of Macaronesia, not only benthic and coastal organisms should be taken into account, but also the pelagic, open ocean fauna should be considered.

Potential role of cephalopods in the carbon cycle of the deep sea of Cabo Verde and Macaronesia

An active pathway of vertical carbon distribution by cephalopods is achieved by vertical migration. Many cephalopods spend the paralarval and juvenile phase of their lifecycle in upper surface waters, benefiting from primary production (Hoving et al., 2014). As they grow and mature, they descend to deeper layers to reproduce, transporting carbon from shallow to deep waters via ontogenetic migration. The second active pathway of carbon distribution is diel vertical migration, a common trait of many deep-sea cephalopods (Hoving et al., 2014; Jereb and Roper, 2010; Judkins and Vecchione, 2020). These cephalopods migrate close to the surface at night for feeding and descend to deeper layers during the day, to potentially avoid visual predators or to maintain their energy reserves in the colder, deeper water (Sutton, 2013). Off Cabo Verde, 31% of the confirmed cephalopod taxa are known to undergo ontogenetic migration such as *Sthenoteuthis pteropus*, *Taningia danae* and most of the cranchiids (Jereb and Roper, 2010; Judkins and Vecchione, 2020). Thirty-five are also known to perform diel vertical migration (e.g., *Sthenoteuthis pteropus, Taningia danae* as well as many enoploteuthids) (Jereb and Roper, 2010).

Carbon can also be distributed passively when cephalopods die after reproduction, either sinking to the seafloor or floating to the surface, depending on the species (Boyle and Rodhouse, 2005; Fields, 1965; Hoving et al., 2017; Martin and Christiansen, 1997; Nesis et al., 1998; Roper and Vecchione, 1996; Stockton and Delaca, 1982). To date, there are no records of cephalopod carcasses on the seafloor in the Cabo Verde region. However, stomach content analysis of deep-sea scavengers off

the Cabo Verde islands have found ommastrephid squid in the stomach contents of the cusk eel (*Neobythites* sp.) and the velvet belly lanternshark (*Etmopterus spinax*) (Clarke and Merrett, 1972). Both scavengers are benthic and likely fed on carcasses rather than actively hunted epi- and mesopelagic squid.

Species in the Cabo Verde area which may form substantial foodfalls when they die (settling to the seafloor) are *T. danae* and *S. pteropus*. *Taningia danae* is suggested to be associated with bottom waters when spawning (Nesis, 1987), indicating that this species may be consumed by benthic organisms after spawning and death. Given the estimated high abundance and large size (~1.70 m mantle length), biomass from this species may contribute significantly to the regional carbon cycle of the deep sea. The orangeback flying squid *S. pteropus* is another abundant and dominant squid species, in terms of numbers of individuals and biomass (instantaneous biomass: 4-6.5 million tons, annual total biomass production = 34 – 52 million tons) in the eastern tropical Atlantic (Jereb and Roper, 2010; Zuyev et al., 2002). *Sthenoteuthis pteropus* can reach maximum mantle lengths of 65 cm and has a broad distribution from Madeira (36°N) in the North to South Africa (32°S) in the South. This species' distribution spans the whole of Macaronesia and individuals are known to migrate thousands of kilometers from Cabo Verde to Madeira and back. Spawning of *S. pteropus* is not linked to bottom waters, but occurs in the epipelagic zone. This species is not neutrally buoyant and individuals sink to the seafloor when they stop swimming. Tissues from this species have high protein concentrations (17%) (Zuyev et al., 2002), rendering them as a valuable food source. *Sthenoteuthis pteropus* undergoes ontogenetic and diel vertical migration down to 1200 m water depth (Moiseev, 1991), resulting in active transport of carbon from the surface to the mesopelagic ocean. Due to its high abundance, wide distribution and migration ability, good nutritional value, large size, spawning style and negative buoyancy, *S. pteropus* may transport carbon from epi- and mesopelagic zones to the deep-sea benthos.

The use of physical, optical and genetic censuses for biodiversity assessment

The results from the three methods employed in this study each indicated differing cephalopod families to be regionally dominant. Mastigoteuthidae followed by Ommastrephidae and Loliginidae were the most commonly detected families with eDNA, whereas with nets, the most commonly

detected families were Cranchiidae followed by Pyroteuthidae and Enoploteuthidae. This is in line with net data from the literature from the eastern North Atlantic, with Cranchiidae (*Liocranchia reinhardti*) being the most commonly detected family in rectangular midwater trawls caught in 1968 and 1972, followed by Onychoteuthidae (*Onychotetuhis banksii*) (Clarke, 2006). However, Pyroteuthidae and Enoploteuthidae only represented 5 and 2 %, respectively, of the samples collected by the trawls described in Clarke (2006). This highlights the potential collection bias towards certain species, as a function of the trawling gear characteristics being used, and therefore that differences in counts can occur within methods (i.e., between different trawls) as well as between methods (i.e., between eDNA and trawl catch analysis). For example, in Clarke (2006), three different types of nets were used with a net opening of between 0.5 m^2 (Multinet) and 480 m^2 (pelagic trawl) and mesh sizes of between 335 µm and 4 mm. In the current study, smaller net openings and mesh sizes, such as those of the IKMT and Multinet, mainly caught paralarvae and small individuals between 4 mm and 190 mm mantle length, whereas the pelagic trawl captured larger individuals of between 14 mm and 240 mm mantle length. However, even the pelagic trawl used here was not able to catch very large squids. Video surveys conducted by different gears also resulted in differing sampling bias. The manned submersible JAGO used in this study is loud under water, potentially scaring organisms away. PELAGIOS on the other hand is attached to a research vessel via cable, causing vibrations in the water column along the cable length, which could also influence observations. Both gears emit light that may attract some species and repel others. The majority of observed cephalopods that could be identified to at least family level was within video data collected with PELAGIOS, whereas within JAGO video data, Cranchiids, which are often less mobile, were more often observed. Within JAGO data cephalopods were often observed in mid escape, moving swiftly and therefore could not be identified to high taxonomic resolution.

The video observation results were in general accordance with the observations from the nets, both detecting the Cranchiidae as the most abundant family. The second most commonly detected family were the Mastigoteuthidae which aligned with the eDNA results. This discrepancy in assessment of the most frequently observed families reported by these three methods may partially be the result of differing characteristics of the sampling methods. Cranchiids are abundant and when small often less mobile, therefore they are more easily caught with nets than some other families. Ommastrephids on the other hand are highly mobile, muscular and often larger squids that may be able to easily escape approaching nets. As a defense strategy, ommastrephids and mastigoteuthids also release large amounts of ink when being threatened. This ink, which contains DNA, may result in

a higher abundance of eDNA in their environment than that associated with some of the other families e.g., cranchiids. Cranchiids have been observed to eject ink inside their mantle cavity for camouflage, therefore not releasing it externally (Hunt, 1996). This may reduce the amount of eDNA released by that cephalopod group. Information on eDNA shedding rates of cephalopods is needed, as the size of an individual does not necessarily reflect the amount of e.g., tissue being shed. Further, differing oceanographic conditions might influence the detectability of eDNA (Pinfield et al., 2019).
The three sampling approaches applied in this study complemented each other in genera and species detections. It seems on first examination of the data that net tows were the most effective sampling method for assessing cephalopod deep-sea diversity, since specimens for reference databases were also collected. However, when comparing the eDNA data with trawl data from all cruises individually, net tows were either more effective, comparable or less effective in finding taxa when compared to the eDNA approach. Between 9-20% of species were detected with both the eDNA and net approach, indicating a considerable contribution of 20 – 43% in species detections when combining eDNA with net trawls. However, direct and quantitative comparisons between and within the three sampling methods applied here was not possible and was not the priority of the current study, as the sampled sites, seasons, years, targeted depths and gear used within and between methods differed substantially, as well as the sample sizes. The volume of water that was sampled with CTD niskin bottles for the eDNA approach was several magnitudes less than that which was physically and optically sampled by nets and video surveys, respectively. As the rarefaction curve for eDNA did not reach the asymptote from the data collected, sampling more stations and depths would likely have resulted in additional detected taxa. Similarly, the addition of more targeted marker genes may have increased the number of species and taxa detected by the eDNA methodological approach. By increasing the sampling size for eDNA samples we can expect eDNA to become progressively more equivalent, or even superior, to the net trawl methodological approach in detecting taxa, as has been the case in other studies focusing on other organisms, such as fish (Boussarie et al., 2018; Govindarajan et al., 2021; Thomsen et al., 2012). However, reference specimens are required from a region to compare with obtained eDNA sequences.

This study highlights that the complementary use of different methods yields the most holistic view of deep-sea cephalopod communities. Nets remain indispensable since reference specimens are needed to detect, compare and validate sequences obtained with eDNA. However, both the eDNA approach and video surveys are non-invasive and appropriate for integration into the surveillance

and management plans of marine ecosystems, but efforts should be increased to incorporate more primers into the analysis procedure and also the seafloor should be sampled for eDNA analysis.

Ethics statement

Cabo Verde has not ratified the Nagoya protocol. To fulfill the national access and benefit-sharing (ABS) regulations of Cabo Verde, we obtained the required permits for sample collection and publication of results based on samples collected in Cabo Verde waters from the Direcção Nacional do Ambiente (National Directorate for the Environment of Cabo Verde).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

HJTH conceived the study; VM, HJTH, TB, TBHR, OP planned the study; VM, JS, JF conducted the lab work, AL, UP, SC, HH, BC, AD, HJTH contributed data, VM, HJTH analyzed the data, VM, HJTH wrote the manuscript; TB, TBHR, OP, JF, JS, AL, HH, UP, SC, BC and AD critically reviewed the manuscript.

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Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (Accession: AY393902.1, MG591434.1, EU735402.1) and in the PANGEAE repository: https://doi.pangaea.de/10.1594/PANGAEA.937568

2.5. References Chapter 2

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Chapter 3

Arctic nekton diversity and distribution uncovered by eDNA metabarcoding

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Abstract

Biodiversity in the Arctic Ocean is unique, but under-studied. Comprehensive baseline studies are essential to detect changes in Arctic biodiversity and to assess the impact of climate change in this region. Due to their high mobility, cephalopods and fishes may be good indicators of the ongoing "atlantification" and "borealization" of the Arctic ecosystem. In addition, when they die, cephalopod and fish may contribute to the local carbon cycle as foodfalls. This study investigated biodiversity and distribution of fishes and cephalopods in the Fram Strait of the Arctic Ocean using environmental DNA metabarcoding of seawater and sediment samples. Water samples were collected at four stations between 50 and 2700 m and sediment at 12 stations with bottom depths ranging between 276 and 5545 m. The samples were analyzed with universal cephalopod (nuclear 18S rRNA gene) and fish (mitochondrial 12S rRNA gene) primers. We detected six cephalopod taxa and 27 fish taxa in the seawater and 18 fish taxa in the sediment. We were not able to detect cephalopods in the sediment. The most commonly detected cephalopod taxa in seawater were *Gonatus* sp. and Gonatidae, the most common squid in the region. For fishes, capelin (*Mallotus villosus*) was detected most often followed by Zoarcidae, Liparidae, *Sebastes* sp., and Atlantic herring (*Clupea harengus*). The native northern range of *M. villosus* is the sub-arctic, but we identified it here as far north as 79.7°N, providing evidence for a range expansion. Comparison of seawater and sediment samples suggested that *M. villosus* may contribute to the carbon cycle as a food falls species. We found no significant differences between the Fram Strait stations in community composition or vertical distribution of neither cephalopods nor fishes. Our study shows that eDNA metabarcoding is a valuable tool for monitoring pelagic diversity and is able to detect range expansions in a rapidly changing Arctic Ocean.

3.1. Introduction

The Arctic Ocean faces unprecedented impacts as a result of global change including the world's fastest warming rates with an average increase in temperature by 1.3°C from 1990 to 2005 (Gille, 2002; Walczowski and Piechura, 2006) and more than 2°C in some areas (IPCC, 2001). The poles experience a more intense warming that is estimated to be 1.5 – 4.5 times higher than in other regions. This phenomenon known as "polar amplification" is leading to further global warming (Holland and Bitz, 2003). The Arctic including Greenland has been shown to be more impacted by polar amplification than central Antarctica (Masson-Delmotte et al., 2006). Between 1970 and 2011, freshwater and marine populations in the Arctic Ocean have declined in abundance by 81% and 36%, respectively (Senapati et al., 2019). Arctic ecosystems are not only impacted by changing water temperatures, but also by a reduction in sea-ice coverage and changing circulation patterns (Fossheim et al., 2015). The process of North Atlantic water (NAW) flowing further northward into the Arctic compared to recent times in combination with the change of Arctic physical conditions becoming more Atlantic is called "atlantification". The NAW separates into two branches, one of them flowing northward towards the Fram Strait and the second one flowing eastward into the Barents Sea (Oziel et al., 2020). In the Barents Sea, the NAW has occupied a larger (two-fold) water volume over the last decades (Oziel et al., 2016). An increased inflow of Atlantic water into the Arctic may lead to the replacement of long-lived and slow growing Arctic organisms with smaller and short-lived boreal species (Walczyńska et al., 2018), a process called "borealization". This has already been observed in zooplankton with the boreal copepod *Calanus finmarchicus* and euphausids expanding northwards with Arctic warming. On the other hand, the Arctic amphipod *Themisto libellula* retreats further north as the temperatures increase (see references in (Polyakov et al., 2020; e.g., Dalpadado et al., 2012; Eriksen et al., 2017; Orlova et al., 2015, 2010). Fishes show a similar pattern with boreal fish species (e.g., Atlantic cod, haddock, capelin) expanding north in the last decade (Fossheim et al., 2015; Haug et al., 2017), while the polar cod declined in distribution area and biomass (Eriksen et al., 2015; Hop and Gjøsæter, 2013). Atlantic species range expansions potentially lead to changes in trophic interactions in the Arctic ecosystem (Johannesen et al., 2012) as a result of increased predation pressure on fish communities (by e.g., Atlantic cod) and intensified competition influencing the food web and ecosystem functioning (Fossheim et al., 2015; Kortsch et al., 2015; Wiedmann et al., 2014). In the light of rapid changes in the Arctic Ocean, comprehensive baseline studies are

essential to detect changes in biodiversity as a result of climate change. Management strategies are needed, especially with additional increasing anthropogenic stressors such as marine shipping (ACIA, 2004), which will be further amplified as soon as the Arctic is ice-free during summer months. The Arctic Ocean is inhabited by a variety of uniquely adapted organisms and its diversity is considered to be at intermediate levels in comparison to the worlds' oceans (Hardy et al., 2011) with around 8000 extant species estimated to occur there (Bluhm et al., 2011). However, new taxa are described frequently and several thousand of species are estimated to be still discovered (Archambault et al., 2010; Darnis et al., 2012; Walczyńska et al., 2018). Especially nekton may be a good indicator of atlantification and borealization, as they are not affected by currents and are able to actively change distribution patterns to find optimal conditions.

In this study, we are therefore focusing on the nekton groups of cephalopods and fish in the Fram Strait. This oceanic region is an area of the Arctic Ocean which provides the possibility to study Atlantic and Polar-Waters in a relatively small geographic area of several hundred kilometers. The Arctic is the habitat of 32 cephalopod species belonging to 15 families known to date, including nine incirrate octopuses, nine sepiolids, three cirrate octopuses, two neritic and demersal squid species and nine squid species, inhabiting meso- and bathypelagic depths (Xavier et al., 2018). Most of these cephalopod species are known to only occasionally occur in the Arctic, as the environmental conditions (low temperatures, high salinity) prevent year-round occurrence (Golikov et al., 2017). Only ten species are known to complete their entire life cycle in high Arctic latitudes, including incirrate octopuses, sepiolids and one pelagic squid species (Xavier et al., 2018 and references therein). *Gonatus fabricii* and *Rossia palpebrosa* are the most abundant cephalopod species in the Arctic, but are assumed to occur in lower abundances than cephalopods in other oceanic regions (Golikov et al., 2017). Nevertheless, *G. fabricii* is main prey for a variety of predators including cetaceans, seals, seabirds, sharks and fishes (Bjørke, 2001; Nesis, 1965; Santos et al., 1999; Wilborg et al., 1984). *Gonatus fabricii* is mostly distributed in mixed Atlantic and Arctic waters and less abundant in coastal and Atlantic waters (Dalpadado et al., 1998), therefore potentially susceptible to the atlantification of its habitat. As a result of climate change, *Gonatus fabricii* is expanding its range poleward and is now found in the eastern part of the Barents Sea and western part of the Kara Sea which previously were too cold for this species (Golikov et al., 2013, 2012). Simultaneously, warm-water cephalopod species have entered colder regions in the Arctic for occasional foraging or underwent permanent range expansions (Golikov et al., 2014). The magnitude of range expansions

in cephalopods is difficult to assess as the Arctic is lacking regular monitoring (e.g., fisheries) surveys. At the same time, cephalopods are hard to sample with nets and video surveys and hence, the Arctic cephalopod biodiversity remains relatively poorly known (Collins and Rodhouse, 2006; Griffiths, 2010; Wormuth and Roper, 1983; Xavier et al., 2016, 2006). This raises the need to apply alternative techniques such as molecular methods to sample diversity in the Arctic Ocean.

Continuous climate change in the Arctic is also expected to cause community-wide changes in fish distribution and abundance on large spatial scales (Fossheim et al., 2015). In the Barents Sea, cold-water species (e.g., *Reinhardtius hippoglossoides, Liparis* spp., *Icelius* spp.) already showed reduced mean abundances, while the abundance of boreal species increased (Fossheim et al., 2015). The same study observed a northward shift of the whole fish community during 2002 and 2012, including the commercially exploited and dominant predators cod and haddock. 242 fish species within 45 families are documented in the entire Arctic and adjacent waters of the Atlantic and Pacific together. Most of these species belong to families included in the suborder Cottoidei (72 species) and Zoarcoidei (55 species), which combined account for more than 52.5% of the species (Mecklenburg et al., 2010). One hundred species of the 242 known fish species (41%) occurring in the Arctic are exclusively arctic species while 142 species (59%) are arctic-boreal, predominantly boreal or boreal species (Mecklenburg et al., 2010).

Arctic benthic communities, except for chemosynthetic communities around hydrothermal vents and cold seeps, rely on organic matter either sinking down from surface layers or being advected from more productive regions (Klages et al., 2004; Lampitt, 1985; Walsh et al., 1989). Vertical carbon fluxes in the Arctic are measured to be lower than in other oceans (Klages et al., 2004). This is due to reduced light intensity by sea-ice coverage resulting in low primary production, which leads to stratified, oligotrophic nutrient concentrations in Arctic surface waters (Anderson et al., 1990; Klages et al., 2004; Subba Rao and Platt, 1984; Wallace et al., 1987). However, primary productivity is comparable to other oligotrophic ocean regions and sufficient to support diverse and abundant benthic life (Klages et al., 2004). Oxygen consumption rates of arctic deep-sea communities suggest that the organic matter supply to the deep calculated by common sediment traps has been underestimated by at least one order of magnitude (Christensen, 2000). One potential source of pelagic carbon that nourishes benthic organisms, but is frequently overlooked, are medium-sized (> 1 cm) foodfalls of fishes and cephalopods. Detecting those foodfalls is very difficult due to rapid

scavenging rates, temporal variability, spatial aggregation, and technical and logistical challenges to find medium-sized foodfalls in the vast ocean (Stockton and Delaca, 1982). In the Fram Strait, the only reported foodfalls are a decapod carcass in the Molloy Deep at 5551 m depth (Klages et al., 2001) and a fish carcass at 1280 m depth west off Svalbard (Soltwedel et al., 2003). The fish carcass was the only medium-sized foodfall observed in the Fram Strait after four years of extensive visual observations with various camera systems (Soltwedel et al., 2003). The rarity of natural foodfall observations in seafloor surveys underlines the need for novel techniques for their detection.

Environmental DNA metabarcoding is a promising tool in biodiversity assessment and species detection and offers major advantages over conventional monitoring methods (Creer et al., 2016). This molecular genetic approach is based on the fact that every organism leaves its DNA in the environment through e.g., feces or mucus. That DNA can be detected in an environmental sample such as seawater or sediment without the source organism being present (Hansen et al., 2018; Sinniger et al., 2016; Taberlet et al., 2018; Thomsen et al., 2016). The analysis of eDNA traces by DNA metabarcoding allows species identification to provide a snapshot of the current species composition in a given area (Ji et al., 2013). Environmental DNA has the potential to detect more or different species than traditional methods (Boussarie et al., 2018). Net and video surveys are susceptible to organism avoidance especially of larger species and thus tend to bias sampling depending on the gear (e.g., mesh size, mouth opening of the net, sound and light intensity of the video system). In Southwest Greenland, eDNA sequencing reads of fish assemblages correlated with biomass and abundance data obtained from trawling and eDNA. That study also detected a high number of eDNA reads stemming from the Greenland shark (*Somniosus microcephalus*), while catching only a single specimen with trawling (Thomsen et al., 2016). Recent eDNA studies in the Arctic have focused on metazoan diversity in coastal ecosystems and in harbors (Grey et al., 2018; Lacoursière-Roussel et al., 2018; Leduc et al., 2019; Sevellec et al., 2021) and fish diversity from continental slope depths (188 to 918 m) in the Davis Strait off Southwest Greenland (Thomsen et al., 2016). The pelagic realm of the Arctic has remained almost unexplored with eDNA, especially with focus on pelagic fauna larger than 1 cm. To date, only two studies have focused on general cephalopod distributions and community compositions in the North Atlantic (Merten et al., 2021; Visser, Merten et al., 2021) and one study investigated the giant squid *Architeuthis dux* in an intra-specific eDNA approach in the Sea of Japan (Wada et al., 2020).

Our area of interest is the Fram Strait, located around 70 to 80° North in the Arctic Ocean. This area is influenced by warm Atlantic Water flowing poleward as the West Spitsbergen current (Gascard et al., 1995; Walczowski et al., 2005) and Arctic Waters flowing southwards as the East Greenland Current. The Fram Strait is particularly interesting for climate change studies, as this region is directly affected by a potentially increased inflow of warm Atlantic water due to the prevailing currents. Zooplankton communities in the Fram Strait are dominated by a small number of species (Weydmann et al., 2014). To closely monitor the Arctic benthic ecosystem and the euphotic zone of the Fram Strait, the Alfred Wegener Institute, Helmholtz-Centre for Polar and Marine Research (Germany) established the deep-sea observatory HAUSGARTEN west of Svalbard (Soltwedel et al., 2005). In the context of the HAUSGARTEN observatory, cruises are conducted annually since 1999 to sample 21 stations resulting in a unique time-series on mega-, macro- and meiobenthic and prokaryotic fauna, as well as biogeochemical and geological processes (Bauerfeind et al., 2009; Bergmann et al., 2009; Hoste et al., 2007). Most faunal studies conducted within the time-series focused on benthic communities and zooplankton, while knowledge on fish and cephalopods in the Fram Strait are fragmentary (Christiansen et al., 2016). Here, we sampled eDNA of cephalopods and fishes in Arctic seawater and sediments in the scope of the HAUSGARTEN observatory. We aim to compare fish and cephalopod eDNA detections in seawater and sediment with existent knowledge on diversity and distribution of fish and cephalopods in this area to (I) establish a biodiversity baseline and to (II) identify current distribution patterns which allow comparison with ongoing climate change and to (III) predict medium-sized foodfall species in the Arctic.

3.2. Material and Methods

Sample collection, filtration and DNA extraction of seawater and sediment eDNA

Seawater samples for eDNA metabarcoding were collected during the cruise PS121 in August/September 2019 (Metfies, 2019) and MSM95 in October/November 2020 in the Fram Strait of the Arctic Ocean (Figure 1). Samples were taken in triplicate between 50 m and above the bottom at three stations (S3, HG4, N4) in 2020 and four stations (S3, HG4, N4, EG4) in 2019 resulting in 177 discrete samples (Figure 2). The depth of the seawater samples taken above the bottom varied between 2250 and 2705 m, depending on the bottom depth of the station. Sampling was conducted using 12-liter Niskin bottles mounted on a CTD rosette. During the cruise PS121, six liters were filled from one Niskin bottle into three 2-liter bottles, that were previously cleaned with bleach and flushed with MilliQ. On cruise MSM95, we had the opportunity to directly filter the water from the Niskin bottles by attaching the tubing needed for filtration directly on the Niskin bottle.

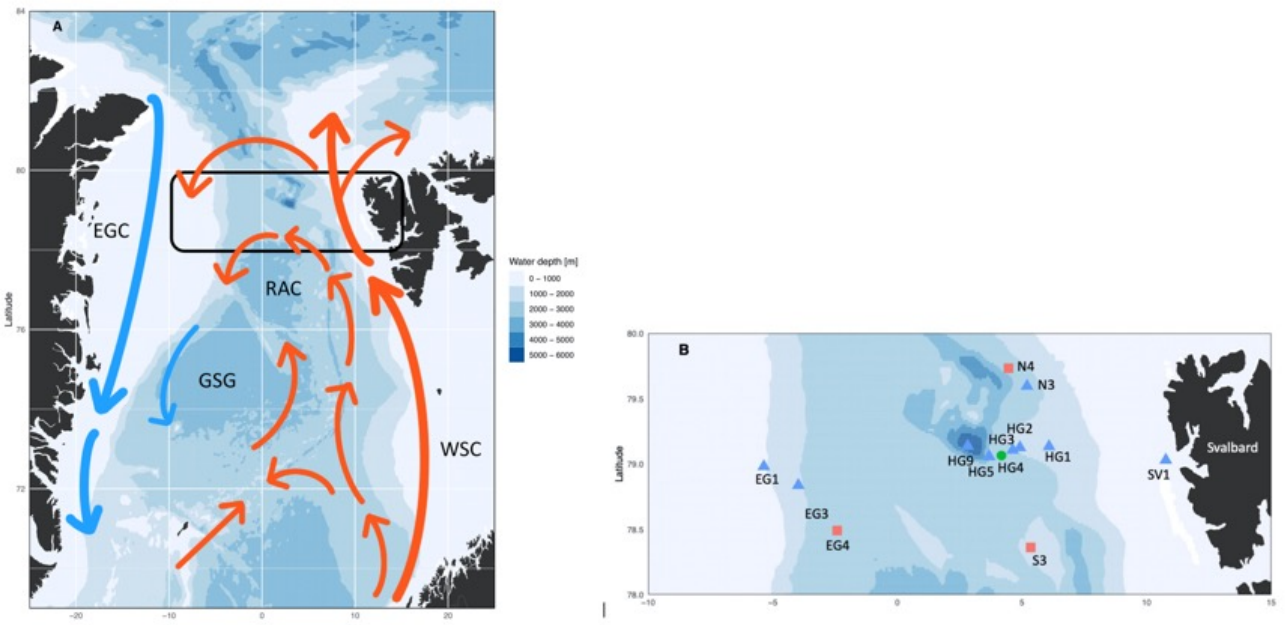

Figure 1| The Arctic Ocean off Greenland and Svalbard. A) The current system of the North Atlantic and Arctic Ocean. WSC: West Spitzbergen Current, RAC: Return Atlantic Current, EGC: East Greenland Current, GSG: Greenland Sea Gyre. The orange arrows indicate warm, Atlantic water and the blue arrows cold, Arctic water. The black square shows the Fram Strait and sampling area. **B)** Sampling sites for collecting eDNA in sediment (blue triangles), seawater (green dot) or both (red square) in the Fram Strait of the Arctic Ocean.

Per 0.22 µm Sterivex-GP filter (Merck Millipore), two liters of water were filtered with a peristaltic pump. For filtration controls, MilliQ water was filtered instead of seawater. The Sterivex filters were stored at -80°C until further processing in the lab. DNA was extracted from the filters using the DNeasy Blood and Tissue Kit (Qiagen) with a modified protocol. Before DNA extractions, each filter was cleaned with RNase Away (Carl Roth) on the outside and then processed under a sterile clean bench to avoid contamination. Then, 720 µl of ATL-buffer and 80 µl of Proteinase K were added directly into the filter and incubated at 56°C for 2 hours with agitation. After incubation, 600 µl of the lysate was transferred from the filter to a sterile 2 ml Eppendorf tube and mixed with 600 µl AL-buffer and 600 µl 99% high grade ethanol. After this step, the DNeasy Blood and Tissue protocol was followed. DNA was eluted in 2x30 µl AE-buffer from the kit. DNA extracts were stored at -20°C until further processing.

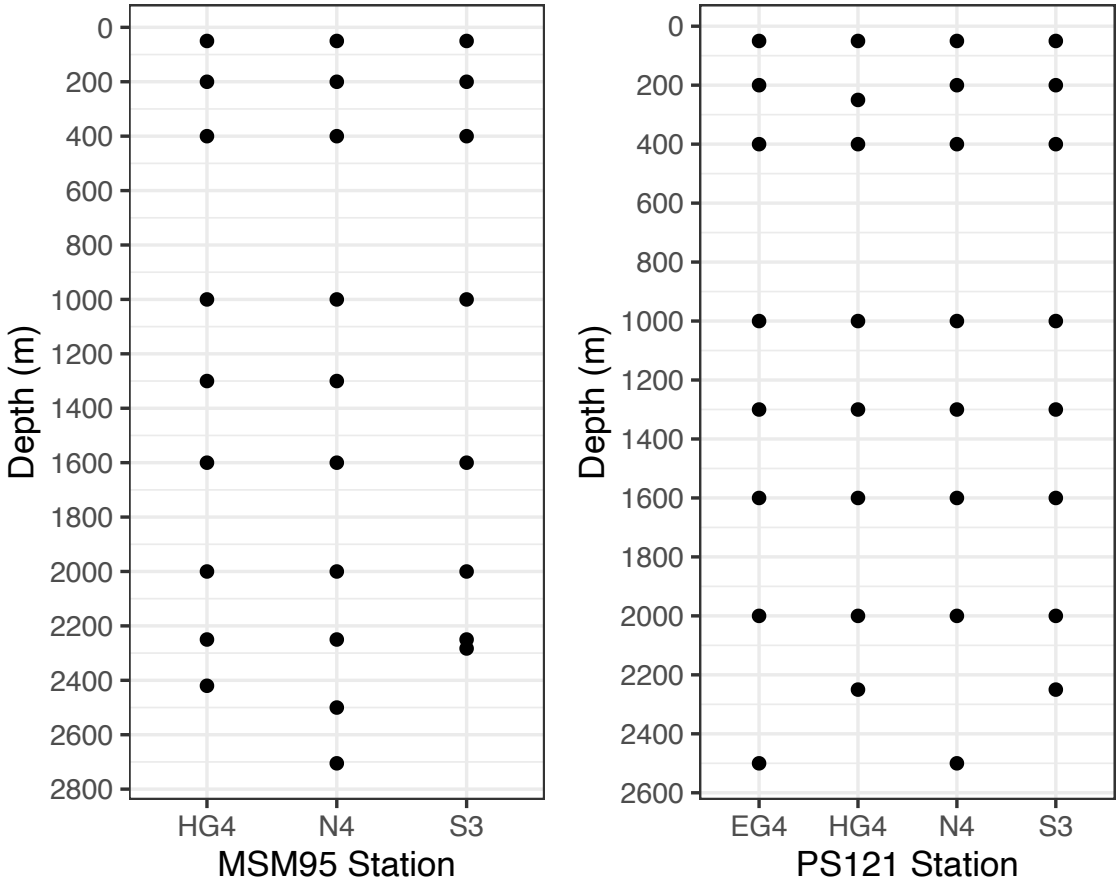

Figure 2| Sampling depths during MSM95 and PS121 for cephalopod eDNA in seawater in the Fam Strait, Arctic Ocean. MSM95 was sampled in 2020 and PS121 in 2019. During MSM95, three stations between 50 and 2705 were sampled and during PS121 the same three stations plus an additional station off the Greenland coast were sampled between 50 and 2500 m depth. The deepest depth sampled for all stations was right above the bottom.

Sediment samples were collected with a Multicorer during the cruise PS121 in August/September 2019 in the Fram Strait of the Arctic with *RV Polarstern* at twelve stations (Figure 1). Sediment samples were taken from three cores from the Multicorer by scooping the first 3 cm of surface sediment into sterile Falcon tubes. The sediment samples were stored at-20°C until further processing. DNA from the sediment was extracted using a DNeasy Power Soil Kit (Qiagen) in combination with a QIAvac 24 Plus Vaccum Manifold following the manufacturers protocol. Sediment DNA was eluted in 2x30 µl Solution C6 (10 mM Tris) and stored at -20°C. To reduce contamination, we only worked with single-use consumables.

For both, seawater and sediment extractions, a DNA extraction control was included to check for potential contamination in the laboratory. As indicated below, positive and negative controls were also included at the PCR stage.

Library preparation and sequencing

The seawater and sediment eDNA were amplified with two universal primer sets, one targeting the nuclear 18S rRNA gene of cephalopods (Ceph18S_forward: 5'-CGCGGCGCTACATATTAGAC-3' and Ceph18S_reverse: 5' -GCACTTAACCGACCGTCGAC-3'; amplicon length = 140 – 190 bp) (de Jonge et al., 2021) and the other one targeting the mitochondrial 12S rRNA gene of fishes (teleo_F: 5'-ACACCGCCCGTCACTCT-3' and teleo_R: 5'-CTTCCGGTACACTTACCATG-3'; amplicon length = 80 – 100 bp) (Valentini et al., 2016). We chose a mitochondrial 12S rRNA primer for fish, as they have a similar taxonomic resolution as the COI marker and provide a high-resolution power for the targeted taxonomic group. COI was not chosen, because its primer binding sites have been shown to often not be conserved enough for metabarcoding studies (Deagle et al., 2014). The Illumina linker, Illumina index, Illumina adapter and a spacer for more variability were directly added to the primer sequence resulting in primer lengths between 86-97 bp (Supplementary Material Table 1). All samples were amplified in triplicates resulting in nine PCR products per sampling depth and site. The PCR reagent mix for the Ceph18S primer had a total volume of 25 µl and included 10 µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 8 µl PCR-grade H_2O, 0.5 µl dsDNAse (ArcticZymes), 0.5 µl dsDNAse buffer DTT (ArcticZymes), 0.5 µl forward primer (10 µM) and 0.5 µl reverse primer (10 µM). The PCR reagent mix for the 12S rRNA gene fish primer had a total volume of 25 µl and included 10 µl TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 8 µl PCR-grade H_2O, 0.5 µl dsDNAse (ArcticZymes), 0.5 µl dsDNAse buffer DTT (ArcticZymes), 0.5 µl forward primer (5 µM) and 0.5 µl

reverse primer (5 µM). Before the eDNA template was added, the reactions were incubated on a thermocycler at 37°C for 15 minutes and 60°C for 15 minutes for activation and inactivation of the DNase, respectively, to remove any contamination by foreign double-stranded DNA prior to PCR-amplification. Subsequently, 5 µl DNA (2-20 ng) extracts were added as well as negative controls containing PCR-grade water instead of DNA template and ppositive controls containing DNA extract from known species (Table 1).

A touchdown PCR program was applied for the Ceph18S primer starting with an initial denaturation step at 95°C for 5 min, 8 cycles of 94°C for 30 sec, 70°C for 30 sec (decreasing this temperature by 1°C after every cycle) and 72°C for 1 min. Subsequently, 32 additional cycles were run with 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min. The program was terminated by a final extension step of 72°C for 5 min. The PCR program for the 12S rRNA gene fish primer started with an initial denaturation step at 95°C for 10 min followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The program was terminated by a final extension step of 72°C for 5 min. Fragment sizes were verified on a 1.5% Agarose gel stained with GelRed (Biotum). The PCR triplicates with the same Illumina index were pooled and their DNA concentration measured with a Qubit dsDNA HS Assay Kit (Molecular Probes Life Technologies) on a Qubit Fluorometer. Following, all samples were pooled to equimolar concentrations resulting in five libraries and therefore five sequencing runs. All cruises and primers were sequenced in individual runs on different flow-cells, except of the seawater eDNA for fish from the two cruises that were sequenced together on one run. The libraries were run on a 2% Agarose gel, purified using the ZymoClean Gel DNA recovery Kit (Zymo Research) and quantified using a Qubit dsDNA HS Assay Kit (Molecular Probes Life Technologies). Insert size distribution was determined with the TapeStation 4200:D5000 ScreenTape (Agilent). The working solution was diluted to 2 nM and loading solution was prepared according to protocol. The library pools were loaded with 8 pm and 20% PhiX spiked in to increase diversity. The sequencing runs targeting cephalopods were done on an Illumina MiSeq with the MiSeq Reagent Kit v3, 600 cycles (PE), 2x 300 bp (Illumina) and the sequencing runs targeting fish were done an Illumina MiSeq with the MiSeq Reagent Kit v2, 300 cycles (PE), 2x 150 bp (Illumina). The sequencing runs targeting sediment were processed in collaboration with the Alfred-Wegener Institute (AWI) in Bremerhaven, Germany, and the remaining sequencing runs were conducted at the Institute of Clinical Molecular Biology (IKMB) in Kiel, Germany.

Bioinformatic analysis

After sequencing, the obtained reads were demultiplexed, sorted by sample and the indexing primer was removed from the sequence. The primers were also removed using cutadapt (version 1.18). Only sequences with both the reverse and forward primer were used for further analysis using the Diverse Amplicon Denoising Algorithm (DADA2, version 1.16.0) pipeline. The sequences were filtered according to the following parameters: sequences with Ns were discarded (maxN=0) and sequences were truncated at the first instance of a quality score less than or equal to 2 (truncQ=2). Sequences with an expected error higher than 3 for the forward and 5 for the reverse reads were discarded (maxEE=3,5). A higher expected error rate was set for the reverse reads in comparison the forward reads, as the quality of the reverse reads was lower than for the forward reads. Forward and reverse reads were merged in amplicon sequence variants (ASVs) with an overlap of at least 80 bp for cephalopods and 20 bp for fish resulting in sequences ranging between 100 and 180 bp for cephalopods and 51 and 125 bp for fish.

For cephalopods, the taxonomic assignment of the samples and all controls were performed as in Merten et al., (2021). Briefly, cephalopod sequences from the SILVA 18S database were searched against the NCBI Genbank database (accessed in June 2020) until no further cephalopod sequences were found resulting in 169 sequences from 119 species. The reference database for cephalopods was then assembled by combining these cephalopod sequences with all other eukaryotic 18S rRNA sequences from the SILVA database, to prevent spurious assignments of non-cephalopod amplicons. For fishes, taxa assignments were conducted with BLAST (Camacho et al., 2009). If the sequence similarity to a query sequence was ≥ 99%, the e-value was less than 10^{-15} and no other taxa had a similar hit, the assembled sequence was assigned to the top-hit species. Assembled sequences with the aforementioned parameters and sequences similarities of 97-99% and 95-97% were assigned to genus or family level, respectively. If the top-hits included more than one taxon, the taxonomic resolution was decreased and the nearest shared relative taken. Taxa that were freshwater species or neither fish nor cephalopods were excluded from the complete dataset. For other taxa in the negative, filtration or extraction controls, we subtracted the maximum number of reads found for that ASV from the corresponding ASV in all other samples. Remaining sequences with less than 10 reads were discarded. We refrained from changing taxonomic assignments that were made on genus level to species level in case that genus only included one species or just one species is known from

the Fram Strait. As the species diversity is under-sampled in the Arctic, we do not know whether those assignments might belong to cryptic species or unknown species and we therefore decided to apply a conservative approach to prevent misinterpretation.

Statistical Analysis

All statistical analyses were conducted in R version 3.6.3. To compare species communities between the different stations sampled for sediments, the stations were binned into areas (N-Sites = Northern Arctic, EG-Sites = Greenland and HG-Sites = Central Arctic). For the seawater eDNA data, the different depths were binned into shallow (0-700 m), meso (800 – 2000 m) and deep (2250 – 2705 m) in order to test whether the taxa composition changes with depth. All analyses were conducted with presence/absence data and the read abundance data for comparison. In order to derive a non-metric multidimensional scaling (NMDS) plot, a Bray-Curtis (for read abundance data) or Jaccard index (for presence/absence data) matrix was created using the package *vegan* (Oksanen et al., 2019). We then performed a permutational multivariate analysis of variance using distance matrices with the function *adonis* to test whether statistically significant differences between the different areas or depths existed. For the comparison between sediment and seawater diversity, we constructed Venn diagrams. To test for a correlation between the number of sequencing reads and eDNA detections per depth and station for fish, we applied Kendall rank correlation coefficient using the function *cor.test*, *method = "kendall".*

3.3. Results

eDNA sequencing results targeting cephalopods and fish in seawater of the Fram Strait

Sequencing results for cephalopod eDNA

Targeting cephalopods in seawater, a total of 6,090,710 sequences were obtained after sequencing with a mean (± standard deviation (SD)) of 56,395 (± 147,115) per sample for MSM95. The number of reads ranged between 13 and 16 in three negative controls belonging to the species used for the positive controls (*Chtenopteryx* sp. and *Abralia veranyi*). The remaining negative controls had no reads. After denoising, merging and removal of chimeras in DADA2, 5,407,110 reads (88%) remained with a mean (± SD) of 50,066 ± 130,431 reads. These sequences were classified into 97 unique amplicon sequence variants (ASVs). After removal of i) ASVs with less than 10 reads, ii) non-identifiable sequences that could not be identified to a phylum, iii) species that are not knowingly occurring in the Arctic or Atlantic Ocean and iv) ASVs from positive controls, 23 ASVs remained (Supplementary Material Table 2). These 23 ASVs could be assigned to six taxa. Three were identified to species (*Filippovia knipovitchi*, *Teuthowenia maculata, Vampyroteuthis infernalis*), one to genus (*Gonatus* sp.), one to family level (Gonatidae) and one to the order Teuthida (Figure 3). The most frequent detected taxa were Gonatidae and *Gonatus* sp. which were represented in 85% and 15% of the sampled depths, respectively (n=23 and n=4, respectively; Figure 3). Gonatidae was also the taxa that was most represented in the sequence reads with 92% (n=183,347; Figure 4). The sequencing run that included eDNA seawater samples from PS121 had to be excluded from the analysis due to extreme contamination from taxa used as positive controls in previous projects.

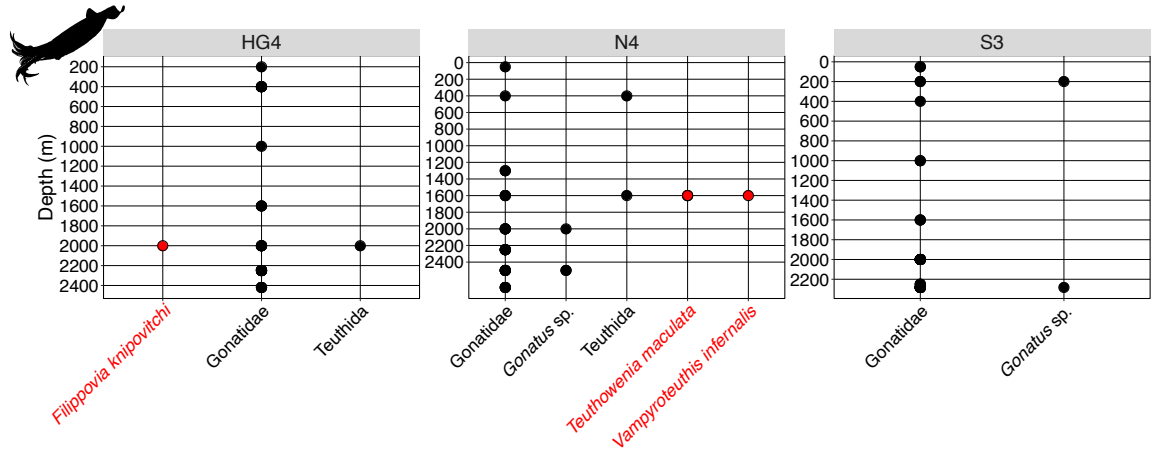

Figure 3| Depth distribution of cephalopod taxa detected with eDNA analysis from cruise MSM95 in 2020. Each panel shows the taxa occurrence and depth distribution of one of the sampled stations (HG4, N4, S3). In total, 23 ASVs could be assigned to six taxa. Bottom depths are 2508 m for HG4, 2727 m for N4 and 2339 m for S3. The taxa depicted in red are very likely false positives due to contamination from either used in positive controls from previous projects (*Filippovia knipovitchi* and *Vampyroteuthis infernalis*) or closely related sister species that cannot be differentiated with the primer used here (*Teuthowenia maculata*).

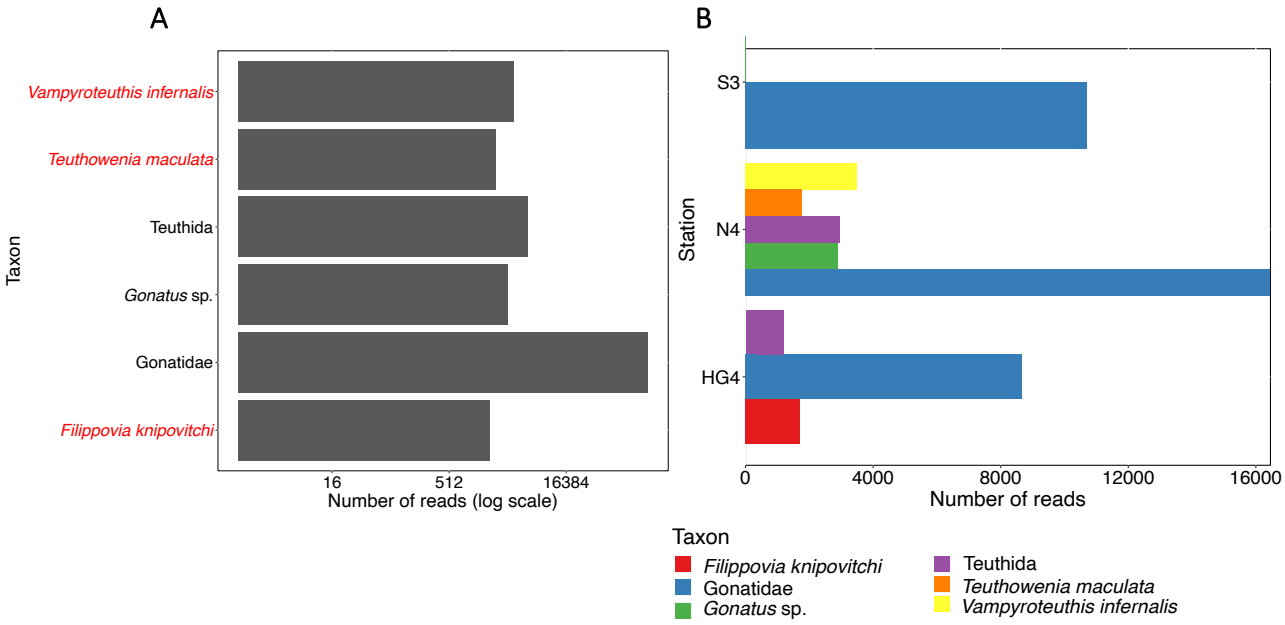

Figure 4| Cephalopod taxa detected with eDNA metabarcoding in seawater samples. A) Number of sequencing reads detected in 2020 during the cruise MSM95 in the Fram Strait, Arctic Ocean. The three stations analyzed have been pooled. The taxa highlighted in red are very likely false positives. **B)** Number of sequencing reads detected in 2020 during the cruise MSM95 in the Fram Strait at the three sampled stations.

Sequencing results for fish eDNA

Comparison between the cruises PS121 and MSM95

The samples of the cruises PS121 and MSM95 were sequenced on the same Illumina MiSeq run, but the sequencing depths of the cruises were substantially different. The cleaned dataset for PS121 included 21,324 reads in total with a mean of 1,185 reads and a standard deviation of ±2,679 reads per sample. In comparison, the sequencing depth for MSM95 was one order of magnitude higher than for PS121. In total, the eDNA samples for MSM95 contained 321,349 reads with a mean of 16,912 reads and a standard deviation of 33,674 reads. Therefore, a direct comparison between the two cruises might be misleading, also because they took place during different seasons (PS121 in 2019 in August/September and MSM95 in 2020 in October/November). The most commonly detected taxon (*Mallotus villosus)* that was also the taxon with most sequencing reads, was similar for both cruises. We identified a high overlap in taxonomic composition between the cruises PS121 and MSM95 (Figure 5) with eleven shared taxa. Seven taxa were only detected during PS121 and nine taxa only during MSM95. Due to the reasons mentioned above, we did not further compare or differentiate the different cruises and years in this study and combined the cruises in the following analysis.

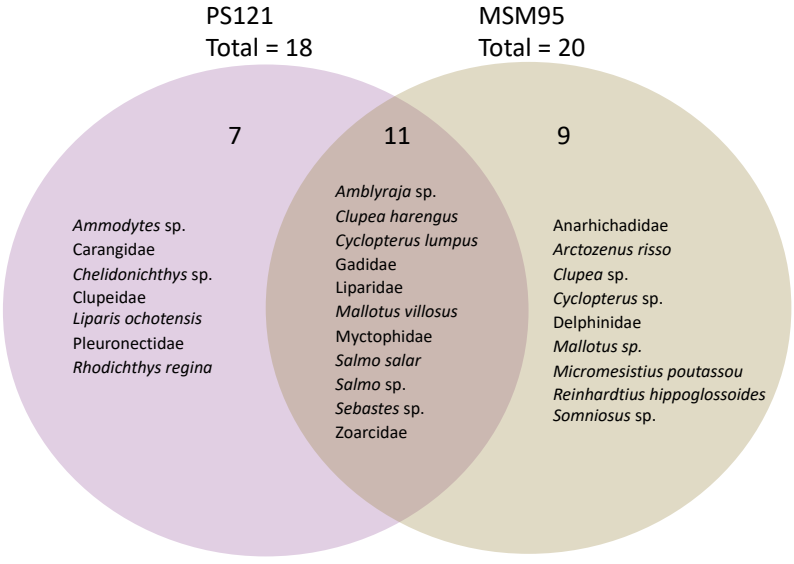

Figure 5| Venn diagram showing the similarities and dissimilarities in taxa composition between the cruises PS121 in 2019 and MSM95 in 2020. Eleven taxa were detected during both cruises and years while seven taxa were only detected in 2019 during PS121 and nine taxa only in 2020 during MSM95.

Analysis of fish eDNA combined for both cruises

After sequencing and without cleaning, a total of 5,701,698 reads were retrieved with an average (± standard deviation (SD)) of 20,363 (± 31,573) reads per sample. After DADA2 cleaning, denoising and chimera deletion, 5,543,889 (97%) of reads were retained with an average of 19,800 (± 30,893) reads that belonged to 174 ASVs. Taxa detected in the negative controls were either *Homo sapiens*, species from the positive control (*Clarias gariepinus* and *Oncorhynchus* sp.), species from positive controls used in previous studies (*Scomber scombrus*), freshwater species (*Barbatula barbatula*, *Gobio gobio,* Leuciscidae) or species held in aquaria in the same building as our labs (*Syngnathus rostellatus*). These taxa were excluded from the dataset. The only species detected in one of the negative controls that is known to occur in the Arctic was *Sebastes* sp., however, the read counts were very low (21 and 43 reads). We took the maximum number of reads for that taxon found in the negative control and subtracted that number from the corresponding ASV read count from every sample. After removing ASVs with i) less than 10 reads, ii) that could not be assigned to a phylum, iii) belonged to the positive controls or iv) were included in the negative controls, 92 ASVs remained. Those 92 ASVs were assigned to 40 taxa. All 40 taxa were checked for their distribution. Three taxa were identified as freshwater species (*Barbus barbus*, *Gobio* sp. and Cyprinidae). Two taxa belonged to fish genera that are kept in aquaria in the same building as our labs (*Aeoliscus strigatus* and *Hippocampus* sp.). Five taxa assigned to birds (*Hirundo* sp., Anatidae, *Anas platyrhynchos* and *Gallus gallus*). Two taxa are known to only occur in the Antarctic Ocean (*Notothenia coriiceps* and *N. rossii*) and two taxa are known only from subtropical and temperate oceans (*Sardinella maderensis* and Exocoetidae). All of the spurious fish taxa mentioned above occurred in only one sample, except of Exocotidae and *Sardinella maderensis* that occurred in four and six samples, respectively. All spurious above-mentioned taxa (fish and vertebrate taxa) were excluded from the dataset, resulting in 27 remaining taxa of which nine could be identified to family (35%), eight to genus (30%) and nine to species level (35%) (Supplementary Material Table 3). The taxon that was most often detected in the Fram Strait of the Arctic (in 41% of the sampled depths, *n*=24) and also had the highest abundance of reads (44% of the total read count, *n*= 150,628) was *Mallotus villosus*. The second most often detected taxa was Zoarcidae (27%, *n*= 16), however, that taxon was only represented by 2% in the read counts (*n*=6,229) (Figure 6). *Sebastes* sp. was detected in 25% of the sampled depths (*n*=15) and represented in 13% (*n*=44,617) of the read counts. Liparidae and *Clupea harengus* followed with 24% (*n*=14) and 20% (*n*=12) of detections, respectively and represented in 9% (*n*=32,091) and 17% (*n*=56,655) of the read counts, respectively (Figure 6).

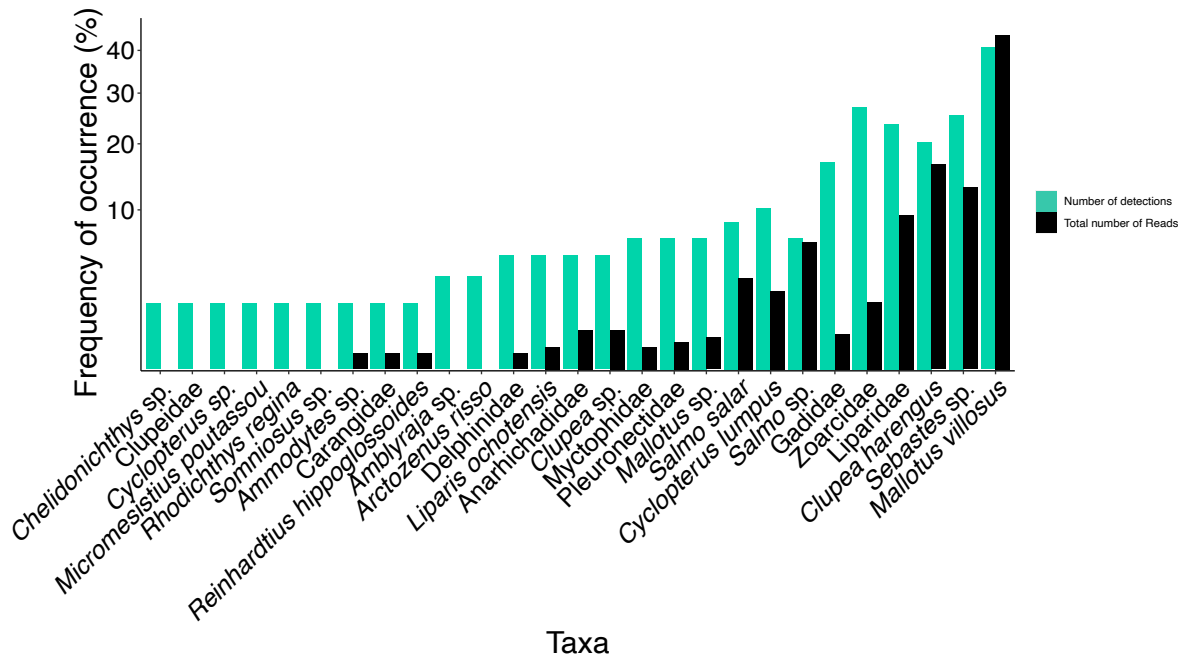

Figure 6| Frequency of occurrence in percent of the fish taxa detected with eDNA metabarcoding. The black bars represent the frequency of occurrence of sequencing reads and the green bars represent the frequency of occurrence of taxa detections.

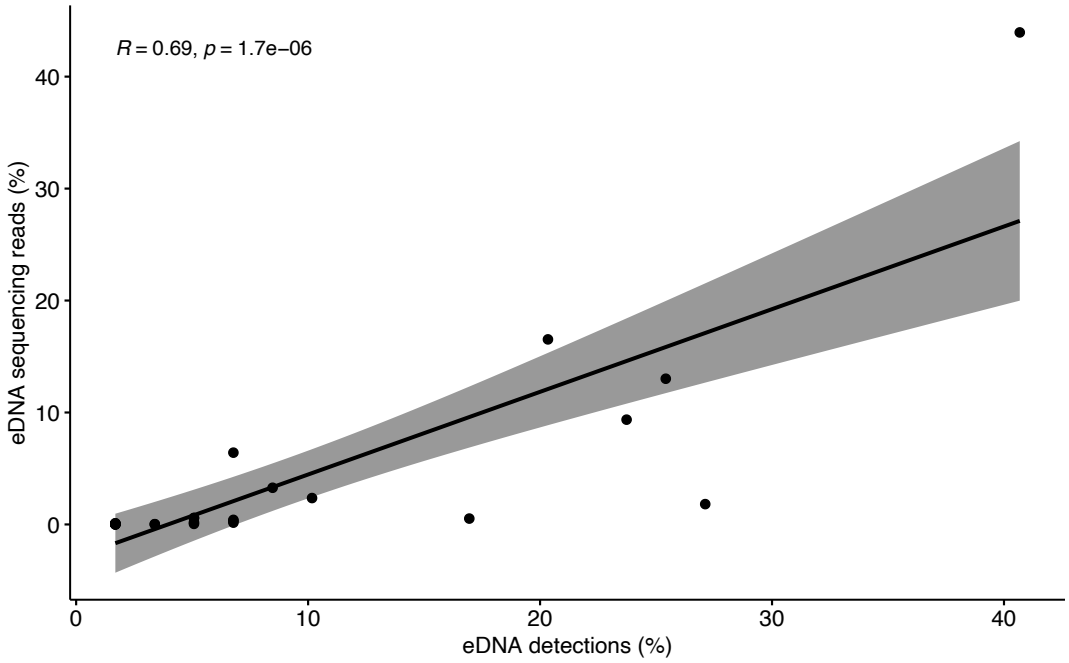

Figure 7| Kendall rank correlation coefficient measuring the association between eDNA sequencing read abundance and eDNA detections. The coefficient showed a significant association between the fish eDNA detections per depth and station and the number of sequencing reads.

All other taxa were detected in less than 17% of the sampled depths. Kendall rank correlation coefficient was used measuring the association between the eDNA sequencing read abundance in percent and eDNA detections in percent at depth and station. A significant positive correlation was detected between the percent of eDNA detections and sequencing reads (*tau = 0.69, p < 0.05,* Figure 7).

A rare taxon detected with eDNA was *Somniosus* sp., which aligned to *Somniosus microcephalus* and *S. pacificus* and therefore was assigned to genus level. However, *S. pacificus* is a pacific species, therefore, we can assume to have detected eDNA of *S. microcephalus,* the Greenland shark, at a depth of 2420 m.

The number of taxa per depth varied between four and 14. Most taxa were detected at 200 m (*n*=11) and 2000 m (*n*=14) (Figure 8). The bottom depth differed between 2250 and 2705 m at the different stations. However, when comparing the individual stations, the peak number of fish taxonomic diversity was present in deeper depths too (Figure 9). EG4 was only sampled during one year and showed the highest number of fish taxa at 2500 m (*n*=5). The other stations were sampled in both years and showed peak numbers of taxa at 2000 m (HG4, *n*=10), 50 and 2500 m (N4, *n*= 7) and 2250 m (S3, *n*=8). The taxa community composition did not change significantly with depth (*adonis* r^2 = 0.22, $p > 0.1$).

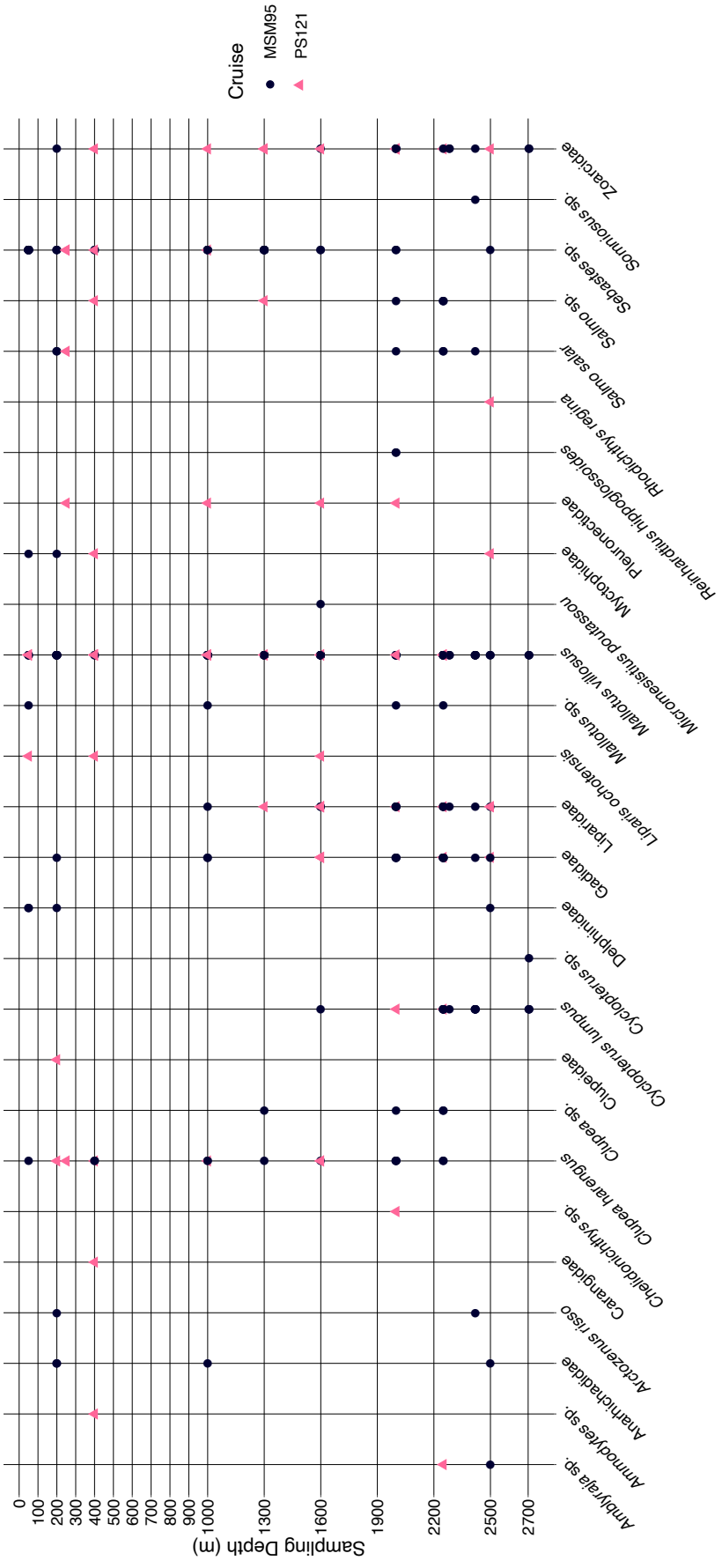

Figure 8| Depth distribution of fish taxa detected in seawater with eDNA metabarcoding in 2019 and 2020 during the cruises PS121 (pink triangles) and MSM95 (black dots) in the Fram Strait, Arctic Ocean. In total, 27 fish taxa were detected between 50 m and 2700 m.

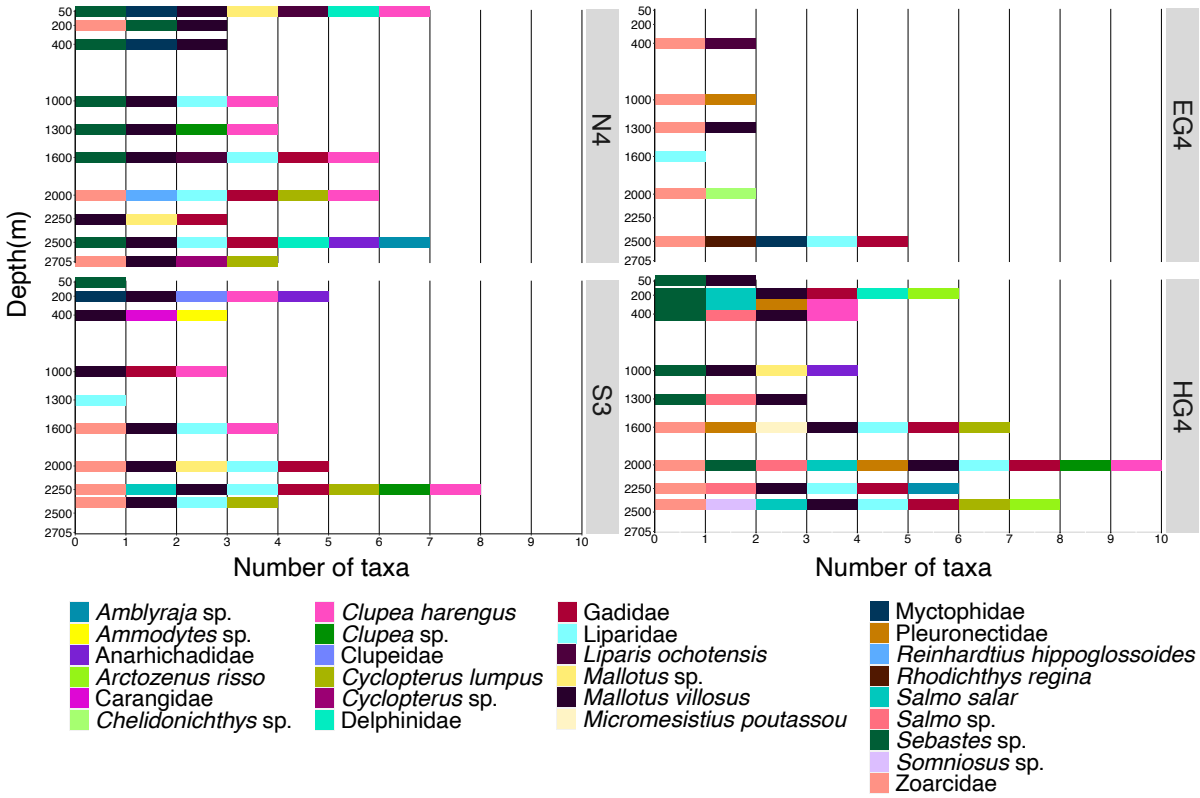

Figure 9| Number of individual taxa detected with eDNA metabarcoding at depths between 50 and 2705 m at four stations in the Fram Strait, Arctic Ocean, sampled during the cruises PS121 in 2019 and MSM95 in 2020. The four panels show the taxa diversity found at the different stations (EG4, HG4, N4 and S3).

eDNA sequencing results targeting cephalopods and fish in sediment of the Fram Strait

The sequencing run targeting cephalopods in the sediment did not yield any cephalopod sequence from the eDNA samples. The positive controls amplified successfully, therefore, we assume that either the cephalopod eDNA concentrations in the sediment were too low for the primer used to be detected or there was no cephalopod eDNA present in the samples.

For fish, a total of 3,746,327 reads were obtained after sequencing with an average (± standard deviation (SD)) of 18,011 (± 55,634) reads per sample. After DADA2 cleaning, 3,647,036 reads (97%) were retained with an average of 17,534 (± 54,540) reads per sample. Those sequences aligned into 285 unique ASVs. After removing ASVs with i) less than 10 reads, ii) that could not be assigned to a phylum, iii) belonged to the positive controls or iv) do not occur in the Arctic or Atlantic Ocean, 23 ASVs remained. Eight of the negative controls contained reads, however, the contamination only

stemmed from species used in the positive controls (*Clupea harengus* and *Scomber scombrus*), *Homo sapiens* or *Gallus gallus* (chicken). All positive controls detected the corresponding species (either *Clupea harengus* or *Scomber scombrus* or both in the mock control). The 23 ASVs were assigned to 18 taxa (Figure 10) of which seven could be assigned to family (39%), four to genus (22%) and seven to species level (39%) (Supplementary Material Table 3). Fish eDNA was detected at all 12 sampled stations. The number of taxa detected per station ranged between three and nine. There were no significant differences between the pooled stations (central Arctic, Greenland and Northern Arctic) using the read abundance matrix (*anosim*: $r^2 = 0.08$, $p > 0.5$) or presence/absence matrix (*anosim*: r2 = .07, $p > 0.4$).

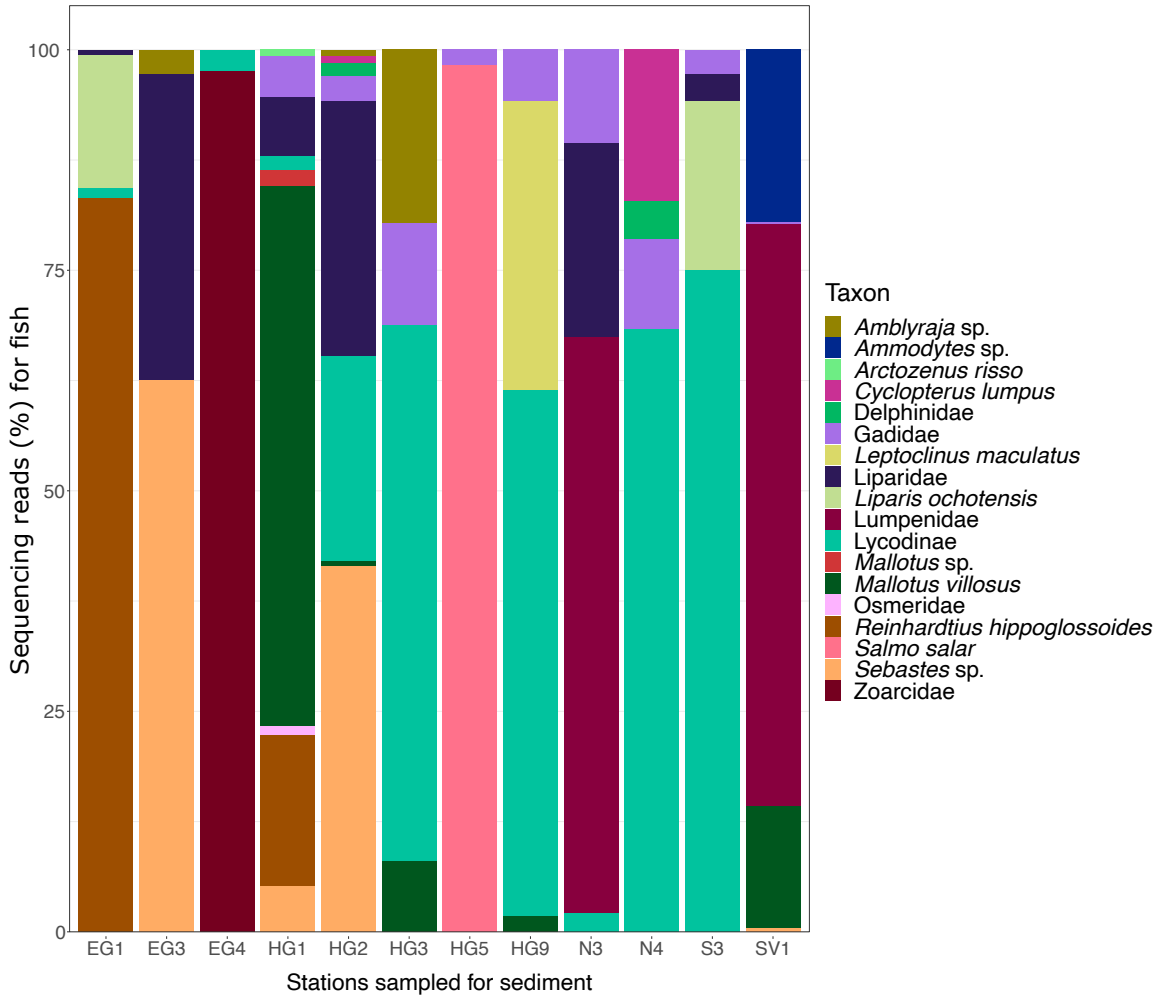

Figure 10| Frequency of occurrence of sequencing reads assigning to fish after eDNA metabarcoding of sediment samples from the cruise PS121 collected in 2019 in the Fram Strait, Arctic Ocean. In total, 18 taxa could be assigned to 23 ASVs.

Comparison of fish eDNA detected in sediment and seawater

For a representative comparison between seawater and sediment fish eDNA, only overlapping stations sampled for both were taken into account. We detected eight taxa in sediment and 27 taxa in seawater (Figure 11). Seven taxa were found in both the sediment and the seawater samples. Five of those taxa were benthopelagic or demersal taxa (*Cyclopterus lumpus*, Gadidae, Liparidae, *Liparis ochotensis* and *Salmo salar*), one of them was a pelagic taxon (Delphinidae) and one a purely benthic taxon (Zoarcidae). One benthic taxon was only found in the sediment (Lycodinae) and 20 taxa only in seawater. The majority of the taxa found only in seawater were pelagic (*n*=10) or benthopelagic/demersal (*n*=8). Two benthic taxa were detected in the seawater (*Amblyraja* sp. and Pleuronectidae), however, Pleuronectidae contains species that are benthopelagic.

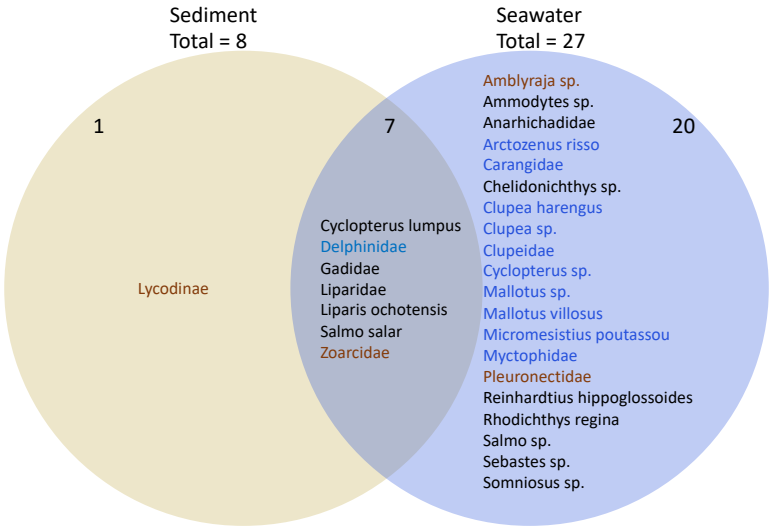

Figure 11| Comparison between taxa diversity found in seawater and sediment eDNA samples. Brown = benthic taxa, blue = pelagic taxa, black = benthopelagic or demersal taxa. For a representative comparison only stations sampling both sediment and seawater were taken into account (EG4, N4, S3, HG4). As sediment was not sampled at HG4, but seawater, we included HG5 in the sediment comparison. HG5 is located directly next to HG4 and should have a similar taxa diversity.

Combining the taxa of all sediment and seawater stations, we were able to detect 31 taxa in the Fram Strait of the Arctic Ocean (Table 1). Twelve of them were identified to family (39%), nine to genus (29%) and ten to species level (32%). The detected taxa belonged to 19 different families. All taxa were known to occur in the Arctic or Northern Atlantic Ocean except of two. The first one was the species *Liparis ochotensis,* which is a Pacific species and occurs in the Bering Sea. The second taxon

assigned to the family Carangidae that mainly involves species inhabiting tropical and temperate waters. Most detected taxa were benthopelagic (35%), followed by pelagic (26%), demersal (16%) and benthic (10%). Another 10% could not be assigned to one group, because they were either benthic/pelagic or pelagic/demersal.

Table 1| Overview of all taxa detected in sediment and seawater with eDNA metabarcoding in this study in the Fram Strait of the Arctic Ocean in 2019 and 2020.

Family	Taxa	Common name	Source	Habitat	Distribution
Rajidae					
	Amblyraja sp.	ray	Sediment, seawater	benthic	North Atlantic and adjacent Arctic
Ammodytidae					
	Ammodytes sp.	Sand lances	Sediment, seawater	benthic and pelagic	North Atlantic and Arctic
Paralepididae					
	Arctozenus risso	spotted barracudina	Sediment, seawater	bathypelagic	Arctic to Antarctic
Cyclopteridae					
	Cyclopterus sp.		seawater	Benthopelagic	up to Greenland
	Cyclopterus lumpus	Lumpfish	Sediment, seawater	Benthopelagic	Eastern Atlantic: Barents Sea, Iceland and Greenland to Spain
Delphinidae	Delphinidae		Sediment, seawater	Pelagic	
Gadidae	Gadidae		Sediment, seawater	benthopelagic	
	Micromesistius poutassou	blue whiting	Seawater	Bathypelagic	North Altantic and Arctic
Lumpenidae	Lumpenidae		Sediment	Benthic	
	Leptoclinus maculatus	Daubed shanny	Sediment	Demersal	Arctic to temperate waters
Liparidae	Liparidae	Snailfishes	Sediment, seawater	Demersal or pelagic	Arctic to Antarctic
	Liparis ochotensis		Sediment, seawater	demersal	North Pacific, Bering Sea
	Rhodichthys regina	threadfin seasnail	Seawater	Bathydemersal	Arctic to northeast Atlantic
Zoarcidae	Zoarcidae		Sediment, seawater	Mostly benthic	Arctic to Antarctic
	Lycodinae		Sediment	Benthic, demersal	Arctic to Antarctic
Osmeridae	Osmeridae	smelts	Sediment	Pelagic	Northern Atlantic, also Arctic

	Mallotus sp.	Smelts	Sediment, seawater	Pelagic,	North Atlantic to Arctic
	Mallotus villosus	Capelin	Sediment, seawater	pelagic	North Atlantic to Arctic
Pleuronectidae	Pleuronectidae		Seawater		Atlantic and Arctic
	Reinhardtius hippoglossoides	Greenland halibut	Sediment, seawater	Benthopelagic	Arctic and temperate waters
Salmonidae					
	Salmo salar	Atlantic salmon	Sediment, seawater	benthopelagic	Up to southern Greenland
	Salmo sp.	Salmon	Seawater	benthopelagic	Up to southern Greenland
Sebastidae					
	Sebastes sp.		Sediment, seawater	Benthopelagic	At least three species occur in the Arctic
Anarhichadidae	Anarhichadidae	wolffishes	seawater	demersal	North Atlantic and Arctic
Carangidae	Carangidae	jacks and pompanos	Seawater	Pelagic	Atlantic (mainly tropical and temperate)
Triglidae					
	Chelidonichthys sp.	gurnard	Seawater	demersal	eastern Atlantic up to Norway
Clupeidae	Clupeidae		seawater	benthopelagic	North Atlantic up to Greenland and Spitsbergen
	Clupea harengus	atlantic herring	seawater	benthopelagic	North Atlantic up to Greenland and Spitsbergen
	Clupea sp.		seawater	benthopelagic	North Atlantic up to Greenland and Spitsbergen
Myctophidae	Myctophidae		Seawater	Pelagic	Arctic to Antarctic
Somniosidae					
	Somniosus sp.	Likely greenland shark	Seawater	benthopelagic	Arctic and North Altantic

3.4. Discussion

Environmental DNA metabarcoding proved successful in the detection of Arctic nekton. We were able to detect three cephalopod taxa in seawater and 31 fish taxa in seawater and sediment in the Fram Strait. In seawater, nine of the fish taxa were detected to species level, while seven fish species were detected in sediment. The low species resolution found here in fish eDNA is potentially attributed to the short gene amplicon being amplified by the primer, which did not allow taxa assignment to species level for 18 and 11 taxa found in seawater and sediment, respectively. However, the number of taxa detected in this study for fish are comparable to a time-series conducted at the continental shelf of East Greenland that established a species list including 3 cartilaginous fish species and 44 bony fish species (Christiansen, 2012). Taking the entire Arctic into account, 99 pelagic fish species have been identified (Mecklenburg et al., 2010), however, the Fram Strait represents only a small fraction of the Arctic and we do not assume that all 99 fish species occur in that area.

The eDNA detections of pelagic origin in sediment suggest *Mallotus villosus* and *Arctozenus risso* to be potential foodfall species. Distribution patterns of the detected eDNA in seawater and sediment contributed evidence to the range expansion of *M. villosus* due to changing ocean conditions. We also detected eDNA of the rare and elusive Greenland shark (*Somniosus microcephalus)* at 2420 m.

Community composition of cephalopods in the Fram Strait of the Arctic Ocean

We detected six taxa with eDNA in seawater from the Fram Strait. The cephalopod taxa with the highest number of detections and read counts were Gonatidae and *Gonatus* sp. The applied primer was not able to identify the corresponding ASVs to species level, but the only known species of the family Gonatidae that occurs in the Arctic is *Gonatus fabricii* (Xavier et al., 2018). Therefore, we could assume that the Gonatidae and *Gonatus* sp. detections here likely belong to *Gonatus fabricii*. However, the North Atlantic is inhabited by another gonatid species, namely *G. steenstrupi* (Xavier et al., 2018), which could potentially have migrated into the Arctic due to ocean warming and resulting borealization. Thus, in this study, we refer to *Gonatus* sp. when talking about eDNA detections of Gonatidae and *Gonatus* sp.

Gonatus sp. was detected between 50 m and close to the seafloor (bottom depths of between 2339 – 2727 m) at all three stations sampled for seawater. This is in line with its known depth distribution in the literature, residing in epipelagic layers as juveniles and undergoing ontogenetic migration to meso- and bathypelagic depths as they grow (Kristensen, 1983). *Gonatus fabricii* feeds on crustaceans as juveniles and shifts towards larger fish and other cephalopods in bathypelagic depths, as adults. As adult individual, it attains among the highest $\partial^{15}N$ (i.e., 14.9 permille) values ever recorded for cephalopods (Golikov et al., 2018). *Gonatus antarcticus* and *Mesonychoteuthis hamiltoni*, the Antarctic colossal squid, are the only cephalopod species exceeding *G. fabricii*'s trophic level (Cherel et al., 2008). Also, among Arctic invertebrates, *Gonatus fabricii* has one of the highest $\partial^{15}N$ values. The few existing exceptions are all scavenging, benthic animals such as gastropods, amphipods and asteroids (Dunton et al., 1989; Hobson and Welch, 1992; Tamelander et al., 2006). *Gonatus fabricii* occupies the same trophic level as Arctic vertebrate top predators such as large piscivorous fishes, seals and toothed whales (Golikov et al., 2018).

Besides Gonatidae and *Gonatus* sp., we detected *Teuthowenia maculata, Vampyroteuthis infernalis* and *Filippovia knipovitchi* in the eDNA seawater samples of the Arctic Ocean. The detection of *Filippovia knipovitchi* was likely a false positive, as it is an Antarctic species and has been used in our lab as positive control in previous studies. It is also unlikely that the nuclear 18S rRNA primer used here failed the correct identification due to closely related species. That is, because there are no species known from the same family (Onychoteuthidae) as *F. knipovitchi* occurring in the Arctic or North Atlantic. Another dubious detection was *Teuthowenia maculata*, which known range distribution is relatively limited and includes waters of the tropical, subtropical eastern North and South Atlantic (Jereb and Roper, 2010). However, another species of that genus, *Teuthowenia megalops*, has been found more than 2000 km to the north of its previous northern range border in the eastern Greenland Sea at about 74°N and the Norwegian Sea at 63°N (Xavier et al., 2018). It is unlikely that *T. maculata* has migrated as far north as 79.4°N as detected here, especially because *T. maculata* is a warm-water squid and not used to cold water, unlike *T. megalops*. It is possible that the primer used in this study is not sufficient to differentiate between species in the genus *Teuthowenia*. The used primer targets the nuclear 18S rRNA gene and although nuclear rRNA genes have a wide taxonomic coverage, they often come with the cost of low taxonomic resolution (Deagle et al., 2014). Therefore, the taxa assignment to *T. maculata* could be a false positive. Another explanation for the occurrence of *T. maculata* in our eDNA data could be sperm whales (*Physeter macrocephalus*) and

other cetaceans feeding on *T. maculata* further south and migrating to the North releasing feces. Defecation is a known source of eDNA and could potentially be detected in seawater (Taberlet et al., 2018). The same holds true for the detection of *Vampyroteuthis infernalis*. Sperm whales (Clarke et al., 1993, 1976; Clarke and MacLeod, 1980) as well as bottlenose dolphins (*Hyperoodon ampullatus*) are known to feed on *V. infernalis* (Clarke and Kristensen, 1980). *Vampyroteuthis infernalis* is a deep-sea species occurring in tropical and subtropical waters and is not known to occur further north than 40°N (Jereb and Roper, 2010). The read and detection frequencies of *V. infernalis* and *T. maculata* might maybe support that hypothesis. Both were detected at only one sampling depth in one replicate at one station (N4) and the number of reads were 3,505 for *V. infernalis* (only represented by one ASV) and between 104 and 1,756 reads (represented by three ASVs) for *T. maculata*. Hence, both species are very rare detections in our dataset.

Only taxa known to occur in the Arctic such as Gonatidae and *Gonatus* sp. were found in biological duplicates (25%) or triplicates (21%). All detections in triplicates belonged to samples taken at 2250 m or directly above the bottom. Although eDNA metabarcoding cannot be used as a measure for abundance or morphology, we hypothesize that the frequent detection at those depths and in triplicates point to an either high abundance of that taxa in those depth or larger individuals potentially shedding more DNA. Additionally, eDNA is more stable in colder waters than sunlit surface layers (Lacoursière-Roussel et al., 2018) and could be preserved for a longer time in the colder Arctic deep water than the warmer surface Atlantic water. Future research is needed to validate how eDNA degrades in deep water versus shallow water in the Arctic and how currents influence eDNA degradation and transport, as well as shedding rates of deep-sea cephalopods.

Biodiversity baseline and distribution of fish in the Fram Strait of the Arctic Ocean

In the samples from PS121 and MSM95, we detected 18 and 27 fish taxa in the sediment and seawater, respectively. Knowledge about the biodiversity and distribution of Arctic marine fishes is incomplete in many areas due to insufficient sampling of the Arctic Ocean. Recent studies have shown that previously established patterns of distribution in Arctic fish by modelling were an underestimate of their real distribution (Mecklenburg et al., 2010). It is difficult to assess whether these revised distribution patterns are due to insufficient sampling or climate change. It has been indicated that

with accelerated warming, boreal species expand their distribution into the Arctic (Fossheim et al., 2015; Polyakov et al., 2020). However, not only warming, but also other conditions such as appropriate trophic structure, substrate and topography need to be taken into account to validate the potential of boreal species to permanently expand into Arctic waters (Mecklenburg et al., 2010). Species with broad physiological limits and fast population growth are able to adapt faster to climate change as seen in the small pelagic species *Mallotus villosus* and *Clupea harengus* (Rose, 2005a). *Mallotus villosus* and *C. harengus* have been shown to have extended their distribution northwards due to climate variability, but also likely climate change (Rose, 2005a). The capelin, *M. villosus*, is known to inhabit the northern boreal oceans at the margins of cold Arctic waters (Rose, 2005b), but its Arctic distribution is not very well documented yet. One specimen was caught in a net trawl for the first time in 2015 at latitudes of 74-77 °N off the Northeast Greenland shelf break (Christiansen et al., 2016). Here, we detected capelin as far north as 79.7 °N, supporting the hypothesis, that eDNA can be used to detect range expansions in nekton species. Throughout history, this species has shown a strong colonizing ability when changes in climate occurred. During interglacial periods it migrated from the North Pacific to the North Atlantic and then further into the Barents Sea and even exceeded to Icelandic and southern West-Greenland waters (Rose, 2005b). Capelin is a small, short-lived pelagic species with high reproductive potential (Murua and Saborido-Rey, 2003) and semelparous reproduction, resulting in the death of both sexes after spawning (Christiansen et al., 2008). In Icelandic and Norwegian waters, it spawns predominantly at depths between 12 and 300 m (Thors, 1981) and usually does not occur deeper than 700 m (Allen and Smith, 1988), although it has been reported at 1086 m in the Davis Strait southern to Baffin Bay (Jorgensen et al., 2005). Spawning sites of capelin are limited by temperature and in the Arctic part of the Norwegian and Greenland Sea are mostly documented off Iceland and the Barents Sea (Thors, 1981). In our study, we found capelin to be the most often detected taxa with eDNA and also the taxa with the most abundant reads. It occurred at all seawater stations up to 79.7° N and in the sediment up to 79.13° N.

We found capelin eDNA in seawater as deep as 2705 m and in sediment at stations with a bottom depth that lie deeper than 1000 m. Therefore, all our sampled stations were located outside of known capelin spawning distributions, assuming that the eDNA signal does not stem from capelin eggs or reproducing individuals. We hypothesize, that the eDNA detections of capelin at great depths and cold waters might stem from predators feeding on them and either biting off body parts or releasing feces. Capelin are important prey for more than 21 fish species such as Greenland halibut and Atlantic cod, 18 seabird species, several seal species, cetaceans (Dawe et al., 1998; Dolgov, 2002) and is also

fed upon by *Gonatus* sp. (Kristensen, 1984). Cephalopods are known to bite off the head of their prey as to not injure their brain that is located around the oesophagus, while swallowing prey. That behavior has been observed in *Loligo reynaudii* (Lipinski, 1987) and *Todarodes pacificus* (Sakurai et al., 2013). *Gonatus* might do the same, resulting in sinking body parts of capelin to the deep seafloor. Additionally, feces of predators is potentially represented in the eDNA data as well and cannot be differentiated from eDNA originating from living organisms. Further research is needed to shed light on the occurrence of capelin eDNA in deep depths as detected in this study.

Our study suggests that capelins are able to migrate far north into the Arctic. As they react quickly to environmental changes and are a key species in the Atlantic and Arctic food web, this species is suggested an "early warning sea canary" for ecosystem changes that might also affect other species (Jákupsstovu and Reinert, 2002; Rose, 2005b). Changes in capelin distribution and abundance likely have major influences on ecosystem structure and productivity of species that feed on them. This link is already visible in northern cod distribution off the coast of Newfoundland and Labrador. In this area, capelin has been observed to migrate southwards due to ocean cooling, resulting in reduced capelin availability for cod. This leads to poor body conditions and lower reproductive success of cod and ultimately diminishing cod populations (Rose and O'Driscoll, 2002). The same phenomenon has been observed in Icelandic and Barents Sea cod stocks (Vilhjálmsson, 1997). Therefore, capelin has to be monitored in more detail in the Arctic Ocean, also in respect to commercial fisheries (Rose, 2005b).

Not only capelins' strong colonizing ability might have major impacts on ecosystem dynamics in the Arctic Ocean. The atlantification and borealization of the Arctic also leads to changes in distribution of other species with major impacts on food webs. In the Barents Sea, the northward shift of boreal species such as Atlantic cod might have led to intensified competition among the few large predators in that area. This includes Greenland halibut (*Reinhardtius hippoglossoides*) and arctic skate (*Amblyraja hyperborean),* which also occur in the Fram Strait. Greenland halibut abundances decreased significantly between 2004 and 2012 in the Barents Sea and this might happen in other ocean areas too. Decreasing halibut populations could explain the low number of detections of this large species in our eDNA data (one detection in seawater at 2000 m water depth and at the stations EG1 and HG1 in the sediment). Not only species' temperature optima might explain the changes in abundance, but also changes in food web dynamics (Fossheim et al., 2015). Arctic zooplankton

species are mostly larger and fattier than Atlantic species (Falk-Petersen et al., 2009). Changes in zooplankton composition and especially the take-over of less energetic Atlantic zooplankton species might be problematic for Arctic fishes (Walczyńska et al., 2018). Ultimately, the carbon cycle is altered as a consequence of ecosystem changes. For instance, the decrease in sea-ice coverage leads to reduced ice-algae production and therefore less organic material that sinks to the seafloor and is available for benthic organism (Fossheim et al., 2015). Reduced sea-ice simultaneously impacts species that are associated with it to survive such as the polar cod (*Boreogadus saida*), which is a key species in the Arctic food web (Hop and Gjøsæter, 2013).

Biodiversity assessments of Arctic marine fishes are needed to establish baseline data in the understudied Arctic Seas (Christiansen et al., 2016). For example, Environmental Sample Processors (ESP) have already been tested successfully in detecting the most abundant fish species in mesocosms, enabling *in situ* water collection, filtration, DNA extraction and qPCR analysis (Hansen et al., 2020). ESP could also be used in the Arctic, where they would allow remote autonomous *in situ* monitoring, without the need of scientists being around. Atlantic cod (*Gadus morhua*), beaked redfish (*Sebastes mentella*) and capelin (*Mallotus villosus*) have been observed for the first time off the northeast Greenland shelf break in 2015 at 74-77 °N (Christiansen et al., 2016). Our eDNA data adds evidence to the observed range expansions by detecting Gadidae, *Sebastes* sp. and capelin as far north as 79.7°N. However, with our chosen marker gene we were not able to differentiate Gadidae and *Sebastes* to species level, stressing the importance to apply several marker genes to reach the highest taxa resolution possible in eDNA studies. The 12S marker gene used here only amplifies a short region which is insufficient to differentiate between some closely related species and genera. To investigate borealization and climate change in the Fram Strait and Arctic Ocean, we do not only require single cruise-based diversity and distribution data, but also time series (Boero et al., 2015; Christiansen et al., 2014) and our data presented here are a first step.

Vertical depth distribution of fish in the Fram Strait

In this study, some of the mostly benthic taxa have been detected with eDNA in shallow depths such as *Liparis* sp. and Gadidae. Environmental DNA does not differentiate between adult and juvenile individuals, and many benthic species have pelagic larvae. Therefore, it seems likely that the shallow eDNA detections of benthic taxa belong to the larvae of the corresponding taxa. For instance,

Gadidae has been detected between 200 and 2500 m with eDNA and while larvae of e.g., cod (Gadidae) reside in shallow depths between 10 to 30 m, adults inhabit the benthopelagic layer (Froese and Pauly, 2000). The same holds true for species from the family Liparidae and the species *Liparis ochotensis* here detected between 50 and 2500 m. *Liparis* larvae preferentially stay between 10 to 200 m and descend to deeper layers as adults (Froese and Pauly, 2000). Therefore, eDNA is a suitable tool to detect pelagic larvae that might be overlooked by traditional methods.

In the Pacific Ocean, pelagic fish diversity peaks at around 200-300 m and then decreases with depth with lowest species richness and number of species below 2000 m (Smith and Brown, 2002). The vertical distribution of fish detected here with eDNA followed a bell-shaped pattern with peak diversities in the upper 200 m and close to the bottom between 2000 and 2500 m. Despite that, no significant differences in taxa community composition were found between the surface and deep depths. The peak in diversity here is in line with the vertical distribution for capelin (100 – 400 m) (Mowbray, 2002) and herring (surface – 400 m)) found in Northern Seas (Huse et al., 2012). These two species are abundant in Northern Seas and support substantial human fisheries (Olsen et al., 2010).

Potential foodfall species contributing to the carbon cycle of the Fram Strait

We were not able to identify cephalopod eDNA in Arctic marine sediments. Therefore, we cannot directly assess potential foodfall species for cephalopods. Yet, we detected *Gonatus* sp. frequently all over the water column. Based on its wide distribution, abundance and importance as both top predator and prey in the Arctic food web, this species likely plays a role in the regional carbon cycle. Females undergo gelatinous degeneration when brooding their naturally buoyant eggs in bathyal depths (Arkhipkin and Bjørke, 1999a; Bjørke et al., 1997; Bjørke and Hansen, 1996; Kristensen, 1981) and potentially fuel the carbon cycle when they die and sink to the seafloor. In the Gulf of California, carcasses from spent female gonatids were observed via ROV on the seafloor with various deep-sea scavengers associated with it (Hoving et al., 2017). Reasons for lacking *Gonatus* detections in our sediment data could be the non-existence of *Gonatus* eDNA in the sediment or low eDNA quantities that were not detected with the primer. *Gonatus* is suggested to only reproduce in a few geographically restricted areas (Arkhipkin and Bjørke, 1999b; Bjørke, 2001; Bjørke and Gjøsæter,

1998) and our eDNA sediment stations were not located in those known breeding areas. Arkhipkin and Bjørke et al. (1999b) stress that outside those areas, extensive sampling has failed to collect large, maturing and mature females in deep waters and except of one breeding area in the center of the Polar Basin, the other breeding areas coincided with large concentrations of the northern bottlenose whale *Hyperoodon ampullatus*, which is the main predator of *G. fabricii*. However, the absence of mature females outside of the known breeding areas could also be due to the difficulties associated with sampling deep-sea cephalopods (Wormuth and Roper, 1983) and deep-sea ecosystems in general. Additionally, observations of cephalopod foodfalls are scarce, as they occur only sporadically in time and space, and are patchily distributed and rapidly consumed (Scheer et al., in press). The sediment samples taken in this study represent only a small fraction of the Arctic seafloor and the probability to sample exactly where a foodfall occurred is low. We need to expand the sampling of deep-sea sediments for pelagic fauna and include known breeding areas of cephalopods to increase the likelihood to detect eDNA of pelagic origin in sediments.

In contrast to cephalopods, we found pelagic fish eDNA in the sediment, namely the spotted barracudina *Arctozenus risso* and capelin *Mallotus villosus*. Due to the high abundance of capelin and its importance in marine food webs, we hypothesize this species to be a foodfall species contributing to the Arctic carbon cycle. The same holds true for the spotted barracudina. Barracudinas are very abundant in midwater and important food sources for salmon, tuna, cod, sharks, albacores, seals, whales (Coad, 2017) and also for some cephalopods (Cherel and Duhamel, 2003). An unexpected detection with our primer was the taxon Delphinidae. We found dolphin eDNA at 50, 200 and 2500 m and in the sediment. The corresponding ASV assigned to the species *Lagenorhynchus albirostris, Peponocephala electra* and *Feresa attenuate.* All species are known from the North Atlantic with a maximum northern range of 40°N, but just the white-beaked dolphin *Lagenorhynchus albirostris* is known to occur in the Arctic Ocean (Galatius and Kinze, 2016). It is possible that we detected remains from this species with eDNA, however, we cannot say whether the eDNA signal in sediment stems from foodfalls or feces sinking to the seafloor.

The use of sediment and seawater eDNA to establish biodiversity baselines

We did not retrieve any cephalopod eDNA from the sediment samples, but the positive controls amplified successfully. Therefore, we can conclude that the sequencing run itself was successful. Additionally, the same primer has been used successfully in other studies to assess cephalopod diversity off the Azores and Cabo Verde (Visser et al., 2021). Most sediment DNA extraction kits are optimized for bacterial DNA analysis and for bacteria only low amounts of sample are sufficient. The sediment DNA extraction kit that we used only included 0.25 g of sediment. Cephalopod abundances are lower in the Arctic compared to other oceans of the world (Rosa et al., 2008; Xavier et al., 2018). Therefore, the amount of sediment being analyzed needs to be as large as possible. Some sediment extraction kits can extract as much as 10 g of sediment from a sample and further research should compare those kits to the kits using low amounts of sample. Additionally, the primer that we used in this study is known to not amplify octopuses very well (de Jonge et al., 2021). That could explain why no benthic octopuses were detected. For a comprehensive survey of the sediment, several primer pairs should be applied to cover a broad taxonomic range and to reduce primer bias by using just one primer. The lack in amplification of cephalopod eDNA in sediment indicates the patchy distribution and fast turnover rate of foodfalls. Further experiments are needed to investigate the persistence and degradation rates of eDNA in surface layers of deep-sea sediments and the PCR detection limits.

A common representative feeding on deep-sea foodfall experiments in the Arctic Ocean is the bathydemersal glacial eelpout *Lycodes frigidus*. We did not detect this species with eDNA, but we did detect the corresponding family Zoarcidae between 200 and 2700 m. The family Zoarcidae includes 230 species and 26 of them occur in Greenland waters (Møller et al., 2010). Zoarcidae was the taxon with the second most eDNA detections. Delineating that family into corresponding genera and species would contribute to the understanding of deep-sea ecosystem functioning in the Arctic. The fish primer used in this study has already been applied successfully off Southwest Greenland, being able to detect 93% of fish families that were also caught by trawling (Thomsen et al., 2016). The number of detected species (37 taxa) was comparable to this study (31 taxa). However, Thomsen et al. faced the same problem that the 12S primer was not able to delineate genera or species inside the family Gadidae, such as Atlantic and Polar cod. Both species are common and important in the Arctic ecosystem (Nygaard and Jørgensen, 2014) and future studies should design or use primer that

are able to separate these species as well as others in the family Gadidae. In this study, 32% of the eDNA detections were identified to species level, while in Thomsen et al. 65% were identified to species level. This highlights the general efficiency of the used primer, although the amplicon length is rather short. According to Thomsen et al., the amplicon length of the 12S primer was around 100 bp, while in our study, the amplicon length was around 70 bp. This could explain the lower resolution to species level in our study compared to Thomsen et al. For future studies, the 12S primer should be combined with primer amplifying a longer gene region to allow a better delineating to species level. The combination of several primer targeting different genes increases taxonomic resolution and species that are missed by one primer might be amplified by another. Another limiting factor is the completeness of the used reference database. For instance, *Lycodes frigidus* is a very rare species and only known from three specimens collected off East Greenland (Møller et al., 2010). That species is not represented in GenBank for 12S and therefore not in our database. To improve the performance of the 12S primer used in this study, blocking primers for abundant nontarget species as e.g., humans could be added to the PCR mixture. Also, a higher sequencing depth may yield in the detection of more rare species.

The sequencing read abundances from this study are significantly associated with the eDNA detections. Although eDNA metabarcoding is not able to provide reliable abundance data, several studies showed a correlation between eDNA reads and biomass (Elbrecht and Leese, 2015; Sevellec et al., 2021; Thomsen et al., 2016). However, eDNA analysis still faces a variety of uncertainties. The amount of eDNA being shed in an environment depends on the organism (e.g., crustaceans have been shown to shed less eDNA than fish (Allan et al., 2021)). The residence time of eDNA depends on physical, chemical and biological factors (Taberlet et al., 2018) and eDNA can be transported by currents or predators. The physical conditions in the deep sea are substantially different in comparison to surface waters and eDNA might behave differently in great depths due to e.g., low temperatures, no light and higher salinity. Therefore, it is difficult to deduce abundance estimates from eDNA metabarcoding data, especially without appropriate experiments. Nevertheless, the read abundance is reflected by the frequency of eDNA detections. That does not necessarily mean that a species is abundant, but that it is widely distributed either horizontally, vertically or both.

Any eDNA study is just as good as its reference database and database gaps greatly hamper eDNA species assignments (Elbrecht et al., 2017; Kwong et al., 2012). Knowledge of the Arctic fish and

cephalopod fauna remain scarce, especially for the pelagic realm (Christiansen, 2012; Xavier et al., 2018). Substantial changes in food web dynamics and composition will affect ecosystem functioning in the Arctic Ocean. We are already facing considerable changes in species composition in the Arctic Ocean, however, a lot of them will stay unrecognized without increasing efforts to implement long-term observations of pelagic fauna and the completions of reference databases.

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Author contributions

HJTH conceived the study. VJM, HJTJ, TB, TBHR and OP designed the study. VJM prepared the eDNA sampling and joined the cruises PS121 and MSM95. VJM collected the samples. VJM led the lab work which was conducted together with JS and JF. KM provided lab facilities and samples. VJM analyzed the data and TB helped with it. VJM and HJTH drafted the manuscript. OP, TB, KM and TBHR critically reviewed the manuscript.

3.5. References Chapter 3

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Chapter 4

A novel metabarcoding primer pair for environmental DNA analysis of Cephalopoda (Mollusca) targeting the nuclear 18S rRNA region

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Abstract

Cephalopods are pivotal components of marine food webs, but biodiversity studies are hampered by challenges to sample these agile marine molluscs. Metabarcoding of environmental DNA (eDNA) is a potentially powerful technique to study oceanic cephalopod biodiversity and distribution but has not been applied thus far. We present a novel universal primer pair for metabarcoding cephalopods from eDNA, Ceph18S (Forward: 50-CGC GGC GCT ACATAT TAG AC-30, Reverse: 50-GCA CTT AAC CGA CCG TCG AC-30). The primer pair targets the hypervariable region V2 of the nuclear 18S rRNA gene and amplifies a relatively short target sequence of approximately 200 bp in order to allow the amplification of degraded DNA. In silico tests on a reference database and empirical tests on DNA extracts from cephalopod tissue estimate that 44–66% of cephalopod species, corresponding to about 310–460 species, can be amplified and identified with this primer pair. A multimarker approach with the novel Ceph18S and two previously published cephalopod mitochondrial 16S rRNA primer sets targeting the same region (Jarman et al. 2006 Mol. Ecol. Notes. 6, 268–271; Peters et al. 2015 Mar. Ecol. 36, 1428–1439) is estimated to amplify and identify 89% of all cephalopod species, of which an estimated 19% can only be identified by Ceph18S. All sequences obtained with Ceph18S were submitted to GenBank, resulting in new 18S rRNA sequences for 13 cephalopod taxa.

4.1. Introduction

Cephalopods, the molluscan class to which squids, octopods, cuttlefish and vampire squids belong, occur in all the world's oceans from the intertidal zone to the deep sea [1–3]. Their high protein content and large populations make them important in commercial fisheries and food–web interactions as both predator and prey [4–8]. Cephalopods are among the giants of the ocean (giant squid *Architeuthis* spp., colossal squid *Mesonychoteuthis hamiltoni*), and the highest diversity at the family level is found in the deep sea [9].

Deep-sea cephalopods have evolved specialized traits to cope with life in continuous darkness, but basic biological data are still lacking for the majority of species. One of the reasons for this paucity of information is that cephalopods are difficult to study with traditional sampling methods like net catches or video surveys [2]. The size of trawled cephalopods is biased by the used mesh and net size and specimens often get entangled and damaged in the mesh which may make it difficult to identify them morphologically [10]. Additional difficulties in sampling cephalopods result from their possibly patchy distribution and their agility which allows them to avoid or escape sampling gear. Video surveys with submersibles and towed cameras require lights that are easily detectable by the well-developed cephalopod eyes, which may result in avoidance behavior [3]. The study of cephalopod remains in the stomachs of predators provides indirect evidence of their presence [11–13], but the digested state of cephalopod remains may hamper species identification, and the selectivity of the predators and limited knowledge of their foraging area introduces bias. The challenges associated with cephalopod sampling, particularly in remote areas such as deep pelagic environments, raise an urgent need for novel monitoring methods.

Environmental DNA (eDNA) analysis constitutes a promising tool to study the distribution and diversity of cephalopods. This technique is based on the idea that organisms leave DNA in the environment, and that this DNA can be extracted and sequenced to identify the species from which it originates [14]. PCR amplification of eDNA in a sample (e.g., filtered from water) can detect the presence of a species or of multiple taxa by targeting a variable gene with either a species-specific or universal primer set, respectively. Biodiversity assessments of an eDNA sample using a universal primer set fall under the broader term metabarcoding [15,16], i.e., parallel identification of multiple taxa from one complex DNA sample. Metabarcoding has its origins in microbiology, paleoecology and

diet analysis [17–21]. Sampling eDNA is relatively easy in the marine environment (from water or sediment) and developments in the field of next-generation sequencing have greatly reduced sequencing costs and dramatically increased sequencing output. Therefore, eDNA analysis represents a non-invasive and cost-effective method for biodiversity assessments, especially for rare or elusive species and in remote areas [14,22–25].

Metabarcoding of eDNA from seawater has mostly been used to identify fishes [26–28] and assess overall (metazoan) eukaryotic diversity [29–32], but to our knowledge has not been used to focus on specific taxonomic groups like cephalopods. Metabarcoding has only been applied to cephalopod eDNA in studies focused on larger taxonomic groups from coastal areas, e.g., eukaryotes [29], metazoans [31] and invertebrates [33]. Even though these studies did not specifically focus on cephalopod distribution, they were able to provide valuable insights on cephalopod distribution. For example, the distribution of *Sepiola tridens*' eDNA suggested that the species' distribution might extend further into the coastal zones of Northern Europe than previously thought [31]. In recent years, there has been a sharp increase in the number of studies that employed eDNA analysis in the deep sea [22,34,35], but none to specifically investigate cephalopod diversity. Recently, a species-specific primer has been developed and used to detect the giant squid, *Architeuthis dux*, in the photic zone of the Sea of Japan [25].

Universal metabarcoding primers, i.e., a single primer set that targets multiple taxa, should have a common annealing site in all taxa and amplify a sequence with enough variation to distinguish between groups at the desired taxonomic resolution, which often is the species level [36]. Ideally, a pair of universal primers will target the largest possible taxonomic group of interest, unambiguously identify all species, while not amplifying non-target taxa. However, due to the often degraded state of eDNA, a target sequence size of less than 300 bp, sometimes referred to as a mini-barcode [37], is preferable [36]. Therefore, the primer pair that amplifies approximately 650 bp of the cytochrome-oxidase-1 (COI) [38,39], which is most commonly used for species identification, is unsuitable for eDNA metabarcoding. In practice, there is a trade-off between the taxonomic range of amplifiable species (i.e., universality), indicated by the coverage index (B_c = n. of amplified taxa/n. of target taxa), and the resolution for identification at species level, indicated by the specificity index (B_s = n. of identified taxa/n. of amplified taxa) [40]. The analysis of eDNA using multiple markers, preferably from different genes, can increase the sensitivity of the technique and overcome the specificity issues

of a single pair of universal primers [29]. So far, two sets of universal primers specifically targeting cephalopods have been published, one targeting the mitochondrial 16S rDNA [41,42] and one targeting the cytochrome b region [43]. However, the latter is not suitable for eDNA metabarcoding due to the long target amplicon size. This study describes the development of a novel set of universal primers for cephalopods targeting the nuclear 18S rRNA region and describes its complementarity to the mitochondrial 16S rDNA primer set [41,42] in a multi-marker approach to study the diversity and distribution of cephalopods through metabarcoding of eDNA.

4.2. Material and Methods

The process for the development of a metabarcoding primer pair encompassed four steps: (i) assembling a reference database, (ii) identifying potential primer sets, (iii) in silico testing, and (iv) empirical testing.

Reference database

Two cephalopod 18S rRNA databases were generated from GenBank and SILVA. The results for a general GenBank query '18S Cephalopoda' included environmental samples and partial sequences from other 18S subregions. To ensure exclusion of environmental samples and inclusion of sequences from the same subregion, the first sequence of the largest subset of partial sequences (the longfin inshore squid *Doryteuthis pealeii*, MH586846, 760 bp) was aligned against the full GenBank database (discontinuous megablast, 9 October 2018) [44] and all matches with full query cover were downloaded. This approach resulted in 31 partial cephalopod 18S rRNA sequences from 24 species. For the SILVA database, all cephalopod 18S rRNA reference sequences were downloaded (11 October 2018) and included 146 sequences from 88 species ranging from 423 to 2610 bp. The NCBI taxonomy dump file was downloaded (18 September 2018) and used for sequence annotation of both the GenBank and SILVA database with unique taxonomic identifications using Python v. 2.7.15 and OBITools v. 1.2.10 [45]. The annotated extended Fasta files were converted to the reference ecoPCR database v. 0.2 format [40].

Identification of potential primer sets

The ecoPrimer v. 0.3 algorithm [16] was used to identify potential primer pairs of 20 bp each that amplify a target sequence of 50–200 bp [46] with ample variation for taxonomic resolution to species level. The settings used on the GenBank reference database did not return any potential primer sets when applied to the SILVA reference database; hence for the SILVA database, the settings were somewhat relaxed (algorithm parameters in table 1). The potential primers were filtered for lowest melting temperature (Tm) between 59 and 69°C, maximum difference between lowest melting temperatures less than 3°C, GC count of 50–60%, a GC clamp with less than four G and/or C, less than four nucleotide repeats and less than four dinucleotide repeats [46]. The relaxed settings for the SILVA database may have resulted in suboptimal primers. Therefore, only primer sets with a B_c equal to or higher than the GenBank derived primer sets when tested *in silico* against the SILVA database were considered and filtered for lowest melting temperature (Tm) (between 45 and 70°C) and a GC count between 45 and 65%.

In silico testing

From the filtered list, eight primer sets with the highest coverage index (B_c) and specificity index (B_s), as estimated by ecoPrimer [16], were chosen for further tests *in silico*, i.e. to predict their effectiveness in PCR amplification. The primers were analyzed for secondary structures (hairpins and primer-dimers) using the online Oligonucleotide Properties Calculator v. 3.27 [47], and for self-complementarity and in silico amplification using online Primer-BLAST [48]. The B_c and B_s of the primer set was calculated with an ecoPCR v. 0.2 [40] *in silico* test against the SILVA database (no mismatches allowed). Three primer sets with optimal characteristics, i.e. no or limited secondary structures and highest B_c and B_s indices, were ordered for empirical testing.

The B_c and B_s of the two mitochondrial 16S rRNA primer sets, *CephMLS* (*CephMLSf1*: 5'-TGC GGTATTWTAACTGTACT-3`,*CephMLSr1*: 5'-TTATTCCTTRATCACCC-3') [41] and *S_Cephalopoda* (*S_Cephelapoda*-F: 5`- GCT RGA ATG AAT GGT TTG AC-3`; *S_Cephalopoda*-R: 5`-TCAWTA GGG TCT TCT CGT CC-3`) [42] were estimated to determine the complementarity of the newly developed and existing primer sets. The target sequence size of *S_Cephalopoda* is 70–73 bp [42] and falls right within the targeted region of *CephMLS*, which has a target sequence size of 212–244 bp [41]. A 16S reference database was obtained by using Primer-BLAST [48] with the respective primer sets, and

subsequently blasting the first match (blastn protocol, limit to cephalopod taxon in nucleotide database, exclude uncultured/environmental samples, query cover greater than 50%) [44] to obtain sequences that were known to contain the targeted region. An ecoPCR v. 0.2 [40] *in silico* test (no mismatches allowed) was used to determine B_c and B_s indices for *S_Cephalopoda* and *CephMLS* using this 16S GenBank reference database.

The 18S primer development process was based on two reference databases: one from SILVA with 146 sequences, and one from GenBank with 31 sequences. The latter has significantly less sequences than the SILVA database, caused by our specific filtering choices to avoid non-overlapping sequences which would have obstructed the development process. We calculated the B_c and B_s indices for our new primer set from the SILVA database during the development process. However, we felt that a comparison between these SILVA-derived 18S indices and the Primer-BLAST-derived 16S indices would be biased. SILVA is specific about which GenBank sequences are admitted into the alignment, and some GenBank sequences might have been left out. To ensure an unbiased comparison between 16S and 18S coverage and specificity indices, we obtained a third 18S database by using the newly developed 18S primer sequence in a GenBank Primer-BLAST [48]. This third GenBank database could not have been obtained at the start when we had not yet developed the primer set.

T**able 1| Parameter settings used for the ecoPrimer v. 0.3 analysis [16].** GB, GenBank reference database (the analysis was run twice with different mismatch and 30 match settings). SV, SILVA reference database (quorum parameters were relaxed relative to the GenBank settings).

parameter	value	description
primer length (bp)	20	required length of forward and reverse primer
Target amplicon length (bp)	50 – 200	required length of the amplified sequence
strict matching quorum	GB: 0.7 SV: 0.5	minimum fraction of sequence records in the reference database with an exact match between the primer and target sequence
sensitivity quorum	GB: 0.9 SV: 0.7	minimum fraction of sequence records in the reference database that exactly match the specified parameters
no. mismatches	GB: 0 and 3 SV: 3	number of allowed mismatches between primer and target sequence
no. 3’ matches	GB: NA and 2 SV: 2	number of strict matches required at the 3’ end

Empirical testing

Cephalopod specimens were collected in the eastern tropical Atlantic in waters near the Republic of Cabo Verde by the *RV* Walter Herwig III in March and April 2015 (cruise ID WH383, permissions obtained from Ministério da Agricultura e Ambiente and Agência Marítima e Portuária of Cape Verde). The sampling net was a pelagic trawl (Engel Netze, Bremerhaven, Germany, length 18 m, 16 x 30 m mouth opening, cod end 20 mm stretched mesh-opening, 1.8 mm inlet sewn into last 1 m of cod end) with a multi-sampler allowing depth-stratified sampling (electronic supplementary material, table S1). The specimens were morphologically identified by H.-J.T.H., and the full specimen (for small individuals) or a part of an arm (for larger individuals) was stored in a 2 ml tube with ethanol. DNA was extracted from the identified cephalopod tissue samples with the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. DNA purity and concentration were measured with NanoDrop (Thermo Fisher Scientific) and Qubit (dsDNA broad range Assay Kit, Thermo Fisher Scientific), respectively. DNA extracts diluted to approximately 10 ng μl^{-1} of three species from different families (*Bathyteuthis abyssicola, Heteroteuthis dispar* and *Liocranchia reinhardtii*), a mixture of these three extracts, negative extraction controls (no tissue) and negative PCR controls, i.e., with PCR grade water instead of DNA template, were amplified with a temperature gradient for all selected potential new primer pairs. A PCR mixture of 40 µl reaction volume of which 10 µl DNA template was prepared with the KAPA Hifi kit (Kapa Biosystems, Roche Inc.) (1X Fidelity buffer [which corresponds to 0.4 mM $MgCl_2$], 0.3 mM KAPA dNTPs, 5% DMSO, 0.02 U μl^{-1} KAPA Hotstart Polymerase, 0.5 µM of each forward and reverse primer). An Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific) was used for the PCR reaction. The PCR program consisted of a 5 min initial denaturation step at 95°C followed by 35 cycles of denaturing at 98°C for 20 s, annealing temperature gradient for 15 s and extension at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min and 4°C on hold. The temperature gradient started at 3°C below the lowest T_m of the primer set and increased with five steps of 3°C each. This temperature gradient was chosen to account for the expected increase in optimal annealing temperature due to the KAPA Hifi kit, and an expected decrease in optimal annealing temperature due to the DMSO in the PCR mix, which together could cause deviation from the theoretical optimal annealing temperature by several degrees Celsius. The PCR products were visualized under UV light on a 1.2% agarose gel using loading

dye, GelRed (Biotium) and a 100 bp ladder (Thermo Fisher Scientific). Once the optimal annealing temperature was determined, the same PCR procedure was conducted on more cephalopod tissue DNA extracts (30 species, figure 4; electronic supplementary material, table S1), DNA extract mixture of the newly tested species, and negative extraction and PCR controls.

PCR products from the primer set producing the most promising results, i.e. clear bands and limited smearing and non-specific bands on the agarose gel, were used for Sanger sequencing. Sanger sequencing was performed in GEOMAR and at the Institute of Clinical Molecular Biology (IKMB) in Kiel using different protocols. In GEOMAR, the PCR products were prepared for sequencing using the Sanger Sequencing Kit (Applied Biosystems). A reaction volume of 12 µl (10 µl PCR product, 0.03 U μl^{-1} FastAP, 0.33 U μl^{-1} ExoI) was used to remove unincorporated primers with the following PCR conditions: 37°C for 20 min, 80°C for 15 min and 4°C on hold. The sequencing reaction was conducted in a volume of 10 µl (0.5 µl cleaned PCR product, 0.5 µl Sequencing buffer, 2.5% BigDye Terminator Mix 3.1, 0.25 µM forward or reverse primer) with the following PCR conditions: 1 min at 96°C, 28 cycles at 96°C for 10 s, primer annealing temperature for 5 s, 60°C for 4 min and hold at 8°C. Finally, the sequencing reaction was purified with a bead-based reagent (62% Sam solution and 13% BigDye XTerminator) by shaking the mixture for 30 min at full speed and centrifuging for 2 min at 1000 r.p.m. At the IKMB, the PCR products were purified in a reaction volume of 10 µl (8 µl PCR product, 0.06 U µl−1 FastAP, 0.30 U μl^{-1} ExoI) with the following PCR conditions: 37°C for 10 min, 75°C for 15 min and 10°C on hold. The sequencing reaction was conducted in a volume of 10 µl (2 µl purified PCR product, 0.75x Sequencing buffer, 7% BigDye Terminator Mix 3.1, 0.32 µM forward or reverse primer) with the following PCR conditions: 1 min at 96°C, 25 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 4 min and hold at 10°C. The sequencing products were purified through Sephadex G-50 fine gel filtration (GE Healthcare Buchler). Low-quality ends and primers were trimmed manually from the Sanger sequences, which were then manually checked and edited using 4Peaks v. 1.8 [49], and subsequently assembled using AliView v. 1.24 [50]. The assembled sequences (or single forward or reverse sequences in cases of failed sequencing) were checked against the online GenBank reference database with BLAST (megablast algorithm, nucleotide collection nr/nt, 29 June 2020) [44].

4.3. Results

Potential primer pairs

All three empirically tested primer pairs amplified cephalopod DNA of the expected length. However, two primer pairs produced electrophoresis gels with side products, i.e. smearing and non-specific bands, potentially due to false priming or greater propensity to form secondary structures. The primer pair with the cleanest amplification was named '*Ceph18S*' (table 2) and selected for sequencing. The other two alternatives were not tested beyond this point (alternative 1: F-5'-GCA CTT AAC CGA CCG TCG AC-3' and R-5'-GTC GCGGCG CTA CAT ATT AG-3'; alternative 2: 5'-ATT AGA CTG AGA CCG ATGCG-3' and R-5'-GAC CGT CGA CAG TTG ATA GG-3'). The *Ceph18S* primer pair targets the V2 variable region of the small-subunit 18S rRNA gene [52] (figure 1). Alignment against *Loligo formosana* (accession code AY557478) shows that the target sequence is located at positions 258–442 (figure 1), but the exact location will depend on the species due to the variation in this region. The *in silico* PCR with *Ceph18S* on the SILVA database shows a target sequence length ranging from 131 bp for *Watasenia scintillans* to 196 bp for *Sepia elegans* (table 2). The alignments of these ecoPCR target sequences and sequences obtained in the laboratory clearly show conserved flanking regions and a highly variable internal region (figure 2). The optimal annealing temperature for *Ceph18S* in the PCR master mix used in this study was found to be 62°C. This differs from the calculated T_m (table 2) as both the KAPA reagents and DMSO in our PCR master mix alter the annealing temperature.

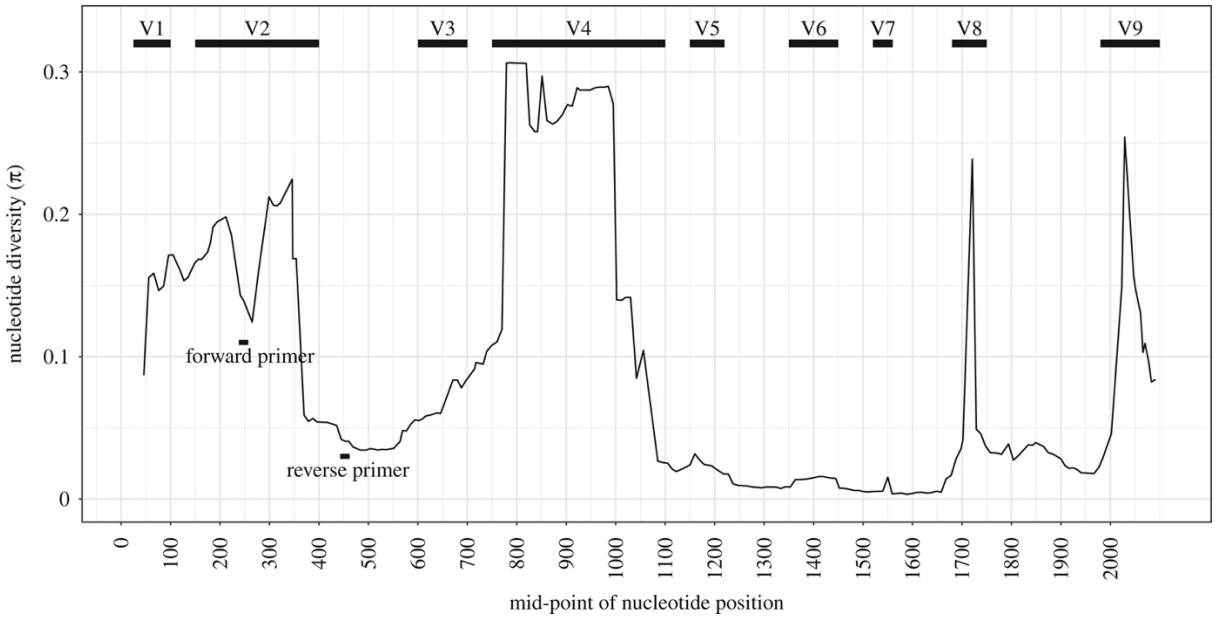

Figure 1| Position of the forward and reverse Ceph18S primers on the cephalopod 18S rRNA gene. Nucleotide diversity (π) was calculated with a sliding-window analysis (window size = 99 bp, step size = 10 bp) over the SILVA alignment (after removal of large indels 0–572, 2004–2411, 6255–6529) and shown based on the reference sequence of *L. formosana* (accession code: AY557478).

Table 2| Properties of the developed universal cephalopod primer pair targeting the 18S rRNA region to be used in eDNA metabarcoding

name	Ceph18S
forward	5‘-CGCGGCGCTACATATTAGAC-3‘
reverse	5‘-GCACTTAACCGACCGTCGAC-3‘
Forward T_m[a]	59.3°C
Reverse T_m[a]	61.7°C
Target length	~150 – 190 bp
B_c[b]	0.85
B_s[b]	0.78

[a] The T_m is estimated using the SantaLucia method [51] for a salt concentration of 0.05.
[b] B_c is *in silico* coverage index (amplified taxa/target taxa) and B_s is *in silico* specificity index (identified taxa/amplified taxa) based on the SILVA database with 146 sequences of 97 unique taxa.

Ceph18S resolution

The power of the *Ceph18S* primer pair to identify cephalopod species was examined with an *in silico* PCR on 88 species plus nine unique genera (i.e. genera without species identification which complemented the genera of the 88 identified species) in the SILVA database. From the 97 unique taxa in the SILVA database, 82 could be amplified in silico (B_c = 0.85). Of these 82 amplified taxa, 64 could be identified unambiguously (B_s = 0.78, figure 3). Therefore, 18 taxa could not be identified unambiguously, i.e. their target sequence was equal to the target sequence of another species: (i) *Leachia atlantica* and *Leachia lemur*, (ii) *Octopoteuthis megaptera* and *Taningia danae*, (iii) *Chiroteuthis veranyi* and *Chiroteuthis calyx*, (iv) *Discoteuthis laciniosa* and *Discoteuthis discus*, (v) *Onykia carriboea* and *Ancistroteuthis lichtensteinii*, (vi) *Selenoteuthis scintillans* and *Lycoteuthis lorigera*, (vii) *Histioteuthis miranda* and *Histioteuthis bonellii*, (viii) *Gonatus antarcticus* and *Gonatopsis* sp., and (ix) *Gonatopsis octopedatus*, and *Okutania anonycha*. Taxa for which some reference sequences were amplified but not all, were *Chtenopteryx sicula*, *Loligo forbesi, Sepia elegans, Sepiella inermis* and *Todaropsis eblanae*. Further inspection revealed that some reference sequences of these species were incomplete, i.e., omitting at least the V2 region around which the *Ceph18S* primer set anneals. Taxa that were not amplified at all due to a lacking reference V2 region were *Eledone cirrhosa, Euprymna scolopes, Hapalochlaena maculosa, Loligo vulgaris, Octopus vulgaris, Opisthoteuthis* sp. and *Rossia macrosoma*. Taxa that were not amplified even though a reference V2 region was available were *Alloteuthis* sp., *Bathypolypus* sp., *Cirrothauma murrayi, Pyroteuthis margaritifera, Sepia pharaonis, Sepioloidea lineolata, Spirula spirula* and *Vampyroteuthis infernalis*.

Subsequently, *Ceph18S* was tested empirically on 75 tissue DNA extracts (figure 4) from 68 specimens (electronic supplementary material, table S1) representing 30 cephalopod species plus two unique genera from specimens that could not be identified to species level (sequences are deposited in GenBank, accession numbers MT680727–MT680790 and MT680792–MT680800). Of the 32 taxa, 14 taxa did not have corresponding 18S rRNA reference sequences in GenBank (figure 4). All tested taxa, except for *Discoteuthis discus* and *Vitreledonella richardi*, could be amplified with *Ceph18S*, although sometimes with suboptimal sequence quality, so empirical B_c = 0.94. The *Ceph18S* barcodes submitted to GenBank add previously non-existent 18S rRNA reference sequences for 13 taxa: *Abraliopsis atlantica, Abralia redfieldi, Bathothauma lyromna, Chiroteuthis* cf. *joubini,*

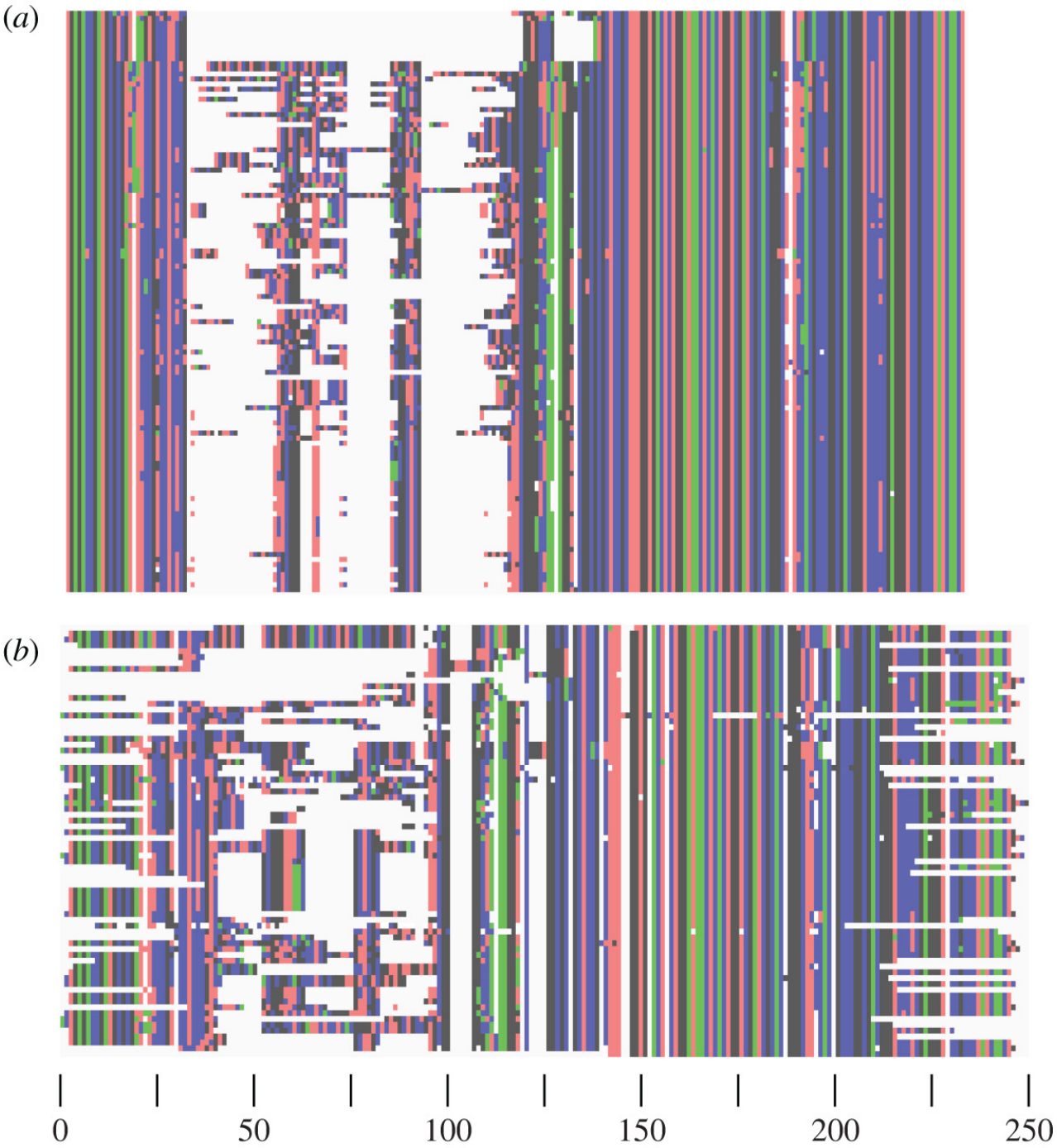

Figure 2| Target sequences alignment. Alignment of (*a*) target sequences obtained through *in silico* PCR and (*b*) sequences of extracted cephalopod DNA, both amplified with *Ceph18*S. Alignment using MUSCLE and image creation done in AliView [50].

Egea inermis, Helicocranchia pfefferi, Heteroteuthis dispar (the existing partial 18S sequence accession code AF034565 is from a different 18S subregion), *Histioteuthis meleagroteuthis, Liocranchia reinhardtii, Ornithoteuthis volatilis, Sthenoteuthis pteropus, Stigmatoteuthis arcturi* and *Teuthowenia megalops*. Of the 15 amplified species with a reference sequence in GenBank

(i.e., excluding the two genus-only taxa and *D. discus*), seven could be unambiguously matched to species level (B_s = 0.47): *Bathyteuthis abyssicola, Cranchia scabra, Enoploteuthis leptura, Histioteuthis reversa, Mastigopsis hjorti, Ornithoteuthis antillarum* and *Vampyroteuthis infernalis*. Some species had an ambiguous BLAST match, i.e., the BLAST match returned multiple species with the same top alignment score including the expected morphologically identified species which, therefore, could not be genetically distinguished from other species. *Leachia atlantica* could not be distinguished from *Leachia lemur, Octopoteuthis danae* could not be distinguished from *Octopoteuthis megaptera* and *Selenoteuthis scintillans* could not be distinguished from *Lycoteuthis lorigera*. Species with a GenBank reference sequence that could not be identified were *Ancistrocheirus lesueurii* (best match with Identity = 96, E-val = 2.00 x 10^{-67} to *Mastigoteuthis hjorti* which belongs to a different family, as identified in GenBank accession code EU735291 by Lindgren [53], but accepted in WoRMS as *Mastigopsis hjorti* [54]), *Histioteuthis corona* (best match with Identity = 100, E-val = 1.00 x 10^{-63} to *Histioteuthis hoylei* as identified in GenBank accession code AY557500 by Lindgren et al. [55], but accepted in WoRMS as *Stigmatoteuthis hoylei* [56]), *Octopoteuthis sicula* (best match with Identity = 100, E-val = 1.00 x 10^{-83} to *Octopoteuthis danae* and *Octopoteuthis megaptera*), *Pterygioteuthis gemmata* (best match with Identity = 99, E-val = 3.00 x 10^{-47} to *Pterygioteuthis microlampas*) and *Taningia danae* (best match with Identity = 93, E-val = 2.00 x 10^{-67} to *Lepidoteuthis grimaldii*, both belonging to octopoteuthid families).

Of the 20 amplified genera with a reference sequence at genus level in GenBank, 15 reference sequence in GenBank at either species or genus level, four could be identified to family level: in the Cranchiidae family (i) *Helicocranchia pfefferi* matched to *Taonius pavo* and *Megalocranchia* sp., (ii) *Liocranchia reinhardtii* matched to *Cranchia scabra*, and (iii) *Teuthowenia megalops* matched to *Taonius pavo*, whereas in the could be identified unambiguously (B_s = 0.75). Of the six species that did not have a reference sequence in GenBank either species or genus level, four could be identified to family level: in the Cranchiidae family (i) *Helicocranchia pfefferi* matched to *Taonius pavo* and *Megalocranchia* sp., (ii) *Liocranchia reinhardtii* mathed to *Cranchia scabra*, and (iii) *Teuthowenia megalops* matched to *Taonius pavo*, whereas in the Histioteuthidae family (iv) *Stigmatoteuthis arcturi* matched to *Histioteuthis hoylei* (as identified in GenBank accession code AY557500 by Lindgren et al. [55], but accepted in WoRMS as *Stigmatoteuthis hoylei* [56]). Additionally, *Taningia danae* was identified as *Lepidoteuthis grimaldii*, which belong to closely related but different families [57]. Taking into account the availability of reference sequences, a total of 18 of the 30 amplified taxa had the best possible expected outcome (60%).

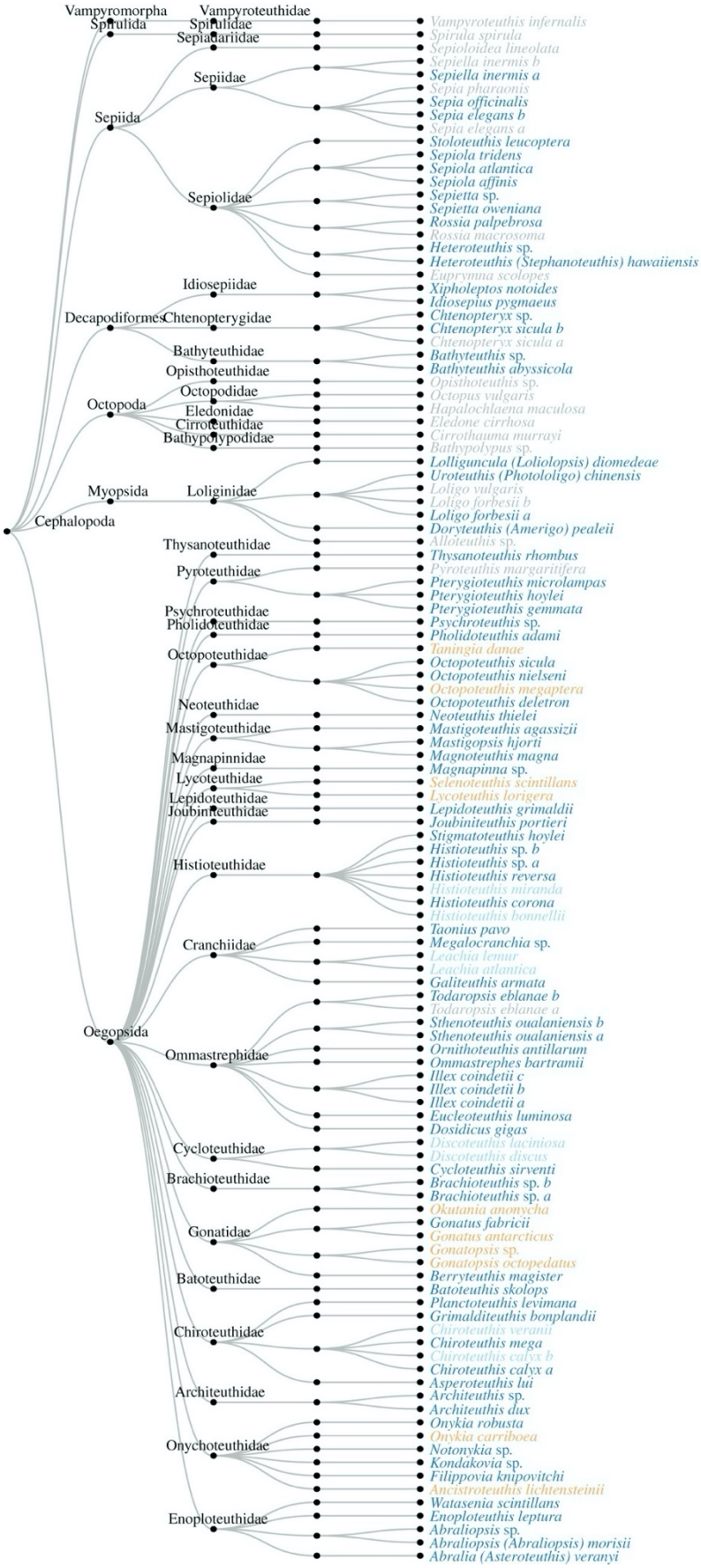

Figure 3| Results of *in silico* amplification of the SILVA database (146 sequences, 97 taxa) with *Ceph18S*. Grey taxa were not amplified *in silico*, whereas dark blue taxa were amplified and unambiguously identified. When taxa had equal target sequences and hence could not be unambiguously identified, colour indicates whether the shared taxonomic level was genus (light blue) or family (orange).

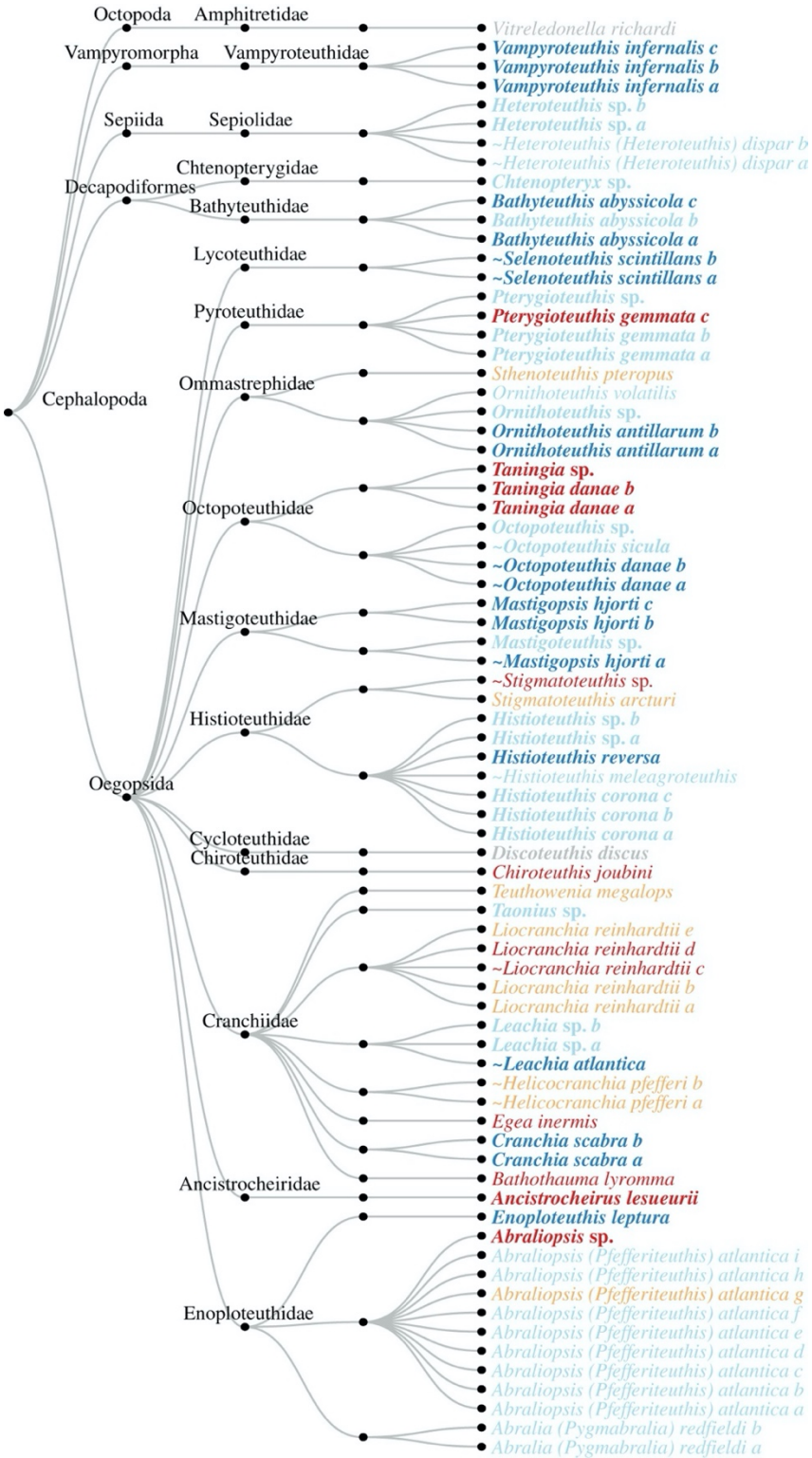

Figure 4| BLAST results of sequences of 75 tissue DNA extracts obtained with the *Ceph18S* primer. Morphological ID names in bold indicate that there was a reference sequence to the same taxonomic resolution (genus or species) in GenBank. Grey specimens could not be amplified. Name colours indicate whether the match between morphological and BLAST ID was to species level (dark blue), genus level (light blue), family level (orange) or other taxonomic level (red). A ~ (tilde) indicates there was another BLAST match with the exact same score, i.e., that the taxon could not be unambiguously identified.

An additional seven taxa could be matched to the next best taxonomic resolution (23%), and three taxa could not be identified to species, genus or family level despite reference sequences being available (10%). A final two taxa could not be identified to species, genus or family level, but also no reference sequences were available, so it is unknown if the lack of references or poor resolution of the primer is responsible (7%).

Comparison to 16S rRNA primer sets

The GenBank nuclear 18S rRNA reference database obtained through Primer-BLAST [48] with *Ceph18S* contained 107 taxa, and was, therefore, similar in size to the SILVA 18S rRNA reference database with 97 taxa. The coverage index and specificity index for *Ceph18S* was similar for this GenBank (B_c = 0.80, B_s = 0.80) and SILVA database (B_c = 0.85, B_s = 0.78). The GenBank mitochondrial 16S rRNA database for both *CephMLS* [41] and *S_Cephalopoda* [42] contained 367 taxa. The coverage index for *CephMLS* and *S_Cephalopoda* was B_c =0.82 and B_c = 0.72, respectively, i.e., of similar size and lower than for *Ceph18S*. The specificity index for *CephMLS* and *S_Cephalopoda* was B_s= 0.69 and B_s = 0.46, i.e., 11–34% lower than for *Ceph18S*. A Venn diagram analysis shows that 95% and 94% of cephalopod taxa can be amplified (figure 5a) and identified (figure 5b), respectively, by a multi-marker approach with Ceph18S, CephMLS and S_Cephalopoda for eDNA surveys.

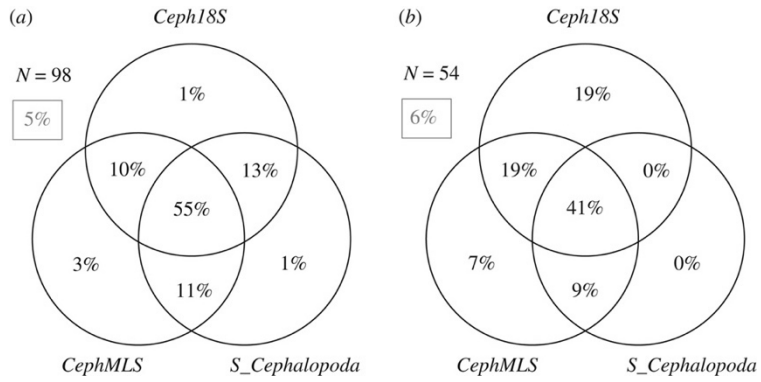

Figure 5| Venn diagram of complementarity in (*a*) amplification and (*b*) identification between *Ceph18S, CephMLS* [41] and *S_Cephalopoda* [42]. *N* represents (*a*) the number of taxa that were present in both the 16S and 18S GenBank reference databases, and (*b*) the number of taxa amplified by all three primer sets (i.e., the middle plane of (*a*) is the subset used for (*b*)). Grey percentage in the box is the number of taxa that could not be (*a*) amplified and (*b*) identified by either primer set. Sum of percentages may deviate ±1% due to rounding.

There is 1% of taxa that can only be amplified by *Ceph18S* and not by *CephMLS* or *S_Cephalopoda*, and 19% of cephalopod taxa can be identified unambiguously by *Ceph18S* but not by *CephMLS* or *S_Cephalopoda*. For comparison, 7% and 0% of taxa can be unambiguously identified only by *CephMLS* or *S_Cephalopoda*, respectively, and 9% can be identified by both 16S primer sets but not by *Ceph18S*. In other words, while the primer sets complement each other only moderately in terms of amplification success, the *Ceph18S* target sequences have a greater taxonomic resolution so that 19% additional taxa can be identified.

4.4. Discussion

A novel universal primer pair named *Ceph18S* targeting the cephalopod nuclear 18S rRNA region was characterized. Due to the relatively small target sequence length of approximately 200 bp, the primer pair is suitable for metabarcoding of cephalopod eDNA and can be applied in field studies. We will discuss the *Ceph18S* primer in the context of metabarcoding, where reliable primers are important to avoid false positives (i.e., wrongly identified taxa) and false negatives (i.e. undetected species) [58].

Application of Ceph18S in field studies

The *Ceph18S* primer pair targets the V2 variable region of the small-subunit 18S rRNA [52] flanked by relatively conserved regions. This region is suitable for taxonomic assignment [59], with enough variation to allow the identification of a variety of taxa. However, clustering algorithms in metabarcoding pipelines can have difficulties with ribosomal target sequences due to the inherent length variation of rRNA variable regions [60]. As this length variation is present in *Ceph18S* target sequences (131–196 bp), it is recommended to omit clustering and use sequence variants directly, for example, with DADA2 [61], so no clustering threshold has to be chosen and a higher taxonomic resolution can be obtained [62].

According to the coverage index estimated in silico, *Ceph18S* should be able to amplify approximately 80–85% of cephalopod species. This coverage index might be slightly underestimated due to missing V2 regions in SILVA reference sequences for some species. Coverage of these species by *Ceph18S* could be checked using tissue DNA extracts if specimens are available. The variation in the annealing sites of the primers indicates that there will be mismatches between the primer and some target

species. A small number of mismatches are not always problematic, as amplification might still occur although perhaps suboptimally [63]. This is confirmed by the fact that all but two tested cephalopod DNA extracts (94%) could be amplified with *Ceph18S* empirically. Case in point is the observation that *Vampyroteuthis infernalis* was not amplified in silico due to a mismatch between the primer and annealing site but was amplified and correctly identified to species level empirically. The distinctive phylogenetic position of *V. infernalis* in the cephalopod phylogenetic tree [53], and thus its distinctive target sequence, allowed the alignment algorithm to distinguish between *V. infernalis* and target sequences from other taxonomic groups even though the annealing mismatch might potentially have resulted in suboptimal amplification. A possibility to increase the universality of the primer would be to create a degenerate version of *Ceph18S*, i.e., a mixture of very similar primers where one or more nucleotide bases are varied in order to limit mismatches with target species [64].

Overall, the tests indicate that an estimated 44–66% (B_C x B_S) of all cephalopod species can be amplified and correctly identified with the *Ceph18S* primer. In comparison, *S_Cephalopoda* [42] and *CephMLS* [41] are estimated to amplify and identify 33% and 56% of all cephalopod species, respectively. Both the in silico and empirical tests indicate that the *Ceph18S* primer pair is not suitable for the detection of octopods, and can give ambiguous results for sepiids, myopsids, octopotheuthids and gonatids. Based on the empirical results, it might appear that the cranchiids are also problematic, but this may also be caused by a severe under-representation of this group in GenBank. If only specificity to genus level is required, *Ceph18S* performs well with an empirically obtained genus-level specificity of 75%. Furthermore, filtering procedures in the metabarcoding pipeline can increase the number of identified taxa. Any assumptions in these filtering procedures should, however, be considered when interpreting the results. For example, it might be possible to filter based on known biogeographic area, i.e. there are multiple possible species matches to a global reference database, but only one species is known to occur in the studied region and this would omit the detection of, for example, any invasive species.

Reference sequences

The specificity index of *Ceph18S* was 0.78–0.80 as estimated in silico, and 0.47 as estimated empirically. However, this estimated empirical specificity index is based on a limited number of identified species (n = 15) for which reference sequences were available in GenBank.

Of the approximately 700 known cephalopod species, only 88 species are present in the SILVA database and fewer than 300 species have a complete or partial 18S rRNA sequence in GenBank. The reference sequences for cephalopods in GenBank that are currently available are mostly mitochondrial sequences, whereas the 18S rRNA gene is nuclear. For almost half of the taxa that were empirically tested with the *Ceph18S* primer, no 18S rRNA reference sequences were available in GenBank. Our barcoding efforts with *Ceph18S* add new 18S rRNA reference sequences for 13 taxa to GenBank. A comprehensive high-quality reference database is important for metabarcoding primer development and testing because it provides a good overview of nucleotide diversity over the gene and can reduce the occurrence of misidentifications and false negatives [58]. An effort to sequence additional species, as we present here, is, therefore, recommended to aid further primer development and the taxonomic resolution of eDNA metabarcoding surveys [65]. If a proper reference library is not available, alternative biodiversity estimates based on sequence diversity (i.e., omitting direct taxonomic assignment) could be used [66]. There are five species for which the *Ceph18S* target sequences did not match to the expected species in GenBank, even though a representative reference sequence was available.

A first explanation could be a wrong morphological identification assigned to the DNA sequence. For example, the taxa in the Histioteuthidae family are relatively difficult to distinguish, which may have caused a misidentification of our *Histioteuthis corona* or its matching GenBank sequence *Histioteuthis hoylei*. However, we deem this explanation unlikely, as all morphological identifications in both this paper and for the GenBank reference sequences were done by cephalopod experts (H.-J.T.H. and Annie Lindgren, respectively). A second explanation could be the existence of cryptic species, where species are morphologically similar but genetically different. Although widespread existence of cryptic oceanic species has been suggested [67] and has been shown for some cephalopod taxa [68,69], no cryptic species complexes have been reported for the species with GenBank mismatches. Additionally, the taxonomy of the Octopoteuthidae is problematic with evidence of genetic similarity between *Octopoteuthis sicula, O. danae* and *O. megaptera*, which does not support the distinction of multiple species [70] and explains our 100% match of *O. sicula to O. danae* and *O. megaptera* with our relatively short *Ceph18S* target sequence. A third explanation for the mismatches is that the relatively short target sequence length of *Ceph18S* in some cases cannot provide enough resolution to account for natural variability for a reliable identification, especially if the species is under-represented in GenBank. Three of the five mismatched species did match to the correct genus.

Target sequences within a taxon can be expected to be relatively similar, so that a couple of different nucleotide bases, either due to natural variability or erroneous base calls in the sequencing process, can induce mismatches especially in short target sequences. The remaining two mismatched species with hits outside the expected genus had low identities to their best match (93%, 96%) and only one representative reference sequence available in GenBank. The quality of all our barcoded sequences was reviewed and approved, and repeated sequencing of the same individuals gave consistent results. For example, the same specimen of *Taningia danae*, which was reliably identified morphologically, was sequenced twice with consistent target sequences and closest match of 93% to *Lepidoteuthis grimaldii*. Therefore, it is likely this sequence of *T. danae* reflects natural variability in this partial 18S rRNA region for the species.

Biodiversity surveys with a multi-marker approach

Extrapolating the *Ceph18S* B_c x B_s value to the known approximately 700 cephalopod species, it is estimated about 310–460 species can be amplified and identified with *Ceph18S*. Additionally, 19% of taxa that are amplified by all three primers can only be unambiguously identified by *Ceph18S* and 16% can only be unambiguously identified by the 16S primer sets. Therefore, a multi-marker approach, where multiple universal cephalopod primers are combined, can increase overall coverage and specificity when applying eDNA analysis for cephalopod biodiversity studies [29]. Note that the 16S rRNA primer set *S-Cephalopoda* from [42] targets a subregion of the target sequence amplified by the 16S primer set *CephMLS* from [41], which are, therefore, similar in their resolution.

Employing a multi-marker approach has several advantages. Firstly, the highest confidence for species detection is obtained when a species is found in multiple samples and by multiple markers [71]. However, solely applying such a stringent definition would omit species that can be detected by one marker only. In the complementary case of 18S and 16S cephalopod primer sets, 35% of cephalopod species would not be detected with confidence under this stringent definition as they are only identified by either one, and not by both. Therefore, we recommend appreciating the complementary nature of primers sets and assigning a confidence value to a species detection rather than fully discarding single marker detections. Secondly, employing multiple markers is useful with limited availability of reference sequences [72]. By searching for multiple markers, the reference database can include species that might have a reference sequence for one region, but not the other.

To successfully employ the multi-marker approach, it is important to understand the limitations of each marker. For example, markers may have different affinities to different taxonomic groups [32] and there might be differences in detectability between nuclear and mitochondrial eDNA [73–75]. Studies into marker specificity and limitations, like the current study, can, therefore, help interpret eDNA metabarcoding results.

If the specificity of certain cephalopod groups is still low even with the combined marker approach, additional universal primer pairs can be developed (e.g., based on the 28S region, [55]), potentially targeting only a subgroup within the cephalopods. Additionally, the usage of slightly varying primer annealing temperatures, like in a touch-down PCR [76], can reveal more taxa than using a single annealing temperature. High temperatures favor rare but perfectly matching sequences, but lower temperatures generally recover broader diversity as it allows annealing mismatches, and taxa found at different temperatures are not strictly subsets of each other and thus add to overall richness [77]. Even though primer sets for specific cephalopod taxa allow identification of specific elusive species [25] and might complement a universal cephalopod primer for problematic taxa, it would be difficult to do cephalopod biodiversity assays using only species-specific primers. For example, regional reported species diversity is 32 in the Arctic [78], 54 in the Antarctic [78], 68 at the Southwest Indian Ocean Ridge [79], 70 around the Kermadec Islands [80], 77 near Bear Seamount [81], up to 85 around the Canary Islands [82]. Additionally, such an approach would require pre-existing knowledge of local cephalopod diversity, which is often lacking. Therefore, an eDNA metabarcoding approach with multiple markers of which the strengths and limitations are tested is a suitable complementary method for further studies into local cephalopod biodiversity patterns.

Conclusion

Despite the pivotal role of cephalopods in marine foodwebs, knowledge on their diversity and distribution still has major gaps, especially in oceanic regions. Metabarcoding of eDNA is a proven powerful technique for biodiversity surveys, but it has not yet been applied to the study of these elusive organisms. This study characterized a new universal metabarcoding primer pair for the analysis of cephalopod eDNA targeting the nuclear 18S rRNA gene in order to complement published mitochondrial 16S rRNA primer sets [41,42]. The developed *Ceph18S* primer pair amplifies an approximately 200 bp target sequence estimated to be able to identify about 310–460 cephalopod species. *Ceph18S* is estimated to amplify and identify 8–31% more cephalopod species than the 16S

rRNA primer sets. Furthermore, 19% of taxa amplified by both the 16S and 18S rRNA primer sets can only be identified with *Ceph18S*, thereby increasing overall taxonomic resolution in a multi-marker metabarcoding approach. *Ceph18S* is not suitable for the detection of octopods, and should be used with caution on sepiids, myopsids, octopotheuthids and gonatids. The submitted barcodes to GenBank add new 18S rRNA partial sequences for 13 cephalopod taxa previously absent in the GenBank database.

The *Ceph18S* primer pair is currently being applied in metabarcoding studies of cephalopod eDNA from epi-, meso- and bathypelagic depths [83]. The preliminary results of this study show that *Ceph18S* can successfully be applied in a metabarcoding workflow on a large dataset and can successfully amplify and identify cephalopod eDNA. Therefore, the *Ceph18S* primer pair, potentially in degenerate form or combined with other universal cephalopod primers, is a useful new molecular tool that can be used alongside other sampling methods for studying cephalopod diversity and distribution from shallow, coastal waters to the pelagic and deep-sea environments.

Ethics

Cabo Verde has not ratified the Nagoya protocol. To fulfil the national ABS regulations of Cabo Verde, we obtained the required permit for the publication of results based on samples collected in Cabo Verde waters from the Direcção Nacional do Ambiente (National Directorate for the Environment of Cabo Verdes). Permission for fieldwork and publication of results was granted by Ministério da Agricultura e Ambiente of Cape Verde and Agência Marítima e Portuária of Cape Verde.

Data accessibility

All barcoded sequences are accessible via GenBank accession numbers MT680727–MT680790 and MT680792–MT680800. The electronic supplementary material includes the Supplementary Data and Code zip-file, which contains the code and data used for data analysis and the creation of figures 1, 3 and 4, and Supplementary Table S1, which contains sample station information.

Authors' contributions

D.S.W.d.J. carried out the molecular laboratory work, performed the bioinformatics and data analysis and drafted the manuscript; V.M. participated in the design of the study, collected field data, helped with the laboratory work and participated in data analysis. T.B. helped with the laboratory work and data analysis. O.P. participated in conceiving and designing the study. T.B.H.R. participated in conceiving and designing the study. H.-J.T.H. conceived and designed the study, collected field data, performed morphologies species identifications and coordinated the study. All authors critically revised the manuscript, gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests

We declare we have no competing interests.

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Chapter 5

Distribution, associations and role in the biological carbon pump of *Pyrosoma atlanticum* (Tunicata, Thaliacea) off Cabo Verde, NE Atlantic

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Abstract

Gelatinous zooplankton are increasingly acknowledged to contribute significantly to the carbon cycle worldwide, yet many taxa within this diverse group remain poorly studied. Here, we investigate the pelagic tunicate *Pyrosoma atlanticum* in the waters surrounding the Cabo Verde Archipelago. By using a combination of pelagic and benthic in situ observations, sampling, and molecular genetic analyses (barcoding, eDNA), we reveal that: *P. atlanticum* abundance is most likely driven by local island-induced productivity, that it substantially contributes to the organic carbon export flux and is part of a diverse range of biological interactions. Downward migrating pyrosomes actively transported an estimated 13% of their fecal pellets below the mixed layer, equaling a carbon flux of 1.96–64.55 mg C m^{-2} day^{-1}. We show that analysis of eDNA can detect pyrosome material beyond their migration range, suggesting that pyrosomes have ecological impacts below the upper water column. Moribund *P. atlanticum* colonies contributed an average of 15.09 17.89 (s.d.) mg C m^{-2} to the carbon flux reaching the island benthic slopes. Our pelagic in situ observations further show that *P. atlanticum* formed an abundant substrate in the water column (reaching up to 0.28 m^2 substrate area per m^2), with animals using pyrosomes for settlement, as a shelter and/or a food source. In total, twelve taxa from four phyla were observed to interact with pyrosomes in the midwater and on the benthos.

5.1. Introduction

Although gelatinous zooplankton are among the most abundant inhabitants of the open ocean[1,2],their roles in marine ecosystems have traditionally been underestimated[3]. Gelatinous zooplankton refers to a polyphyletic group of marine organisms, characterized by high water content of their tissues (~ 95%) and a planktonic existence (carried by the currents). This group includes taxa such as ctenophores, medusae, siphonophores and pelagic tunicates (i.e. salps, pyrosomes, doliolids and larvaceans)[1,3,4]. Since many of these animals possess delicate bodies that are easily damaged by net collections, it was not until the advent of underwater technologies such as bluewater SCUBA and submersibles that their ability to seasonally dominate pelagic midwater communities was noted[1,3,4]. With this discovery, a more complex picture of their roles in marine ecosystems soon emerged. Gelatinous zooplankton are now increasingly recognized as important players in the global carbon cycle, accumulating and transporting organic carbon to the seabed[5–7]. Nevertheless, at present day, only a small fraction of our ocean has been explored and gelatinous zooplankton remain poorly studied, particularly in deep pelagic ecosystems[2,8]. Pyrosomes are abundant gelatinous zooplankton in open ocean environments and continental shelf slopes, but relatively little is known about their general biology[9–12]. Most studies on the role of pyrosomes in ocean ecosystems focus on *Pyrosoma atlanticum* Peron, 1804, a species common throughout the Pacific, Atlantic and Indian Oceans[13]. *Pyrosoma atlanticum* has been shown to markedly redirect primary production with clearance rates measured up to 35 L h^{-1} for a single colony[12]. Additionally, dense shoals were estimated to clear up to 53% of the phytoplankton stock[14]. Since *P. atlanticum* possesses one of the highest carbon contents measured among gelatinous zooplankton (35% of dry weight)[15], sinking moribund or dead colonies can transport substantial amounts of organic matter to the deep[15–17]. Mass mortality of pyrosome blooms, for instance, can result in so called 'jelly-falls', providing abundant food for a variety of benthic fauna[15,17]. Together with living pyrosomes, decrepit colonies were shown to be consumed by echinoderms, actinarians, crustaceans[15,17], fishes, turtles[18], marine birds[19,20] and even marine mammals[21,22]. Few of these studies, however, have addressed the ecology of *P. atlanticum* in the water column, and rarely via in situ observations[15–17,23]. In addition to being a food source, living pyrosome colonies further add to the downward flux of carbon by the fast production of fecal pellets, which may have as much as 22% carbon per unit dry weight[14]. The rate of this downward carbon flux

may be further enhanced through *P. atlanticum's* diel vertical migrations, by which they can actively transport fecal material below the mixed layer[10]. The extent of this transport, however, has only been estimated theoretically[10,14] and it remains unknown whether the fecal material reaches greater depths in practice. To our knowledge, pyrosome fecal pellets have not been reported from sediment traps, even though the sedimentation of salp fecal material has been well documented (e.g.[24,25] and references therein). A novel molecular method that may provide an effective tool to confirm this transport is the detection and barcoding of environmental DNA (eDNA)[26]. eDNA is defined as the genetic material that organisms shed in the form of dead tissue cells, feces or mucous, which can be extracted from their environment as it usually remains in the water for a period of time without direct presence of the animal. Although eDNA analyses are becoming increasingly popular to document biodiversity of marine communities[26,27], the technique has never been implemented to detect pyrosome DNA (from colonies or fecal pellets) at depth. During two research cruises to the Cabo Verde region of the eastern tropical North Atlantic (ETNA), we encountered large aggregations of *P. atlanticum*, which allowed us to investigate its ecological role in this system. Even though the open ocean surrounding the Cabo Verde archipelago is oligotrophic, geographic features such as seamounts and islands induce local upwelling and therefore enhance biological productivity[28,29]. In addition, northeasterly trade winds force eddy formation in the wake of the islands and enhance upwelling depending on season[30,31]. The region features a weak mesopelagic oxygen minimum zone (OMZ), centered around 450 m depth with dissolved oxygen concentrations reaching ~ 40 µmol kg^{-1}[;32,33]. By combining multinet sampling with genetic molecular tools and in situ video observations from a manned submersible and two towed camera platforms, our aim was to investigate: (i) the abundance and vertical distribution of *P. atlanticum* in the water column in relation to environmental drivers, (ii) its role in the transport of organic carbon, and (iii) associations with other pelagic and benthic organisms.

5.2. Material and Methods

Sampling stations

Pyrosoma atlanticum were sampled during two R/V Poseidon research cruises from February 14–March 1, 2018 (POS520) and February 4–24, 2019 (POS532). Sampling stations were located off the Cabo Verde Archipelago and included the Cabo Verde Ocean Observatory (CVOO)[34] in the open ocean windward of the islands, two cyclonic eddy cores, and coastal and oceanic deployment sites in the lees of the islands of Santo Antão and Fogo (Fig. 1). Most stations were sampled during both day and night, with the exceptions of the eddy station in 2018, CVOO in 2019, and the oceanic station near Fogo in 2019, which were only sampled during the day. Eddies were identified and tracked with the help of satellite altimetry based on their negative sea level anomaly, reduced sea surface temperature and enhanced chlorophyll-a (chl-a) concentrations[31].

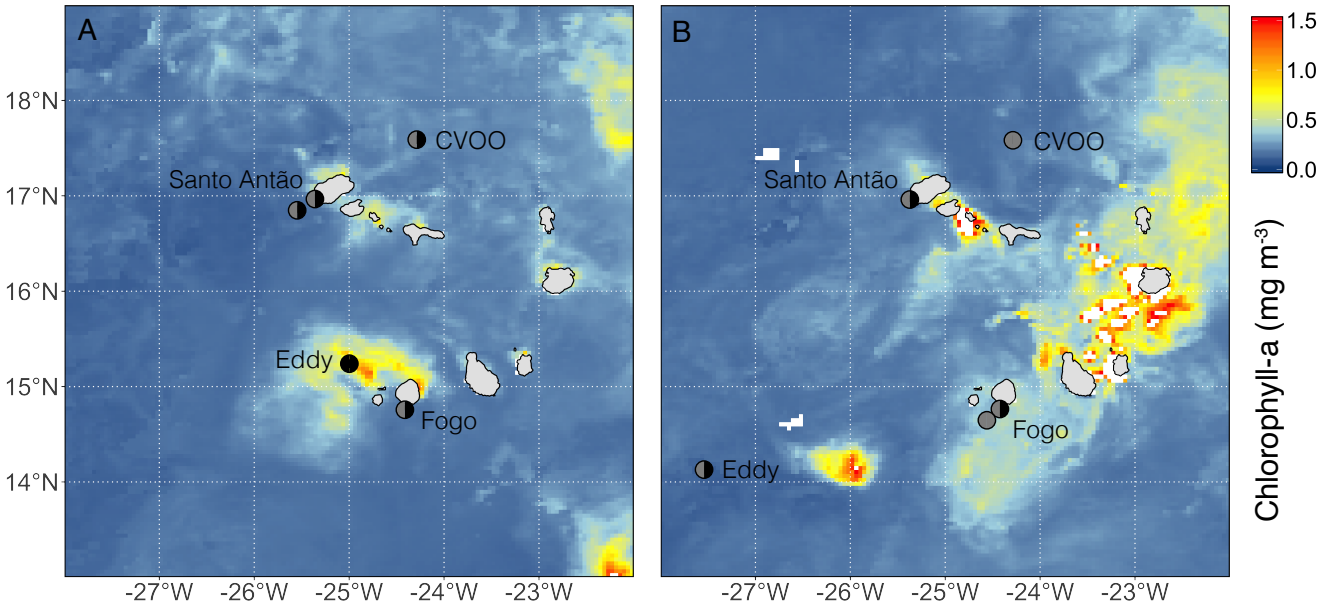

Figure 1| Sampling stations during the (A) POS520 cruise in 2018 and the (B) POS532 cruise in 2019, showing respective mean monthly chl-a concentrations (mg m^{-3}) from the MODIS-aqua database[35].
Since cloud cover obstructed chl-a measurements in February for both years, monthly measurements are shown for March in 2018 and January in 2019. As a result, the MODIS-aqua data does not align with our cyclonic eddy sampling points. White fields indicate missing data due to cloud cover. Circles indicate day (grey) and night time (black) sampling.

Eddy cores were targeted using the ship's thermosalinograph (TSG) and acoustic Spectroradiometer (MODIS) aqua database[35]. To confirm the depth range of pyrosome material, and to learn if the presence of pyrosomes can be confirmed through molecular methods, eDNA was Doppler current profiler (ADCP). CTD profiles (i.e., temperature, salinity, oxygen and chl-a concentrations) were collected at each sampling station, while additional chl-a surface concentrations were obtained from the Moderate Resolution Imaging sampled at varying depths in the cyclonic eddy (at 400, 600, 1000, 1900, 2200 and 2500 m) and CVOO station (at 1000, 1300, 1900, 2500 m) in 2019. The latter acted as a reference station where we did not observe pyrosomes. Water samples for the eDNA analyses were collected with 10 L Niskin bottles attached to a CTD rosette, while on board each depth was sampled in biological triplicates consisting of 2 L seawater. Samples were immediately filtered by letting the water drip through filters (0.22 µm pore size sterivex™ filters MERCK), which were then stored at -80 °C. Back in the laboratory ashore, samples were barcoded for pyrosome eDNA as described in the Supplementary Material. Cabo Verde has not ratified the Nagoya protocol. To fulfill the national ABS regulations of Cabo Verde, we obtained the required permit for the publication of results based on samples collected in Cabo Verde waters from the Direcção Nacional do Ambiente (National Directorate for the Environment of Cabo Verde).

Vertical depth distribution, abundance and substrate area

The vertical distribution and abundance of *P. atlanticum* were investigated using two methods. First, oblique multinet hauls were used to collect *P. atlanticum* colonies at different depth strata (Supplementary Table S1) using a Hydrobios Maxi multinet (0.5 m^2 in aperture, 2 mm mesh size, nine nets and electronic flow meters) towed at approximately 2 kn. Pyrosome length, width and wet weight were quantified as described in the Supplementary Methods. The weighted mean depth (WMD) for each haul was calculated using the WW of pyrosomes per multinet depth bin (g) and median depth (d in meters) of each bin per sampling station (i):

$$WMDi = \frac{\sum (WWi \cdot di)}{\sum WWi}$$

antitative video footage of pyrosomes at various depths (Supplementary Table S2). PELAGIOS was towed in a stair-step trajectory at the ship's average speed of approximately 0.51 m s^{-1}, resulting in

horizontal and vertical video transects. The sampling volume (2.54 m^3 s^{-1}) was estimated by mounting the PELAGIOS camera to the multinet (i.e. the MuViNet) at the Fogo and Eddy stations in 2018 and comparing video counts and net catches using a linear model following calculations by Hoving et al.[36] (Supplementary Figure S1). Video recordings were annotated using the Video Annotation and Reference System (VARS) developed by the Monterey Bay Aquarium Research Institute[37]. The depth-integrated substrate area provided by *P. atlanticum* aggregations was calculated per station as described in the Supplementary Methods.

Fecal pellet production and respiratory carbon flux

The downward active carbon flux from *P. atlanticum* fecal pellet production, linking primary production to depth, was based on nighttime multinet biomass. Since we did not catch any pyrosomes above the mixed layer during the day, we assume that all *P. atlanticum* migrated downward. The fecal pellet production (FP; in mg C $colony^{-1}$ day^{-1}) was calculated following the formula by Henschke et al.[10] based on observations from Drits et al.[14]: FP = 0.25 * CC where CC is the carbon content of *P. atlanticum* WW biomass (i.e. 3.92% of WW)[10,15]. To calculate the number of fecal pellets that are actively transported by migrating colonies, we used the gut turnover rate (GTR i.e. the time it takes for *P. atlanticum* to digest food particles, in hours) and the time needed to vertically migrate below the mixed layer (DM in hours). The GTR was obtained from Perissinotto et al.[12], who reported a gut processing time of 1.43 h for *P. atlanticum* at presumably 13.7 to 17.8 °C, which, although not directly reported, was the temperature range for all other experiments in their study, and similar to the temperatures measured here. Since temperature is known to affect metabolic rate, we chose not to use the GTR reported by O'Loughlin et al.[38], who measured a GTR of 2.6 h for *P. atlanticum* at a much colder 12 °C. Additionally, the faster GTR by Perissinotto et al.[12] results in most conservative estimates for the fecal pellet carbon flux (FPF) as it implies that most gut content will be evacuated before migrating out of the productive upper water layers. The DM was calculated based on the swimming speed of 0.05 m s^{-1} reported by Henschke et al.[10] and the distance from the average night WMD to the mixed layer depth at approx. 100 m (i.e. 54.2 m). The active fecal pellet carbon flux (FPF in mg C $colony^{-1}$ day^{-1}) was calculated as follows[10]:

$$FPF = \frac{FP}{24} \cdot (1.43 - DM)$$

To calculate hourly rates from daily rates, the FP was divided by 24 h. To obtain the total amount of fecal pellet carbon produced in the mixed layer at night, the hourly rates were multiplied by 8.5, which was the approximate time spent near the surface. Besides the fecal pellet flux, pyrosomes actively transport carbon below the mixed layer through the respiratory release of carbon dioxide[10]. This respiratory flux was quantified from daytime multinet biomass when pyrosomes were present below the mixed layer. For this, the respiratory carbon equivalent (RC, in µg C m^{-2} day^{-1}) was calculated according to the formula by Al-Mutairi and Landry[39] using the respiration rate (R, see Eq. (5)), the respiratory quotient (RQ, i.e., 1.16)[40], the molar weight of carbon (12) and the molar volume of an ideal gas at standard pressure and temperature (22.4):

$$RC = R \cdot RQ \cdot \left(\frac{12}{22.4}\right)$$

The R and RQ terms were derived from Henschke et al.[10]. Since there is no respiratory quotient for pyrosomes available, the respiratory quotient for salps was used. The oxygen respiration (in ml O2 $colony^{-1}$ $hour^{-1}$) for *P. atlanticum* was calculated as follows, using the average WW (g) per station:

$$R = 0.0046 WW^{1.2284}$$

To get to the final respiratory carbon equivalent, the respiration rate was multiplied by the time spent below the mixed layer and the depth-integrated abundance.

Organic carbon transport and biological associations

The instantaneous organic carbon flux as a result of pyrosome mortality was estimated by quantifying carcasses on the seabed at the Santo Ant o and Fogo stations in 2019. High-definition video recordings of these pyrosome-falls were made with the manned submersible JAGO[41] and the towed Ocean Floor Observation System (OFOS) equipped with the same camera and telemetry as PELAGIOS[36], two lasers for size reference, and a CTD for depth recording. OFOS was towed just above

$$CC = 12.54 TL^{1.90}$$

the seabed between 1500 to 200 m depth at the ship's average speed of 0.51 m s^{-1}. JAGO was restricted to < 400 m depth and either drifted with the currents or motored purposely to qualitatively investigate species associated with pyrosomes in the water column and on the seafloor. JAGO was also deployed in 2018 for additional midwater surveys. Observations of crustaceans and other animals associated with pyrosomes were made using all three platforms. To identify some of the crustaceans and the tissue they were attached to, select specimens were sampled with the suction samplers or acrylic collecting cylinders mounted to JAGO. Live animals were photographed on board using a Canon EOS 5DS R camera with a Canon 65 mm f/2.8 1–5 x macro lens. In the laboratory ashore, the samples were barcoded for DNA as described in the Supplementary Methods. The video recordings were annotated using VARS as above for midwater observations[37]. Next, carbon content (CC;C in µg) of the pyrosome carcasses was calculated following the equation by Lavaniegos and Ohman[42] using the average total length of colonies in 2019 (TL, 80.9 16.4 mm):

Bathymetric data from the Cabo Verde region were obtained from the GEBCO database[43] and used to plot the relative concentrations of pyrosomes on the seabed.

Statistical analysis

To investigate what environmental conditions led to increased *P. atlanticum* abundance, both linear and median quantile regression were used respectively for the multinet spatial and vertical distributions. Since the latter dataset deviated from normality, a non-parametric model was implemented. At the stations and depths where no pyrosomes were caught in the multinets, absence was recorded as zero values in both models. In the spatial model, depth-integrated abundance was tested against average sea surface temperature, minimum oxygen concentrations and depth-integrated chl-a concentrations at each station. In the vertical distribution model, the integrated abundance per depth stratum was correlated to the latter variables recorded at mid-depth of each depth bin, introducing day and nighttime observations as nested factor. Due to large differences in absolute abundance, the integrated abundance per depth stratum was normalized as a fraction of the total integrated abundance per station. In both analyses, the Akaike's Information Criterion (AIC) was used to determine the most parsimonious model based on combinations and single terms of the variables. All data were analyzed using R 3.5.2.

5.3. Results

Oceanographic conditions.

All sampling stations were characterized by relatively high surface temperatures, ranging between 17.5 ± 2.9 (s.d.) and 22.1 ± 1.1 °C in the upper 100 m of the water column for both years (Supplementary Table S3). Below the thermocline, these temperatures gradually decreased to reach ~ 6 °C around 400 m depth (Supplementary Figure S2). Lowest dissolved oxygen concentrations were recorded between 300–400 m depth (i.e. the OMZ core), with a minimum concentration of 42.8 µmol kg^{-1}. The mean monthly chl-a data from the MODIS-aqua database (Fig. 1) showed that the water masses surrounding the Cabo Verde Islands were characterized by relatively high productivity. In both years, the highest integrated (upper 100 m) chl-a concentrations were measured in the eddy during the night (reaching 46.0 mg m^{-2} in 2018 and 44 mg m^{-2} in 2019) and lowest at the CVOO during the day (dropping to 21.0 mg m^{-2} in 2018 and 16.4 mg m^{-2} in 2019; Supplementary Table S3). Although non-significant, the most parsimonious model to explain *P. atlanticum* spatial distribution (using integrated abundance) included sea surface temperature as the main driver of abundance (Linear regression, R2 adj. = 0.170, df = 13, F = 3.856, p = 0.071; Supplementary Figure S3A and Table S4). Here, lower temperatures correlated with greater pyrosome abundances. For the vertical distribution, the most parsimonious model only included chl-a, with increased chl-a concentrations correlating significantly to increased abundance at night (Median quantile regression, chl-a vs. nighttime abundance, df = 4, $p < 0.000$; Supplementary Figure S3B and Table S4).

Vertical distribution, abundance and substrate area.

Pyrosoma atlanticum was recorded at all sampling stations with exception of the CVOO in 2018, and was only observed in the cyclonic eddy and at the coastal Fogo station in 2019. During the day, *P. atlanticum* was present below the mixed layer (mixed layer depth 100 m), while most colonies migrated to the surface at night (Fig. 2). During these diel vertical migrations, pyrosomes migrated on average 313 m, with an average daytime weighed mean depth of 359 m and 46 m at night (Supplementary Table S5). It should be noted that the daytime distribution coincided with the OMZ core between 300 and 400 m depth. The deepest pyrosomes observed were recorded with PELAGIOS

at the Santo Ant o oceanic night station in 2018, where seven colonies were observed between 1300 and 2500 m depth. These colonies did not have the straight cylindrical shape typical for most pyrosomes, but instead were bent or almost spherical. They did have a similar color to living pyrosomes as they were semi-transparent pink to purple, rather than the opaque grey frequently observed in dead colonies. The multinet and PELAGIOS platforms recorded slightly different depth-integrated abundance and densities, with PELAGIOS generally observing higher numbers of colonies (Supplementary Table S6, Fig. 2). In 2018, PELAGIOS observed highest depth-integrated abundances at the coastal Fogo station (110.51 and 8.37 colonies m^{-2} during the day and night, respectively), with maximum concentrations of 8.27 colonies 10 m^{-3} between 400 and 500 m (day). The multinet data, on the other hand, showed highest abundances at the Santo Antão oceanic station in 2018 (17.85 colonies m^{-2} at night, no daytime numbers available), but maximum concentrations of 0.64 colonies·10 m^{-3} between 350 and 450 m (day) at the Fogo station. Surprisingly, the multinet did not catch any pyrosomes during the night at Fogo, while PELAGIOS' 'night' sampling had already commenced at 16:30 h (UTC) with maximum densities reaching only 0.72 colonies·10 m^{-3} between 300 and 400 m. In 2019, PELAGIOS observed maximum abundances in the cyclonic eddy (1.07 and 15.93 colonies m^{-2} during the day and night, respectively), with maximum densities of 16.07 colonies 10 m^{-3} at 30 m (night).

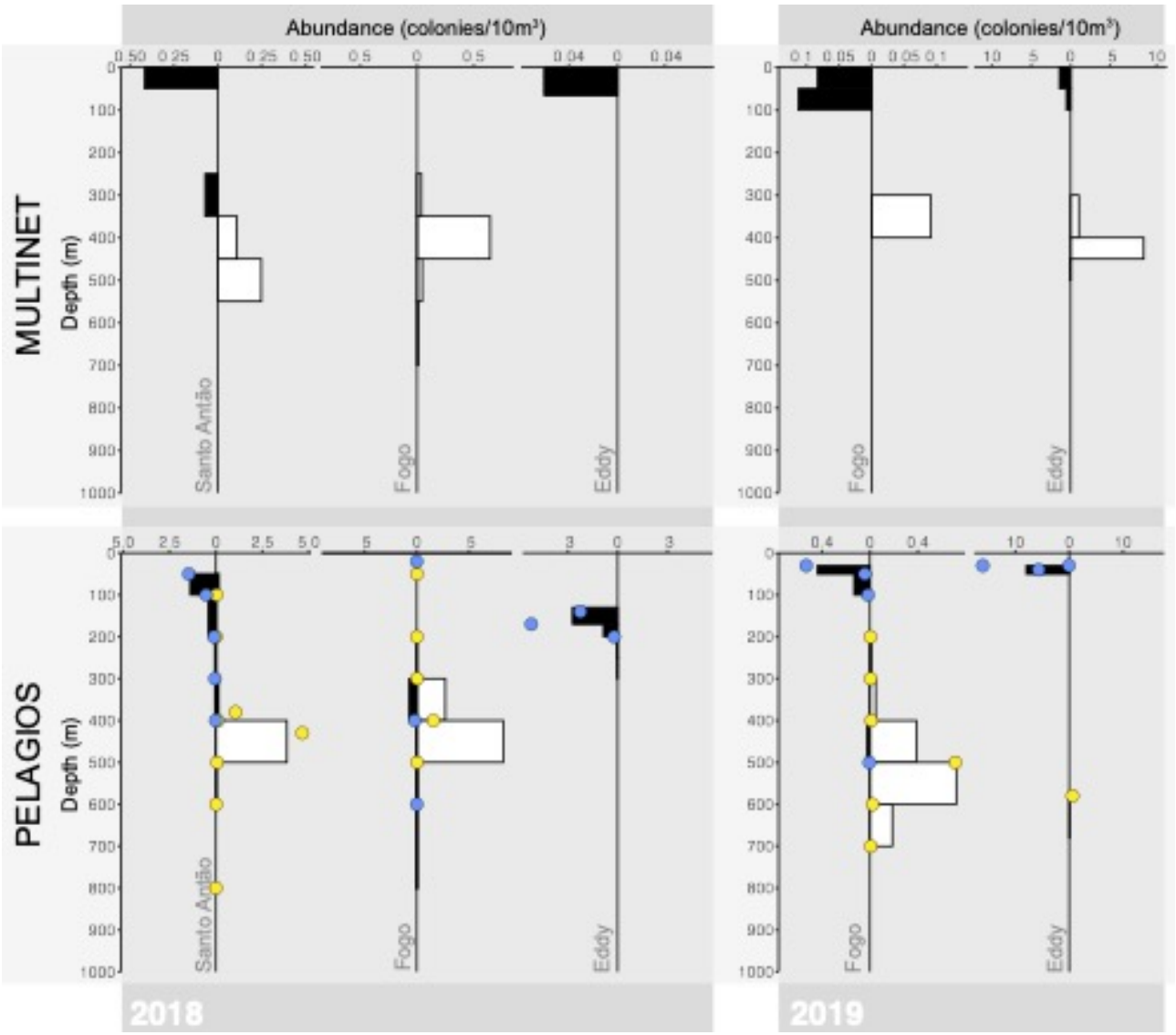

Figure 2| Vertical distribution of *P. atlanticum* (colonies 10 m^{-3}) in the eastern tropical North Atlantic, as caught with the multinet (upper plots) and observed with PELAGIOS (lower plots) in 2018 (left) and 2019 (right). Observations were made at Santo Antáo, Fogo and two cyclonic eddies. For the PELAGIOS observations, bars indicate abundance counted during vertical descents of PELAGIOS, while dots indicate horizontal transects. Both day (yellow dots, white bars) and nighttime (blue dots, black bars) observations are shown.

At the same station, the multinet showed 56.75 and 10.12 colonies m^{-2} during the day and night, respectively, with maximum concentrations reaching 9.28 colonies 10 m^{-3} between 400 and 450 m (day). The horizontal distribution of *P. atlanticum* was heterogeneous and

neither multinet nor PELAGIOS ever recorded a similar abundance at a station twice. The depth-integrated substrate area provided by the pyrosome aggregations ranged between 17.0–368.0 cm^2 per m^2 water surface in 2018 and 28.3–2820.4 cm^2 per m^2 in 2019 (Supplementary Table S6).

Fecal pellet production, respiratory carbon flux and eDNA.

The production of fecal pellets at night in the mixed layer accounted for an estimated 14.75–295.51 mg C m^{-2} day^{-1} in 2018 and 19.12–485.57 mg C m^{-2} day^{-1} in 2019 (Supplementary Table S5). It was estimated that, during the downward migration at dawn, pyrosomes transport 13.3% of these fecal pellets below the mixed layer, corresponding to 1.96 to 64.55 mg C m^{-2} day^{-1} (Supplementary Table S5). The amount of respiratory carbon released below the mixed layer accounted for only 0.0003 to 0.0053 mg C m^{-2} day^{-1} in 2018 and 0.0004 to 0.0393 mg C m^{-2} day^{-1} in 2019 (Supplementary Table S5). Since the oceanic station in the lee of Santo Ant o and the Eddy in 2018 only included nighttime sampling, the respiratory carbon flux for these stations was based on nighttime biomass, as we assume that these pyrosomes migrated below the mixed layer given our observations from other stations (Fig. 2). The respiratory carbon flux accounted for approx. 0.03 0.02% of the fecal pellet flux. Our eDNA analysis showed that the presence or traces of pyrosomes can be detected in water samples (Supplementary Figure S4), although we only detected four sequences in total. In the cyclonic eddy in 2019, the station of maximum pyrosome abundance, water samples collected at 400 m (n = 2), 600 m (n = 1) and 1000 m (n = 1) had a 100% Blastn match with 100% coverage to sequences from *P. atlanticum*, *Pyrostremma spinosum* (Herdman, 1888), *Pyrosomella verticillata* (Neumann, 1909) and *Pyrosoma godeauxi* van Soest, 1981 (Supplementary Table S7). In comparison, PELAGIOS only recorded pyrosome colonies down to 700 m and the multinet caught colonies down to 500 m in the cyclonic eddy (Fig. 2). No pyrosome eDNA was detected at the CVOO, which is in correspondence with our observational data.

Organic carbon on the seabed

All *P. atlanticum* carcasses were found on the shallower end of the island slopes (Fig. 3), with a total of 404 pyrosomes observed at Santo Ant o and 140 at Fogo (Supplementary Table S8). The deposited pyrosomes observed between 500 and 213 m had a similar color to living colonies (i.e. pink to purple), with many more floating > 1–2 m above the seabed (i.e. 133 seen at Santo Ant o and 136 at Fogo).

Relative carbon depositions were similar at both locations, accounting for up to 41.71 and 39.75 mg C m−2 at the Fogo and Santo Ant o stations, respectively (Fig. 3, Supplementary Table S8).

Biological associations

Different organisms were observed to interact with *P. atlanticum* in the water column and on the seabed, most likely involving feeding (Table 1). In the pelagic realm, eleven unidentified shrimps of the Oplophoridae Dana, 1852 (220–360 m, Fig. 4A) and the medusa *Drymonema gorgo* Müller, 1883 (50 m, Fig. 4B) were seen feeding on *P. atlanticum*. Juvenile stages of the penaeid shrimp were observed within and on pyrosomes (Fig. 5D–E). These shrimps have distinctive red and white patterns on their antennae, telson and uropods and were often observed inside a colony with antennae projecting. DNA barcoding identified them as *Funchalia villosa* (Bouvier, 1905) (COI 92–100% coverage; voucher USNM1524835). Furthermore, PELAGIOS in situ observations revealed four occasions where a small fish was situated inside *P. atlanticum* (20–30 m, Fig. 4C). Hyperiid amphipods *Phronima* Latreille, 1802 and *Hyperia* Latreille, 1823 were the most common associates with living *P. atlanticum* (Fig. 4F). Smaller hyperiids were frequently seen as white dots on the pyrosomes (Figs. 4D, E; 5A, H; i.e. about the same size as a pyrosome zooid ~ 3 mm, Fig. 5B,C), which on closer inspection had excavated individual zooids to take their place in the colony. These hyperiids were juveniles of an unknown oxycephalid (Fig. 5F,H), most closely related to *Streetsia* Stebbing, 1888 and *Leptocotis* Streets, 1877. More specific identification was not possible due to absence of similar sequences and the early development stage of the specimens, which lack established taxonomic characteristics. In addition, several *Phronima* were observed feeding on, or having fashioned their barrel from *P. atlanticum* (Fig. 5I,J). In one case, the *Phronima* was left with the pyrosome it was feeding on overnight in chilled seawater and within eight hours it had consumed nearly 20 mm2 of the colony (Fig. 5G). Barcoding revealed that two of the six collected *Phronima* were *Phronima sedentaria* (Forsk l 1775) and eight barrels were confirmed as pyrosome tissue. On the seabed, an unidentified decorator crab (382 m, Fig. 4G), five Anomura (361 m, Fig. 4H), two Actiniaria (287 m, Fig. 4I,J), a gastropod (307 m) and five *Pandalopsis* Bate, 1888 (230 m, Fig. 4L) were seen consuming pyrosome carcasses. Two specimens of *Primno* Guérin-Méneville, 1836 were observed and collected with a *P. atlanticum* colony lying on the sea floor (312 m). This hyperiid generally fits the description of *Primno evansi* Sheader, 1986 but barcodes indicate they belong to an as yet undocumented/unsequenced species of *Primno*. In addition, a *Dondice* Er. Marcus, 1958 nudibranch

(315 m, Fig. 4K) was observed moving over a colony, although a trophic interaction could not be confirmed.

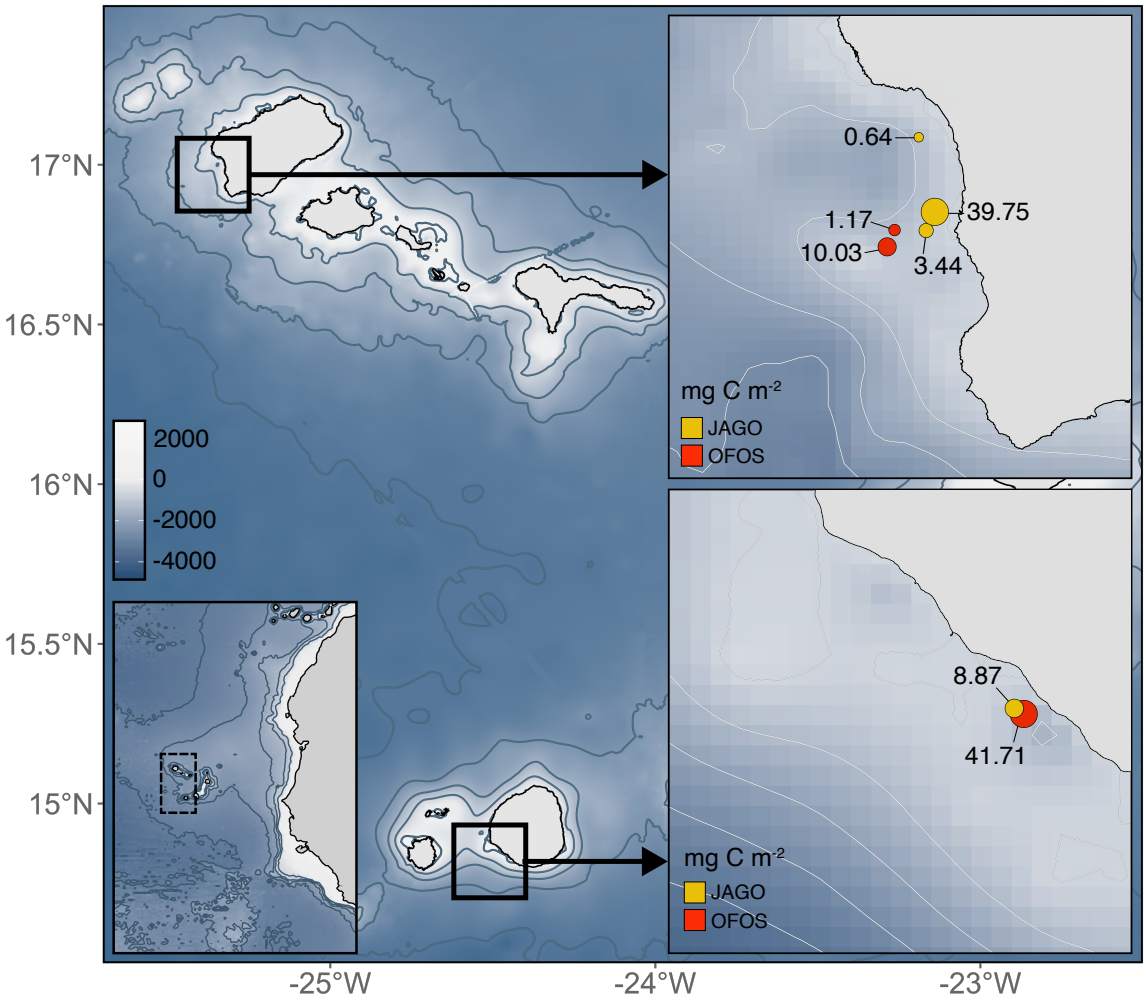

Figure 3| Carbon depositions of *P. atlanticum* in mg C m^{-2} near Cabo Verde Archipelago (left lower panel, dashed line) on island slopes of Santo Antão (right upper panel) and Fogo (right lower panel). Observations were made with the manned submersible JAGO (yellow) and towed camera platform OFOS (red). Bathymetric data was obtained from the GEBCO database[43].

5.4. Discussion

Abundance and organic carbon flux

Our statistical analyses showed that increased pyrosome abundances were correlated with higher chl-a concentrations and reduced surface temperatures. This suggests that the island- and eddy-induced upwelling surrounding the Cabo Verde landmasses created a favorable environment for *P. atlanticum* blooms in 2018 and 2019. Even though temperature proved to be a less strong predictor of abundance than chl-a, both environmental variables are inversely linked in upwelling events. Our observations are further confirmed by the fact that pyrosomes have rarely been caught in the oligotrophic ocean surrounding the islands, but were observed in the equatorial upwelling at 23 °W and in a cyclonic eddy east of the archipelago (Hauss, unpublished data). Productivity as a driver for pyrosome proliferation was also confirmed by Henschke et al.[10], who investigated *P. atlanticum* abundance in three eddies in the Tasman Sea. In contrast, Schram et al.[44] reported increased *P. atlanticum* abundance in temperatures associated with reduced phytoplankton productivity in the northern California Current (NCC, i.e. 12–15 °C). However, the latter authors only measured chl-a concentrations at 5 m depth, whereas this study and Henschke et al.[10] measured chl-a in the upper 100 m of the water column. When assessing the impact of pyrosome blooms, both local productivity (e.g. upwelling events) and temperature appear to be important factors in determining their abundance and concomitant effect on ocean ecosystems.

Pyrosoma atlanticum colonies were found to vertically migrate from the surface at night to depths of approximately 360 m during the day. The highest pyrosome densities were recorded during the night (1.61 colonies m^{-3}) and were within the range of reported maximum concentrations in the Atlantic Ocean near the Guinea Dome (0.1–2 colonies m^{-3})[45], but much lower than densities recorded off the Congo River mouth (9.5–41 colonies m^{-3})[14] and Oregon coast in 2017 (i.e. NCC, up to 5 colonies m^{-3})[44]. In contrast, the Cabo Verde blooms greatly exceed the maximum densities reported in the Mediterranean Sea (0.187 colonies m^{-3})[46], the Oregon coast in 2018 (i.e. NCC, 0.07–0.7 colonies m^{-3})[38] and a cyclonic eddy in the Tasman Sea (0.003 colonies m^{-3})[10]. The pyrosome abundance measurements in our study differed slightly with sampling gear. PELAGIOS probably provided a slightly higher resolution, considering the continuous transects and wide field of view of the

PELAGIOS camera versus the small multinet opening[36]. The tools nicely complemented each other as the multinet allowed us to collect specimens while PELAGIOS showed more detailed depth distributions and biological associations that would have otherwise been missed with the multinet. Our study thus illustrates the need for congruent collection of in situ observations and net samples.

On average, *P. atlanticum* in the Cabo Verde region traveled greater vertical distances (~ 313 m) when compared to *P. atlanticum* in the North Pacific (~ 60 m)[11] and eddies in the Tasman Sea (~ 222 m)[10]. The diel migration near the Cabo Verde Islands, however, was shallower than the ranges recorded in the Ligurian Sea (410–515 m)[46,47] and off the Canary Islands (650 m)[48]. It is possible that such regional variation is caused by differences in sunlight attenuation[49], although food availability, predation, temperature and oxygen concentrations are also known to affect migration ranges[49,50]. We here report one of the deepest records of pyrosomes in the pelagic realm yet, with *P. atlanticum* observed between 1300 and 2500 m depth. Even though these pyrosomes appeared to be alive due to their healthy coloration, they were abnormally shaped when compared to their mesopelagic counterparts. We cannot conclude whether these malformations resulted in some sort of swimming dysfunction that resulted in their abnormal depth, or if the colonies were simply moribund and sinking to the deep. It is particularly interesting that the highest *P. atlanticum* daytime abundance corresponded to the depth with the lowest oxygen concentrations in the OMZ. Various species of gelatinous zooplankton have been reported to tolerate such low oxygen environments[51–54], even though these low concentrations make the OMZ inaccessible for many animals[55]. Purcell et al.[51] suggested that some species of gelatinous zooplankton use the OMZ to relieve predation pressure[18]. At the same time parasitism may also be reduced by temporary visits in low oxygen regions. Nevertheless, pyrosome migration depth may be independent from oxygen, as *P. atlanticum* in the North Pacific was not found to migrate as deep as the OMZ centered around 700 m depth.

During the night, we estimated that *P. atlanticum* released a substantial amount of fecal carbon within the mixed layer (14.75 to 485.57 mg C m^{-2} day^{-1}). These rates are somewhat lower than, but within the range of, values estimated in the Atlantic Ocean near the Congo River mouth (87.4 to 1035 mg C m^{-2} day^{-1})[14]. Drits et al.[14] hypothesized that most of this carbon would be remineralized in the upper water column, as they showed that P. atlanticum fecal pellets degrade relatively quickly in warm surface waters (60% of their carbon content was lost after incubation for 45 h at 23 °C). We calculated that at least 13% of the fecal pellets produced (1.96–64.55 mg C m^{-2} day^{-1}) were actively

transported below the mixed layer by migrating colonies. Considering the relatively fast sinking rate of pyrosome fecal pellets (70 m·day-1)[14] and the immediate temperature drop below the mixed layer to approx. 14–19 °C slowing microbial degradation), this suggests a substantial flux of organic carbon during pyrosome blooms. Although we only provide an estimate of the fecal export flux, our values seem highly probable as they are within the range of the fecal pellet flux reported for salps in the Sargasso Sea (8.5-137 mg C m^{-2} day^{-1})[56]. We estimated that the P. atlanticum population released an additional amount of 0.0003 to 0.0393 mg C m^{-2} day^{-1} in the form of respiratory carbon once pyrosomes had reached their daytime depth. Even though this flux exceeded the respiratory flux for *P. atlanticum* reported in the Tasman Sea by several orders of magnitude (0.00006–0.12 10^{-3} mg C m^{-2} day^{-1})[10], it only contributes a fraction to the respiratory flux estimated for zooplankton communities in the North Atlantic near the Canary Islands (3.4 mg C m^{-2} day^{-1})[57], and micronekton and zooplankton southeast of the Cabo Verde Islands (22.2 mg C m^{-2} day^{-1})[58]. In spite of this, the combined fecal and respiratory flux are over three times higher than the particulate organic carbon flux reported in the ocean surrounding the Cabo Verde Islands (averaging between 9.3–18.1 mg C m^{-2} day^{-1} at 200 m)[59]. The excretory flux, as a result of *P. atlanticum* blooms, thus accounts for a considerable pulse of organic carbon released to deeper water layers.

Table 1| Biological associations of *P. atlanticum* in the eastern tropical North Atlantic.

Species	Association	Type	Date	Start dive (UTC)	Station	Depth (m)
Chordata						
Unidentified Actinopterygii	Swimming inside *P. atlanticum*, observed 4 times.	Pelagic: Trophic, shelter	22/02/18, 22/02/19	21:21, 21:04	Fogo, Eddy	20, 30
Crustacea						
Unidentified Oplophoridae sp.	Sitting on and eating *P. atlanticum*, observed 11 (2018) and 5 (2019) times.	Pelagic: Substrate, trophic	22/02/18, 17/02/19, 18/02/19	09:28, 15:56, 07:15	Fogo	220 – 300, 290 – 330, 360
Funchalia villosa	Sitting inside and on *P. atlanticum.*	Pelagic: Substrate	18/02/19	07:15	Fogo	320
Pandalopsis sp.	Eating *P. atlanticum*, observed 5 times.	Benthic: Trophic	17/02/19	15:56	Fogo	230
Phronima sp.	Holding cut *P. atlanticum*.	Pelagic: Trophic, reproduction	08/02/19	21:30	Santo Antão	70
Primno sp.	Attached to *P. atlanticum* on seafloor, observed 2 times.	Benthic: Trophic	10/02/19	09:24	Santo Antão	312
Hyperiid amphipods	Holding, sitting/swimming in and on *P. atlanticum.* (i.e. including *Hyperia* and Oxycephalidae).	Pelagic: Substrate, trophic	All	-	All	All
Unidentified decorator crab	Eating *P. atlanticum.*	Benthic: Trophic	10/02/19	09:24	Santo Antão	382
Unidentified Anomura	Five Anomura eating *P. atlanticum.*	Benthic: Trophic	12/02/19	09:16	Santo Antão	361
Mollusca						
Dondice sp.	Sitting on *P. atlanticum.*	Benthic: Trophic	10/02/19	09:24	Santo Antão	315

Unidentified gastropod	Eating *P. atlanticum.*	Benthic: Trophic	17/02/19	15:56	Fogo	307
Cnidaria						
Drymonema gorgo	Twelve *P. atlanticum* caught in tentacles.	Pelagic: Trophic	26/02/18	09:14	Fogo	50
Unidentified Actiniaria	Eating *P. atlanticum*, observed 2 times.	Benthic: Trophic	10/02/19	09:24	Santo Antão	287

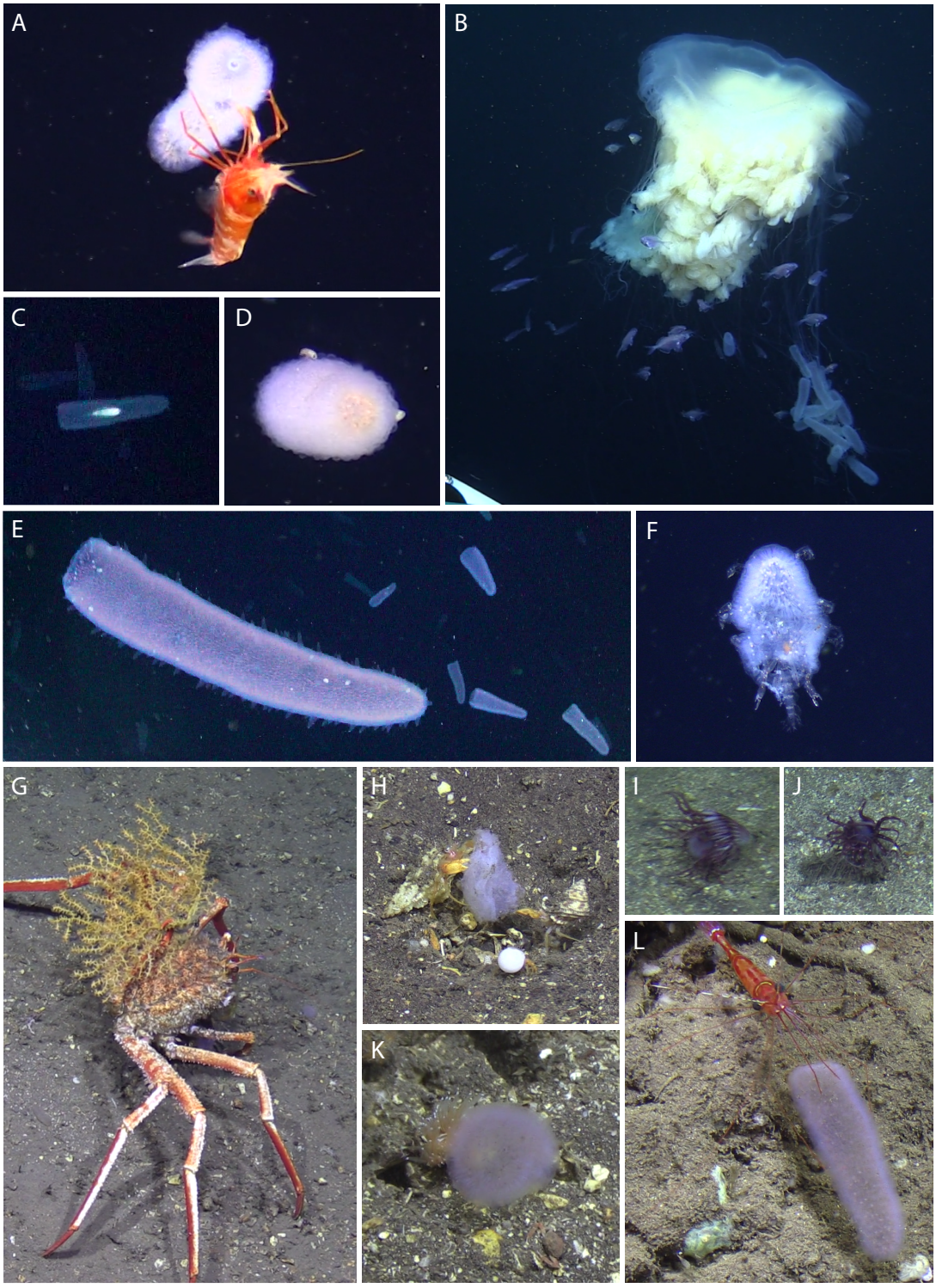

Figure 4| Pelagic (A–F) and benthic (G–L) animals associated with *P. atlanticum* in the eastern tropical North Atlantic, observed in February 2018 and 2019. (A) Unidentified Oplophoridae and (B) *Drymonema gorgo* feeding on pyrosomes. (C) An unidentified fish swimming inside pyrosome. (D,E) Unidentified hyperiid amphipods and (F) *Phronima* sp. using *P. atlanticum* as substrate, potentially feeding and reproducing on it. (G) An unidentified decorator crab, (H) three unidentified *Anomura*, (I,J) two unidentified *Actiniaria*, (K) *Dondice* sp. and (L) *Pandalopsis* sp. feeding on pyrosomes. All images were recorded with JAGO with exception of (C) and (E), which were recorded with PELAGIOS.

The detection of pyrosome eDNA down to 1000 m depth confirmed the downward transport of pyrosome material well below their normal depth distribution. At present, we cannot determine if this material originated from fecal material or sinking colonies, but it does show that large pyrosome blooms may affect water layers deeper than their relatively shallow migration range. Since the analysis of eDNA only provides qualitative results, comparison to the quantitative net and video observations is limited to the presence or absence of pyrosome material at certain depths. Future research could be focused on quantifying the amount of pyrosome eDNA through quantitative PCR in relation to bloom density to further substantiate the extent of the downward transport. Nevertheless, more specific primers are required, as the custom primers used for the *Phronima* barrels were not specific enough to amplify the pyrosome genetic material in the eDNA samples. In addition, the primers used for eDNA analysis only amplified a short region and were not as specific as desired. Despite Blastn matches to *P. spinosum*, *P. verticillata* and *P. godeauxi*, we assume that all eDNA detected in this study belonged to *P. atlanticum* because of its high abundance in the sampled area and since the former species have not been reported in the eastern tropical North Atlantic[9,13].

In addition to the organic carbon excreted by living *P. atlanticum*, colony mortality contributed actively to local bentho-pelagic coupling. The carbon deposited by these pyrosome-food falls (0.64 to 41.71 mg C m^{-2}) exceeded depositions of pyrosomes along the continental margin of the Mediterranean (0.3 to 1.4 mg C m^{-2})[16], but were substantially lower than those observed on the continental slope off the Ivory coast, which were reported to average > 5000 mg C m^{-2} (with maximum values reaching 22,000 mg C m^{-2})[15]. In the present and latter studies, pyrosome carcasses generally accumulated along the continental and islands slopes, especially in regions with increased geographic complexity, which acted as a 'trap' for sinking colonies[15,16]. Considering that most pyrosomes on the benthos appeared healthy in coloration, with many seen floating a few meters above the seabed, it is possible that colonies were not actually moribund, but were blocked by the island slope during their diel vertical migration. This obstruction of diel vertical migrators by bathymetric features is a well-known phenomenon of seamounts and has been termed "topographic blocking"[60,61].

Biological associations.

While pyrosomes are known to be prey for a range of oceanic predators, few studies have investigated the biological interactions between pyrosomes and other members of the pelagic food web via in situ observations[23]. Here, we estimated that *P. atlanticum* formed an abundant substrate in the water column (reaching up to 2820 cm^2 substrate area per m^2).

In particular, *Pyrosoma atlanticum* formed an important substrate for hyperiid amphipods, which are common associates of gelatinous zooplankton (e.g.,[62,63]). *Hyperia* amphipods are most often associated with various medusae and ctenophores[64], with this being the first report of an association with pyrosomes. The juvenile oxycephalids appeared to have a species-specific association with their pyrosome host as they all belonged to a single species and were encountered on pyrosomes from different locations. Moreover, the oxycephalids did not leave the host even with strong physical stimuli. Though most often associated with ctenophores[64,65], oxycephalids have been reported from a diversity of other gelatinous zooplankton including cnidarians[66], salps and heteropods[63], yet this is the first report of any oxycephalid associated with pyrosomes.

Phronima are typically considered parasites of pelagic tunicates and are well known for the "barrels" they create from the bodies of their hosts to house their developing young[67]. *Phronima* is primarily found on salps, with few reports of phronimids living or feeding on siphonophores, medusae, and ctenophores[66,68,69]. *Phronima* leave minimal clues to the origin of their barrels, as they remove zooids and smooth the surface. Most barrels in this study could only be identified with the help of DNA barcoding and were found to be constructed from pyrosome tissue.

The fishes we observed inside a pyrosome colony may belong to the genus *Tetragonurus* Risso, 1810 (squaretails), which are known to occur in the Atlantic and reported to occupy the body cavities of salps and pyrosomes[70]. In laboratory experiments, squaretails preferred pelagic tunicates over a variety of different gelatinous zooplankton, a result substantiated by field observations and stomach content analysis[70]. In the case of pyrosomes, Janssen and Harbison[70] observed squaretails to enter colonies through the shared aperture and to easily exit by swimming backward or forward. In addition to using the colony as shelter, squaretails micropredate on pyrosomes by biting off small pieces and also consume hyperiid amphipods. This would make the interaction partly mutualistic, as the fish can free the colony of its hyperiid parasites.

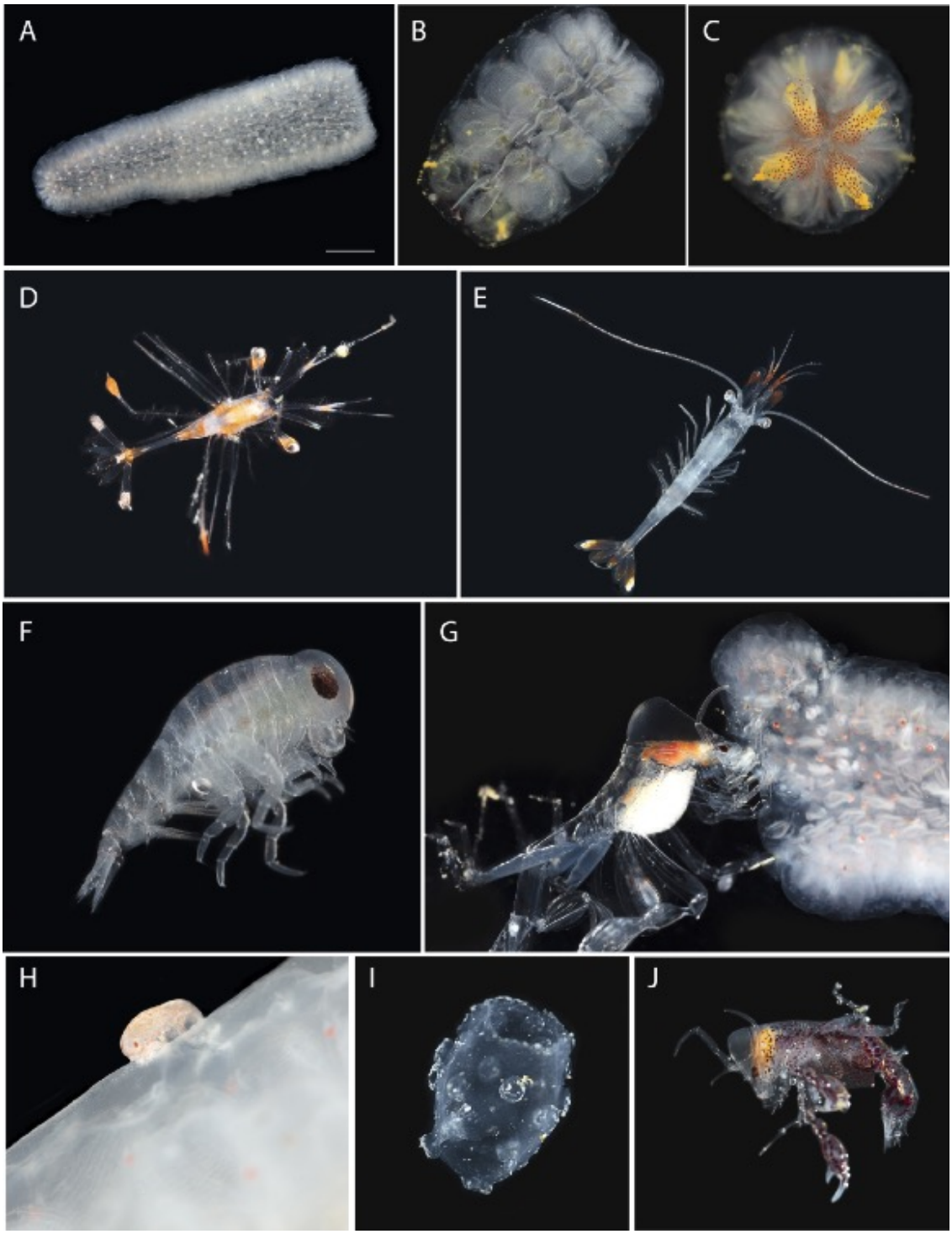

Figure 5| Macro photographs of live animals on board R/V Poseidon. (A–C) *P. atlanticum*, scale bar 1 cm. Individual zooids visible in B and C, colonies respectively ~ 1 cm in length and < 3 mm in diameter. (D,E) Early (< 1 cm) and late (> 2 cm) juvenile *Funchalia villosa* collected from *P. atlanticum*. (F) Unknown juvenile oxycephalid collected off *P. atlanticum*, body length < 2 mm. (G) *Phronima* specimen feeding on *P. atlanticum* colony overnight and undeterred by the camera strobes. (H) Unknown juvenile oxycephalid still attached to *P. atlanticum*, body length < 1 mm. (I–J) *Phronima*-modified barrel made from a *P. atlanticum* colony that was inhabited by the individual in J, barrel was just under 2 cm in length.

A medusafish, *Icichthys lockingtoni* Jordan and Gilbert, 1880, was also reported inside a *P. atlanticum* colony[21], although this species has not been reported in the North Atlantic. To our knowledge this is the first published report of the medusa *Drymonema gorgo* in the Cabo Verde region, and of this species or any *Drymonema* scyphozoan feeding on *P. atlanticum*. Medusozoa are known predators of pelagic tunicates but the available observations mostly involve predation on salps[18,71]. It is possible that *D. gorgo* passively caught the pyrosomes during their upward diel migration, which was also suggested as a hunting strategy for several siphonophores and ctenophores[72,73].

The benthic invertebrates feeding on *P. atlanticum* in this study were similar to the taxa observed by Lebrato et al.[15] off the Ivory Coast, with exception of the *Dondice* nudibranch that was moving across the colony. Even though we did not see these mollusks feeding on pyrosomes, other nudibranchs are commonly reported to prey on other species of gelatinous zooplankton[74] and a similar association may apply here.

Conclusion

The present results illustrate the important ecological role of *P. atlanticum* in the ocean surrounding the Cabo Verde archipelago, impacting both pelagic and benthic ecosystems. Local island-induced upwelling appeared to favor pyrosome proliferation, and it was estimated that a substantial amount of organic carbon was transported to depth via *P. atlanticum*'s fecal pellet production and carcass deposition. Moreover, pyrosomes are here shown to be important components of the Cabo Verde pelagic community during their bloom formation, functioning as a food source and biological substrate in the midwater column. Our study emphasizes the need to complement quantitative net catches with in situ observations to increase spatial resolution, and to allow biomass estimates with additional information on behavior and interactions.

Data availability

All data is available on the PANGAEA Data Publisher database https://doi.pangaea.de/10.1594/PANGAEA.918915.

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Author contributions

H.J.H. and H.H. conceived the project and carried out the fieldwork with K.J.O., B.H.R., V.M., S.S. and R.F. V.I.S. processed and analyzed all data, while P.N. helped with video annotations. Figures and tables were prepared by V.I.S. with K.J.O. providing Fig. 5. Molecular analyses were carried out by K.J.O., V.M., S.S., and V.I.S. The main manuscript text was written by V.I.S., H.J.H., H.H. and K.J.O., and was reviewed by all authors. Funding Open Access funding enabled and organized by Projekt DEAL.

Competing interests

The authors declare no competing interests.

Additional information

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5.5. References Chapter 5

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Synthesis and Perspective

Synthesis

The work in this thesis contributed to the development and optimization of an environmental DNA metabarcoding pipeline to monitor presence and diversity of deep-sea metazoans, in particular cephalopods. It used eDNA data to test hypotheses in ecology and biogeography in hotspots of diversity and climate change. The work involved the first efforts to specifically investigate cephalopods with eDNA metabarcoding, and therefore added a powerful and widely applicable tool with which to investigate marine diversity. Fish eDNA diversity assessment allowed the confirmation of a poleward range expansion. Additionally, this thesis contributed the first steps towards the qualification (presence/absence) and identification of nekton foodfall species, as derived from samples of deep-sea sediments, contributing to the discussion on the importance of medium-size foodfalls to local carbon budgets.

The first objective of this thesis was to establish an eDNA pipeline for the detection of deep-sea cephalopods, from primer design to species assignment, and to then apply this pipeline to water and sediment samples from the deep sea (Chapter 1-3). This objective was approached with the design of a universal cephalopod primer targeting the nuclear 18S rRNA gene (Chapter 4). *In silico* and empirical tests with the 18S rRNA primer estimated an amplification of 310-460 species of the 800 extant cephalopod species known today (44-66%). The 18S rRNA primer in combination with two previously published cephalopod primers (mitochondrial 16S rRNA gene), are suggested to amplify and identify ~89% of all cephalopod species. Applied alone, the 18S rRNA primer is estimated to amplify and identify 8-31% more cephalopod species than the 16S rRNA primer. In Chapter 1, the eDNA pipeline was further developed and optimized for the application of the novel 18S rRNA primer in combination with a mitochondrial 16S rRNA primer to target cephalopod eDNA in seawater samples collected off the Azores, from depths of between 50 and 1600 m. Environmental DNA metabarcoding resulted in the detection of 39 cephalopod taxa representing 17 families. Of the 39 taxa, 59% could be identified to species, 21% to genus and 20% to family level or lower. In Chapter 2, the eDNA pipeline was used on seawater samples collected from off Cabo Verde, from depths of

between 100 – 2500 m. Here, the 16S primer failed, but the 18S primer detected 32 taxa of which 38% were identified to species, 31% to genus and 22% to family level. For the same study, a species-specific primer targeting *Taningia danae* was designed that detected this elusive squid species recurrently from depths of between 100 and 2500 m. The application of the eDNA pipeline in Chapter 3 yielded in the detection of three cephalopod taxa in seawater samples from the Arctic. The cephalopod eDNA pipeline was also developed to test the hypothesis that traces of pelagic origin can be detected in deep-sea sediments. The analysis of Arctic sediments for cephalopod eDNA yielded no results. This stressed the patchy distribution and high turnover rates of cephalopod carcasses, but also the limitations of the method. The 18S primer is known to have difficulties to amplify octopus eDNA (Chapter 4) and the amount of sediment used for eDNA analysis was very low. Additional primers and larger amounts of sample need to be processed to glean positive results from further studies.

The second objective of this thesis was to establish biodiversity and distribution patterns of key organisms in the deep sea, and to relate these patterns to ecology and biogeography (Chapter 1, 2, 3). In Chapter 1, cephalopod diversity and distribution was assessed for two bathymetrically distinct environments off Terceira, Azores. These environments are used as foraging grounds for Cuvier's beaked whales (*Ziphius cavirostris*, CBW) and Risso's dolphins (*Grampus griseus*, RD). These cetacean top predators both feed predominantly on cephalopods, but hunt in different habitats. Biologging shows that CBW hunt further offshore and in deeper depths (900 – 1700 m) than RD, which hunt closer to shore and in shallower depths (surface to 600 m). By reconstructing the cephalopod prey spectra in the foraging habitats and zones of these two cetaceans, we aimed to test the hypothesis that niche segregation in these cetaceans is driven by cephalopod diversity. However, our eDNA results showed no significant differences in cephalopod species composition between CBW and RD foraging habitats and zones, but instead indicated a strong overlap in distribution of the species. These findings suggest that the observed niche segregation between the two cetacean species is not driven by distinct community compositions of their prey alone. This led to the alternative hypothesis that CBW hunt further offshore and in deeper depths for large, maturing cephalopods which migrate from shallow to deeper waters as a result of ontogenetic migration. The RD target the younger individuals of the same species, which occur at shallower depths and closer to shore. To test this hypothesis, net trawls and video surveys in addition to eDNA sampling needs to be conducted off the Azores, to correlate cephalopod size and maturity with depth and to investigate

species abundances. Within this study, 21 of the 83 cephalopod species that have been reported in waters around the Azores were detected with a sampling effort of only 3.5 days, with two new species for this region, *Chiroteuthis mega* and *Cycloteuthis sirventi* and the rarely encountered giant squid *Architeuthis* also identified. The congruent combination of eDNA, and biologging as pioneered in this study can be transferred to other predator-prey systems to predict "prey-scapes" and to unravel deep-sea food webs.

In Chapter 2, different censuses to document cephalopods off Cabo Verde were applied to establish total diversity and to test the hypothesis that Cabo Verde cephalopod fauna is distinct from other Macaronesian islands (Freitas et al., 2019). We combined submersible and towed camera observations with net and eDNA sampling. The results showed that the waters around the oceanic archipelago of Cabo Verde are a cephalopod diversity hotspot. In total, 63 confirmed species were detected, of which six species and three genera were documented for the first time in this area. One of these was the elusive bathypelagic squid *Magnapinna* sp. Each method contributed between 7 to 54% of taxa to the total diversity that was not detected by any of the other methods. This highlights the need for integrating differing methodologies to achieve the most accurate biodiversity assessments. Cabo Verdes' overall cephalopod community composition was similar to communities at the Canary Islands and Azores, but differed in benthic octopus diversity. These results support the hypothesis that Cabo Verdes' benthic and coastal diversity is biogeographically separated from other islands included in Macaronesia, due to physical barriers formed by strong currents and upwelling. However, it appears mobile, muscular squid are able to migrate between the archipelagos.

Chapter 3 aimed to establish a fish and cephalopod diversity baseline for the Fram Strait in the Arctic Ocean to detect range expansions and shifts in community composition. The Fram Strait is a gateway to the Arctic and hence, changes in Atlantic currents increasingly transporting warm waters into the Arctic resulting in "Atlantification" and "Borealization" (Fossheim et al., 2015; Polyakov et al., 2020) will be first observed there. Nekton such as cephalopods and fish, as mobile fauna, have been hypothesized to show range expansions moving into the Arctic. Contrary to the high diversity in cephalopod eDNA which was detected in seawater off the Azores (21 species, Chapter 1) and Cabo Verde (32 species, Chapter 2), only six cephalopod taxa were detected in seawater of the Fram Strait. Of those six taxa, three detections were likely due to lab contamination. However, the endemic cephalopod *Gonatus* was the taxon with the highest frequency of reads in our Fram Strait eDNA data, which is in line with the fact that this cephalopod is the most abundant squid species within the Arctic (Xavier et al., 2018). The presence of eDNA of the additional three taxa in the Arctic is doubtful, as

they are not known to occur in the northern North Atlantic. Challenges with cephalopod eDNA detections in Arctic seawaters are the generally low cephalopod diversity (32 species) and biomasses (Xavier et al., 2018) reported for the area, potentially resulting in low DNA concentrations and the limited specificity of the 18S rRNA primer (Chapter 4). This primer is not specific enough to amplify octopus eDNA reliably, which is the main cephalopod group inhabiting the Arctic (Xavier et al., 2018). In seawater and sediment, 31 fish taxa were detected, of which 12 were identified to family, nine to genus and ten to species level. A rare species detected with eDNA from water depths of 2420 m was *Somniosus microcephalus*, the Greenland shark. Environmental DNA of the widely distributed fish species capelin (*Mallotus villosus)* was most frequently detected followed by Zoarcidae, Liparidae, *Sebastes* sp., and Atlantic herring (*Clupea harengus*). This study was able to support the range expansion of the sub-arctic capelin to as far north as 79.7°N, which indicates that eDNA analysis can be used to investigate range expansions in Arctic and deep-sea ecosystems. No significant differences were found in community composition of fishes or cephalopods between the different stations or depths analyzed. This was expected as all analyzed stations were mainly located within the warm Atlantic water current. Future studies should ideally also sample the colder Arctic currents to investigate differences in species composition associated with differing current systems and physical conditions. Additionally, to identify range expansions, a time-series is needed, and additional primers targeting different genes applied to increase the species resolution.

The aim of the third objective was to identify taxa that potentially contribute to the vertical transport of carbon. In Chapter 1, nine of the cephalopod families detected with eDNA off the Azores are known to perform ontogenetic migration. These include important prey species for CBW, therefore transporting biomass and carbon into deeper waters. Coupled with carcass deposition after terminal reproduction for the species (Hoving et al., 2017), this indicates substantial transport of nutrients and carbon to deeper layers (Arkhipkin, 2013).

In Chapter 2, *Taningia danae* and *Sthenoteuthis pteropus* were suggested as foodfall species off Cabo Verde. *Taningia danae* is a very abundant squid that can reach mantle lengths of up to 1.70 m and is associated with the benthopelagic environment when spawning (Nesis, 1987). In the Atlantic, *T. danae* has only been observed a few times *in-situ,* including our observations with the towed camera system PELAGIOS (Gomes-Pereira and Tojeira, 2014; Hoving et al., 2019; Kubodera et al., 2007), although it is an important prey species for whales as shown by stomach content analysis (Clarke, 1967; Okutani and Satake, 1978). With the design of a species-specific *T. danae* primer in this thesis,

this squid has been detected recurrently between 100 and 2500 m water depth and may contribute significantly to the local carbon cycle following death and settling to the seafloor. The same is suggested for the orangeback flying squid *S. pteropus*. This species is also abundant, with a broad distribution from Madeira in the North to South Africa in the South (Jereb and Roper, 2010; Zuyev et al., 2002). It is not neutrally buoyant and sinks to the seafloor on death (Zuyev et al., 2002). Its high protein concentration of 17% renders this squid species a potentially valuable food source for benthic organisms (Zuyev et al., 2002). To validate the role of these two species and other squids in the transport of carbon into deeper water layers, not only pelagic samples need to be analyzed, but also sediment samples. A comparison of eDNA in pelagic and sediment samples allow prediction as to which species may contribute to the deposition of pelagic carcasses on the seafloor.

Chapter 3 successfully traced pelagic metazoan eDNA in sediments. The comparison of pelagic fish eDNA in sediments and seawater revealed the abundant capelin (*Mallotus villosus*) and barracudina (*Arctozenus risso*) to be potential foodfall species in the Arctic Ocean. Both species are pelagic and their eDNA has been detected in sediments collected from different stations at bottom depths deeper than 2000 m, with a high number of sequencing reads. The high number of sequencing reads across stations contradicts the hypothesis that feces being the source of capelin eDNA detections, and strengthens support to the counter hypothesis that the reads are actual eDNA originating from foodfalls following individual mortality. An unexpected detection in sediment of the Arctic was dolphin eDNA that was also detected within the water column. The dolphin eDNA in the sediment potentially stemmed from a dolphin carcass, but could also originate from dolphin feces sinking to the seafloor. Dolphin eDNA has only been detected at two stations and with low sequencing reads. The fact that cephalopod eDNA was not detected in sediments of the Arctic depicts the patchy distribution and quick turnover rate of cephalopod carcasses in the deep sea. Yet, we would have expected to detect *Gonatus* eDNA in sediment as this species is reported as the most abundant cephalopod species together with *Rossia palpebrosa* in the Arctic (Golikov et al., 2017) and known to form foodfalls following postbrooding gonatid female mortality in the Gulf of California (Hoving et al., 2017). However, *Gonatus fabricii* is suggested to only reproduce in a few geographically restricted areas (Arkhipkin and Bjørke, 1999; Bjørke, 2001; Bjørke and Gjøsæter, 1998) and the stations sampled for sediment here were not located in those known breeding areas. Additional steps to validate the potential of eDNA to detect foodfall species, especially for *Gonatus*, need to expand sampling efforts into known breeding areas.

Chapter 5 focused on the distribution of the pyrosome *Pyrosoma atlanticum* in waters of Cabo Verde, their role in the local carbon cycle and ecological interactions. Pelagic *in situ* observations revealed them as abundant substrates in the water column for hyperiid amphipods, fishes and medusae using them as settlement, shelter and/or food source. On the seafloor, pyrosome carcasses were scavenged upon by decapods, gastropods and sea anemone. Living pyrosomes were observed to perform diel vertical migration and thereby covered distances of 313 m on average. The here designed and applied species-specific primer allowed the detection beyond the reach of observational and net surveys. Environmental DNA of *Pyrosoma* was detected as deep as 1000 m, which is beyond their known migration range as observed with a towed video system. These detections confirmed the vertical transport of pyrosome material into deeper layers well below their normal depth range, potentially stemming from fecal pellets or dead sinking pyrosome colonies, showing that large pyrosome blooms may affect water layers deeper than their migration range.

This thesis highlights the potential of eDNA metabarcoding for biodiversity monitoring of pelagic metazoans. However, we also faced challenges and limitations. Challenges in the field of eDNA analysis have been repeatedly reviewed in recent years (Barnes and Turner, 2016; Beng and Corlett, 2020; Deiner et al., 2017). A major disadvantage of eDNA analysis is that due to it being a relatively recently developed technique, there are no standardized protocols as yet. Despite this, the number of published eDNA studies has increased enormously over the last years (Beng and Corlett, 2020). Information on the abundance or biomass of a species cannot reliably be retrieved from eDNA metabarcoding data and information on a species life-history, sex ratio or breeding status is completely lacking. Yet, on a regular basis new studies appear that focus on the question on how to further develop and improve this methodology so that it can be used for abundance and biomass estimates (Beng and Corlett, 2020). Abundance data provides more information on the status of an ecosystem and potential differences between ecosystems than only species presence/absence data. Large scale abundance estimates are still difficult to obtain due to different shedding rates of organisms and the lack of knowledge on how eDNA behaves in seawater, in particular within the deep sea. Shedding rates of fish eDNA are positively correlated with temperature and the relationship between eDNA concentration and fish abundance were less pronounced in cold water than in warm water (Lacoursière-Roussel et al., 2016).

The occurrence of false positives and false negatives in eDNA studies also has to be investigated in more detail (Ficetola et al., 2016, 2015; Guillera-Arroita et al., 2017; Lahoz-Monfort et al., 2016;

Stoeckle et al., 2016). Although increased PCR replicates can minimize the probability for false positives and false negatives (Piggott, 2016), costs for eDNA studies rapidly increase simultaneously and errors can still occur. In Chapter 1-3 false positives were detected in some eDNA samples. That contamination mostly stemmed from PCR positive controls or animal species that were kept near the lab. Although the sequencing read abundance of those false positives not detected in negative controls was mostly low in our studies, false positives do occur and have to be taken into account when analyzing eDNA data sets. We also detected false negatives in our datasets. For example, *Taningia danae* was observed during video transects in Cabo Verdean waters, but we were not able to detect it with the universal cephalopod primer that we applied. We therefore designed a species-specific primer which enabled the detection of *T. danae* recurrently. This highlights the fact that taxonomic knowledge of target groups or species is needed to reliably interpret eDNA data and that primers need to be tested thoroughly before being applied. When we designed the universal 18S cephalopod primer, we tested it *in-silico* and *in-situ* on various cephalopod tissue samples from voucher specimens. Due to this rigorous testing, we were aware that our universal cephalopod primer had difficulties with amplification of Octopoteuthidae, the familie to which *T. danae* belongs. We could, therefore, correct for that issue and specifically design a primer targeting our species of interest.

Perspective

Sampling eDNA

The amount of seawater available for eDNA analysis is often limited when sampling from the deep sea. Several research groups commonly participate in a cruise and need seawater for different analyses, so that the available seawater collected by CTD rosettes needs to be distributed among them. Therefore, it is often not possible to filter unlimited amounts of seawater for eDNA analysis. However, the more water that is filtered, the higher the eDNA yield and the more primer can be applied. To circumvent this problem, *in-situ* filtration systems such as the Environmental Sample Processor (ESP) have been developed (Yamahara et al., 2019). This device can either be attached to AUVs or operate as a stand-alone system to repeatedly sample water at a specific station without the need to first collect the water. Simultaneously, the use of an ESP reduces contamination, because the filtration takes place in the investigated environment and not in a lab. So far, ESPs are limited in the number of samples they can collect. In the future, *in-situ* filtration systems should be developed or

optimized to collect large numbers of samples and should be fitted with chemical and physical sensors to observe complex biological processes and interactions. They should also be easy to attach to different kinds of underwater vehicles such as AUVs or ROVs. Also, *in-situ* processing of the filtered samples would be beneficial for year-around monitoring surveys without the need of human presence (Hansen et al., 2020).

In the laboratory

It is worth investing efforts in optimizing newly developed amplification methods such as digital droplet PCR (ddPCR) (Everts et al., 2021), loop-mediated isothermal amplification (LAMP) (Williams et al., 2017) and capture hybridization (Wilcox et al., 2018) for eDNA. For species-specific approaches, ddPCR has proven to be more effective and sensitive than quantitative PCR (Doi et al., 2015a). As no reference curves need to be measured in ddPCR, this method is less error prone. This method has been used successfully to estimate abundances of cetaceans (Baker et al., 2018; Hunter et al., 2018), invertebrates (Everts et al., 2021) and fish (Capo et al., 2020; Doi et al., 2015b).

Future efforts in the lab should be directed towards circumventing amplification of the eDNA molecules via PCR, as PCRs can introduce bias (Elbrecht and Leese, 2015; Taberlet et al., 2012). Loop-mediated isothermal amplification allows direct species-specific detections from eDNA samples without the need for DNA extractions or PCRs. Although DNA extraction-based methods and PCR are still more sensitive, LAMP can be used efficiently for rapid screening of an environment for a specific species and can be used in the field (Williams et al., 2017). For instance, an environment could be screened for a specific species of interest by using eDNA-LAMP analysis, and in case of positive detections, additional methods such as video systems could be deployed to investigate the species or species associated with it *in situ*. Another promising new method for eDNA detections circumventing primer bias is “capture by hybridization” (Wilcox et al., 2018). With this method, the target DNA is enriched with probes. Probes are single-stranded sequences of DNA that attach in different locations to the target DNA. They therefore allow recovery of longer amplicon fragments than via eDNA metabarcoding with primers and by applying several probes, they can also cover a much wider diversity, improving taxonomic assignment (Günther et al., 2021). Longer amplicon fragments are especially beneficial when reference databases contain large gaps, and they additionally also render phylogenetic reconstructions possible (Denonfoux et al., 2013; Gasc and Peyret, 2017). Capture enrichment has been suggested to outperform conventional PCR in detecting

more mammal species and retracting a maximum mitogenome coverage of 99.8% in water samples of a shared waterhole (Seeber et al., 2019). However, other studies estimate hybridization capture to be comparable, but less sensitive, than PCR-based enrichment methods, especially with low DNA concentrations (<0.1 ng of total genomic DNA) (Wilcox et al., 2018). Another limitation is the amount of DNA extract that needs to be used. The PCR-based approach in our study used 5 µl of DNA extract (between 2 – 20 ng of library), however, for capture hybridization, > 50 µl of template DNA (Wilcox et al., 2018) or ~500 ng of library (Günther et al., 2021) are needed. This exceeds by far the DNA concentrations available from deep-sea cephalopod eDNA. Albeit with those limitations, capture based hybridization is a promising tool that needs to be optimized for samples with low DNA concentrations.

Future efforts should focus on the design and optimization of additional primer pairs for cephalopods and gelatinous zooplankton. Primers vary in their specificity, sensitivity and efficiency and it is impossible to design an unbiased primer that is able to amplify all taxa in a specific group equally well (Deagle et al., 2014), raising the need for the application of multiple primers in eDNA studies. Lastly, the eDNA community needs to work towards standardized lab protocols to be able to compare different studies and to increase the robustness of the method.

Bioinformatics

Currently, approximately 761 cephalopod species are known and described (Appeltans et al., 2012). A literature review on how many more species are likely to be discovered in the future resulted in 500 additional species for cephalopods (Appeltans et al., 2012). For fish, this estimate was even higher and ranged between 4200 and 4300 species remaining at present undiscovered, with 16,733 species currently described. A major component in biodiversity assessments aside from the identification of known species, is the detection of new, unknown species. So far, eDNA analysis is unable to detect new species and heavily relies on comprehensive reference databases of voucher specimens. However, many deep-sea species have been documented on video footage, but specimens captured in nets are rare or absent, so that no DNA samples are available for eDNA reference databases to compare eDNA sequences with. A solution could be the deployment of specialized lures to attract deep-sea species in combination with tools to take tissue samples from the attracted animal and video footage for its identification.

In the scope of this thesis, we were able to extend public databases by contributing 29 newly sequenced cephalopod taxa for the 18S rRNA gene. We are continuously working and collaborating

on collecting new voucher specimens, their morphological identification also in collaboration with taxonomists and barcoding for future eDNA studies.

In the future, bioinformatic pipelines need to be standardized to be able to compare different studies targeting different ecosystems and organisms and to ensure standardized qualities in eDNA data. The handling of contamination in eDNA data and the cleaning thereof differs widely between studies. For example, some studies use relative thresholds to clean for contamination while others use absolute copy number thresholds or exclude entire taxa from sample batches (Beng and Corlett, 2020). Many studies still use molecular operational taxonomic units (MOTUs) which were initially developed for bacterial sequences and clusters sequences that differ by less than a fixed dissimilarity threshold (Beng and Corlett, 2020, Taberlet et al., 2018). However, the use of amplicon sequence variants (ASV) has been shown to be advantageous over MOTUs, as fine-scale variations can be resolved more accurately and fewer incorrect sequences are kept (Callahan et al., 2016). The next step in the bioinformatic pipeline is the species assignment. For that, different algorithms can be used to assign species to query sequences. Some popular classifiers are BLAST, MAPSeq, QIIME, SINTAX, SPINGO and the RDP Classifier, but IDTAXA has been demonstrated to have higher accuracies than the before mentioned (Murali et al., 2018). This variety of methods resulting in differing qualities that can be applied depict the inconsistencies in eDNA studies that are currently prevailing. Environmental DNA consortia need to work towards the development of standardized protocols and guidelines to ensure comparable and high-quality analysis and publications.

Other applications

Environmental DNA analysis can not only be used to elucidate biodiversity, it can also evaluate intraspecific genetic diversity, as the eDNA of multiple individuals may coexist in a sample. For example, Uchii et al. quantified the relative proportion of two different genotypes of common carp (*Cyprinus carpio*) based on a single-nucleotide polymorphism (SNP) (Uchii et al., 2017). Two other studies were able to identify intraspecific genetic diversity in whale shark (*Rhincodon typus*) (Sigsgaard et al., 2017) and harbor porpoise (*Phocoena phocoena*) populations (Parsons et al., 2018) with eDNA, that have been identified previously from tissue-derived DNA by Sanger sequencing. A recent study developed a novel analytical method called HaCeD-Seq (Haplotype Count from eDNA) which allowed the detection of 94% of existing haplotypes of tuna in a tank. The advantage of the HaCeD-Seq method is the reduction of PCR- and sequencing-errors, that could lead to false haplotypes by adding unique molecular identifiers (UMI) to every DNA molecule (Yoshitake et al.,

2021). This method allows the computation of abundance and haplotype diversity of specific species. However, this method also needs sufficient DNA concentrations that are often not met within deep-sea samples. Nevertheless, the sequencing data obtained during this thesis could potentially be used for the construction of haplotype diversity networks. The data is particularly useful as it covers a wide geographical range from the Azores to Cabo Verde, which shares the same abundant cephalopod species. Future efforts should be made to investigate if the here collected data is sufficient for the future analysis of genetic connectivity between cephalopod species between both archipelagos.

Experimental studies

Experimental studies on species shedding rates and degradation of eDNA in deep-sea ecosystems are needed to further understand the ecology of eDNA. Additionally, the dispersal of eDNA is basically unknown. A study modelling eDNA dispersal suggested eDNA tends to stay within tens of meters of the depth at which the eDNA was originally shed. However, the experimental data that model relies on is limited (Allan et al., 2021). For instance, eDNA shedding rates depend on various abiotic and biotic factors (Lacoursière-Roussel et al., 2016) and differ depending on the species. Settling rates of eDNA are unknown and the model used values based on marine snow. Also, the decay rate of eDNA is not a fixed parameter, but depends on e.g., temperature and bacterial community and the strength of currents, which differ, temporally, by area and by depths investigated (Allan et al., 2021; Taberlet et al., 2012). To answer some of these questions, artificial DNA could be released in a specific oceanic habitat (specific location and depth). By sampling seawater for this artificial DNA in different temporal and spatial scales, actual data could be obtained for the modelling of eDNA dispersal in a specific area and depths.

Conclusion

Traditional methods as well as eDNA analysis have all their own limitations and strengths and provide different information. The application of environmental DNA is constantly optimized and we continuously learn more about this genetic technique. The goal of eDNA studies should not be to outcompete other methods, but to be used as complement to fill limitation gaps and to add additional strength. It can also be used as stand-alone method, when it has been chosen as the most appropriate approach to answer a well-defined question with keeping its limitations in mind.

Synthesis and Perspective

In this thesis, eDNA metabarcoding was applied alone and in complementation with other methods and proved to be a valuable tool to investigate diversity, distribution and ecological dynamics of pelagic metazoans. This thesis contributed to the advancement of knowledge in deep-sea cephalopod diversity, distribution and ecology, showing that eDNA is a powerful methodology to investigate these notoriously difficult to sample organisms.

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Supplementary Material Chapter 3

Table 1| Primer sequences used for cephalopods (18S) and fish (Teleo) with the Illumina linker, Illumina index, Illumina adapter and a spacer.

Target Gene	Primer Name	Sequence
Ceph_18S	18S_F1	AATGATACGGCGACCACCGAGATCTACACTATAGCCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTACGCGGCGCTACATATTAGAC
Ceph_18S	18S_F2	AATGATACGGCGACCACCGAGATCTACACATAGAGGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGACGCGGCGCTACATATTAGAC
Ceph_18S	18S_F3	AATGATACGGCGACCACCGAGATCTACACCCTATCCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCACGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F4	AATGATACGGCGACCACCGAGATCTACACGGCTCTGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F5	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTGGACGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F6	AATGATACGGCGACCACCGAGATCTACACTAATCTTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTGGCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F7	AATGATACGGCGACCACCGAGATCTACACCAGGACGTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGTTATCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F8	AATGATACGGCGACCACCGAGATCTACACGTACTGACTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F9	AATGATACGGCGACCACCGAGATCTACACTGAACCTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F10	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTATCTACGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F11	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGAACGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F12	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F13	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCACGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F14	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATAGCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F15	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCTCGCGGCGCTACATATTAGAC
Ceph_18S	F18S_F16	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTACGCGGCGCTACATATTAGAC
Ceph_18S	18S_R1	CAAGCAGAAGACGGCATACGAGATTAAGGCGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATTGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R2	CAAGCAGAAGACGGCATACGAGATCGTACTAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R3	CAAGCAGAAGACGGCATACGAGATAGGCAGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R4	CAAGCAGAAGACGGCATACGAGATTCCTGAGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R5	CAAGCAGAAGACGGCATACGAGATGGACTCCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R6	CAAGCAGAAGACGGCATACGAGATTAGGCATGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGCACTTAACCGACCGTCGAC

Ceph_18S	18S_R7	CAAGCAGAAGACGGCATACGAGATCTCTCTACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGAAGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R8	CAAGCAGAAGACGGCATACGAGATCAGAGAGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R9	CAAGCAGAAGACGGCATACGAGATAGCGATAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGTGGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R10	CAAGCAGAAGACGGCATACGAGATCGAGGCTGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGTATGGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R11	CAAGCAGAAGACGGCATACGAGATAAGAGGCAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R12	CAAGCAGAAGACGGCATACGAGATGTAGAGGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGTAGAGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R13	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R14	CAAGCAGAAGACGGCATACGAGATATTACTCGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGAGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R15	CAAGCAGAAGACGGCATACGAGATTCCGGAGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTGAGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R16	CAAGCAGAAGACGGCATACGAGATCGCTCATTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGAGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R17	CAAGCAGAAGACGGCATACGAGATGAGATTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAGTGGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R18	CAAGCAGAAGACGGCATACGAGATATTCAGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTATGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R19	CAAGCAGAAGACGGCATACGAGATGAATTCGTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCACTTCTGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R20	CAAGCAGAAGACGGCATACGAGATCTGAAGCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTACTCTGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R21	CAAGCAGAAGACGGCATACGAGATTAATGCGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGTGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R22	CAAGCAGAAGACGGCATACGAGATCGGCTATGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R23	CAAGCAGAAGACGGCATACGAGATTCCGCGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCACTTAACCGACCGTCGAC
Ceph_18S	18S_R24	CAAGCAGAAGACGGCATACGAGATTCTCGCGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCACTTAACCGACCGTCGAC
Fish_12S	teleo_F_1	AATGATACGGCGACCACCGAGATCTACACTATAGCCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTGACACCGCCCGTCACTCT
Fish_12S	teleo_F_2	AATGATACGGCGACCACCGAGATCTACACATAGAGGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGAACACCGCCCGTCACTCT
Fish_12S	teleo_F_3	AATGATACGGCGACCACCGAGATCTACACCCTATCCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAACACCGCCCGTCACTCT
Fish_12S	teleo_F_4	AATGATACGGCGACCACCGAGATCTACACGGCTCTGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTAACACCGCCCGTCACTCT
Fish_12S	teleo_F_5	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACACCGCCCGTCACTCT
Fish_12S	teleo_F_6	AATGATACGGCGACCACCGAGATCTACACTAATCTTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTATACACCGCCCGTCACTCT
Fish_12S	teleo_F_7	AATGATACGGCGACCACCGAGATCTACACCAGGACGTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACCGCCCGTCACTCT
Fish_12S	teleo_F_8	AATGATACGGCGACCACCGAGATCTACACGTACTGACTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACACACCGCCCGTCACTCT
Fish_12S	teleo_F_9	AATGATACGGCGACCACCGAGATCTACACTGAACCTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACACCGCCCGTCACTCT
Fish_12S	teleo_F_10	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCACACCGCCCGTCACTCT
Fish_12S	teleo_F_11	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTACACCGCCCGTCACTCT

Fish_12S	teleo_F_12	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGCAACACCGCCCGTCACTCT
Fish_12S	teleo_F_13	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACCGCCCGTCACTCT
Fish_12S	teleo_F_14	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTCACACCGCCCGTCACTCT
Fish_12S	teleo_F_15	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAATAGACACCGCCCGTCACTCT
Fish_12S	teleo_F_16	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGTACACCGCCCGTCACTCT
Fish_12S	teleo_R_1	CAAGCAGAAGACGGCATACGAGATTAAGGCGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_2	CAAGCAGAAGACGGCATACGAGATCGTACTAGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTACTTCCGGTACACTTACCATG
Fish_12S	teleo_R_3	CAAGCAGAAGACGGCATACGAGATAGGCAGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_4	CAAGCAGAAGACGGCATACGAGATTCCTGAGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTTCCGGTACACTTACCATG
Fish_12S	teleo_R_5	CAAGCAGAAGACGGCATACGAGATGGACTCCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATAGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_6	CAAGCAGAAGACGGCATACGAGATTAGGCATGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAGACTTCCGGTACACTTACCATG
Fish_12S	teleo_R_7	CAAGCAGAAGACGGCATACGAGATCTCTCTACGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_8	CAAGCAGAAGACGGCATACGAGATCAGAGAGGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_10	CAAGCAGAAGACGGCATACGAGATCGAGGCTGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_11	CAAGCAGAAGACGGCATACGAGATAAGAGGCAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTTCCGGTACACTTACCATG
Fish_12S	teleo_R_12	CAAGCAGAAGACGGCATACGAGATGTAGAGGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_14	CAAGCAGAAGACGGCATACGAGATATTACTCGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_15	CAAGCAGAAGACGGCATACGAGATTCCGGAGAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAACTTCCGGTACACTTACCATG
Fish_12S	teleo_R_16	CAAGCAGAAGACGGCATACGAGATCGCTCATTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATACTTCCGGTACACTTACCATG
Fish_12S	teleo_R_17	CAAGCAGAAGACGGCATACGAGATGAGATTCCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAATGCCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_18	CAAGCAGAAGACGGCATACGAGATATTCAGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_19	CAAGCAGAAGACGGCATACGAGATGAATTCGTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_20	CAAGCAGAAGACGGCATACGAGATCTGAAGCTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_21	CAAGCAGAAGACGGCATACGAGATTAATGCGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATCCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_22	CAAGCAGAAGACGGCATACGAGATCGGCTATGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_23	CAAGCAGAAGACGGCATACGAGATTCCGCGAAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTATCTTCCGGTACACTTACCATG
Fish_12S	teleo_R_24	CAAGCAGAAGACGGCATACGAGATTCTCGCGCGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTTCCGGTACACTTACCATG

Table 2| Species assignments for eDNA of cephalopods detected in seawater in the Fram Strait of the Arctic Ocean. Species were assigned according to the IDTAXA algorithm. *Sum_reads* is the sum of all reads in one distinct sample. *N_reads* is the number of reads of one specific ASV in one sample.

Query	Taxon	identified_to	Station	Station	Depth	sum_reads	n_reads	Cruise
5	Gonatidae	Family	MSM95/26-1	S3	2283	11079	6716	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	1600	48	19	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	1000	2558	2541	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	1000	24	11	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	400	810	794	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	2283	8695	8621	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	200	8799	8687	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	50	2090	884	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2420	408	362	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2420	294	292	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2420	2272	1612	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2250	6154	5798	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2250	10397	4937	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	2283	8070	3989	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2250	830	790	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	2000	9206	8660	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	1600	2143	1274	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	400	4288	1166	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	400	5724	5642	MSM95
5	Gonatidae	Family	MSM95/36-1	HG4	200	302	245	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	2250	1410	1394	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2705	666	664	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2705	1683	1541	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2705	1924	1897	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2500	5499	1604	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2500	3885	3573	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2250	8618	8549	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2250	3149	3122	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	2250	2349	2336	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2250	6832	1143	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	2000	16555	16456	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	1600	5442	5369	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	1300	7248	6521	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	2000	10762	17	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	400	1124	1113	MSM95
5	Gonatidae	Family	MSM95/41-1	N4	50	411	406	MSM95
5	Gonatidae	Family	MSM95/26-1	S3	2000	21411	10501	MSM95
8	Gonatidae	Family	MSM95/26-1	S3	2283	11079	2641	MSM95

8	Gonatidae	Family	MSM95/26-1	S3	1600	931	917	MSM95
8	Gonatidae	Family	MSM95/26-1	S3	1600	48	27	MSM95
8	Gonatidae	Family	MSM95/26-1	S3	50	2090	1161	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	2420	2272	642	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	2250	6154	320	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	2250	10397	3375	MSM95
8	Gonatidae	Family	MSM95/26-1	S3	2283	8070	4000	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	1600	1942	1925	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	1600	2143	852	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	1000	1316	1290	MSM95
8	Gonatidae	Family	MSM95/36-1	HG4	400	4288	3099	MSM95
8	Gonatidae	Family	MSM95/41-1	N4	2705	1683	120	MSM95
8	Gonatidae	Family	MSM95/41-1	N4	2500	3885	262	MSM95
8	Gonatidae	Family	MSM95/41-1	N4	2250	6832	5640	MSM95
8	Gonatidae	Family	MSM95/26-1	S3	2000	10762	10510	MSM95
8	Gonatidae	Family	MSM95/26-1	S3	2000	21411	10714	MSM95
17	Vampyroteuthis infernalis	Species	MSM95/41-1	N4	1600	6796	3505	MSM95
20	Gonatus sp.	Genus	MSM95/26-1	S3	2283	11079	14	MSM95
20	Gonatus sp.	Genus	MSM95/26-1	S3	200	8799	13	MSM95
20	Gonatus sp.	Genus	MSM95/41-1	N4	2500	5499	2884	MSM95
20	Gonatus sp.	Genus	MSM95/41-1	N4	2500	3885	13	MSM95
20	Gonatus sp.	Genus	MSM95/41-1	N4	2000	16555	15	MSM95
23	Teuthida	Order	MSM95/41-1	N4	400	2960	2957	MSM95
38	Teuthowenia maculata	Species	MSM95/41-1	N4	1600	6796	1756	MSM95
40	Filippovia knipovitchi	Species	MSM95/36-1	HG4	2000	2920	1718	MSM95
41	Gonatidae	Family	MSM95/36-1	HG4	2250	10397	1582	MSM95
41	Gonatidae	Family	MSM95/26-1	S3	2000	10762	14	MSM95
41	Gonatidae	Family	MSM95/26-1	S3	2000	21411	43	MSM95
43	Gonatidae	Family	MSM95/26-1	S3	2283	11079	829	MSM95
43	Gonatidae	Family	MSM95/26-1	S3	2283	8695	14	MSM95
43	Gonatidae	Family	MSM95/36-1	HG4	2000	9206	409	MSM95
43	Gonatidae	Family	MSM95/41-1	N4	2000	16555	16	MSM95
43	Gonatidae	Family	MSM95/41-1	N4	1600	5442	13	MSM95
43	Gonatidae	Family	MSM95/26-1	S3	2000	21411	19	MSM95
45	Teuthida	Order	MSM95/41-1	N4	1600	6796	1211	MSM95
46	Teuthida	Order	MSM95/36-1	HG4	2000	2920	1188	MSM95
49	Gonatidae	Family	MSM95/26-1	S3	2283	11079	809	MSM95
49	Gonatidae	Family	MSM95/26-1	S3	2283	8070	14	MSM95
49	Gonatidae	Family	MSM95/26-1	S3	2000	10762	14	MSM95
49	Gonatidae	Family	MSM95/26-1	S3	2000	21411	22	MSM95
50	Gonatidae	Family	MSM95/26-1	S3	2283	11079	18	MSM95
50	Gonatidae	Family	MSM95/26-1	S3	2283	8695	14	MSM95
50	Gonatidae	Family	MSM95/36-1	HG4	400	5724	12	MSM95

50	Gonatidae	Family	MSM95/41-1	N4	1600	5442	14	MSM95
50	Gonatidae	Family	MSM95/41-1	N4	1300	7248	674	MSM95
50	Gonatidae	Family	MSM95/26-1	S3	2000	21411	34	MSM95
51	Gonatidae	Family	MSM95/26-1	S3	2283	8695	12	MSM95
51	Gonatidae	Family	MSM95/36-1	HG4	2000	9206	18	MSM95
51	Gonatidae	Family	MSM95/41-1	N4	2500	5499	640	MSM95
51	Gonatidae	Family	MSM95/41-1	N4	2500	3885	12	MSM95
51	Gonatidae	Family	MSM95/41-1	N4	2250	8618	13	MSM95
51	Gonatidae	Family	MSM95/41-1	N4	2000	16555	13	MSM95
51	Gonatidae	Family	MSM95/26-1	S3	2000	21411	12	MSM95
56	Gonatidae	Family	MSM95/26-1	S3	200	8799	33	MSM95
56	Gonatidae	Family	MSM95/36-1	HG4	2250	6154	11	MSM95
56	Gonatidae	Family	MSM95/36-1	HG4	2250	10397	367	MSM95
56	Gonatidae	Family	MSM95/26-1	S3	2283	8070	13	MSM95
56	Gonatidae	Family	MSM95/36-1	HG4	2000	9206	12	MSM95
56	Gonatidae	Family	MSM95/36-1	HG4	400	5724	19	MSM95
56	Gonatidae	Family	MSM95/41-1	N4	2000	16555	19	MSM95
63	Gonatidae	Family	MSM95/41-1	N4	2500	5499	360	MSM95
65	Gonatidae	Family	MSM95/26-1	S3	2000	10762	191	MSM95
65	Gonatidae	Family	MSM95/26-1	S3	2000	21411	13	MSM95
67	Teuthowenia maculata	Species	MSM95/41-1	N4	1600	6796	213	MSM95
73	Gonatidae	Family	MSM95/26-1	S3	2283	11079	11	MSM95
73	Gonatidae	Family	MSM95/41-1	N4	2250	8618	15	MSM95
73	Gonatidae	Family	MSM95/41-1	N4	2000	16555	16	MSM95
73	Gonatidae	Family	MSM95/26-1	S3	2000	21411	14	MSM95
74	Gonatidae	Family	MSM95/26-1	S3	2250	108	108	MSM95
75	Teuthowenia maculata	Species	MSM95/41-1	N4	1600	6796	104	MSM95
81	Gonatidae	Family	MSM95/36-1	HG4	2000	9206	61	MSM95
82	Gonatidae	Family	MSM95/36-1	HG4	2250	10397	59	MSM95

Table 3| Species assignments for eDNA of fish detected in seawater and sediment in the Fram Strait of the Arctic Ocean. The eDNA sequences were run against the nucleotide database in Genbank with the BLAST algorithm. *Sum_reads* is the sum of all reads in one distinct sample. *N_reads* is the number of reads of one specific ASV in one sample. S = Sediment, SW = Seawater

Query	Percent Identity	Query length	e-value	Bit score	Taxon	sum_reads	n_reads	Cruise	Station	Sample Type	Depth
8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	1429	343	PS121	EG1	S	

8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	188	90	PS121	EG1	S
25	96.774	62	5.04e-19	104	Liparidae	1376	37	PS121	EG1	S
29	100	61	8.1e-22	113	Liparis ochotensis	1608	781	PS121	EG1	S
8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	1376	792	PS121	EG1	S
8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	4182	3434	PS121	EG1	S
25	96.774	62	5.04e-19	104	Liparidae	430	194	PS121	EG3	S
20	100	63	6.69e-23	117	Sebastes sp.	1508	397	PS121	EG3	S
10	100	62	2.33e-22	115	Lycodinae	5336	15	PS121	EG4	S
34	100	64	1.92e-23	119	Leuciscidae	209	39	PS121	EG4	S
41	98.387	62	1.08e-20	110	Zoarcidae	5336	601	PS121	EG4	S
10	100	62	2.33e-22	115	Lycodinae	550	60	PS121	EG1	S
29	100	61	8.1e-22	113	Liparis ochotensis	550	78	PS121	EG1	S
8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	550	71	PS121	EG1	S
25	96.774	62	5.04e-19	104	Liparidae	290	27	PS121	EG3	S
83	100	62	2.33e-22	115	Amblyraja sp.	464	17	PS121	EG3	S
10	100	62	2.33e-22	115	Lycodinae	155	30	PS121	HG2	S
26	100	62	2.33e-22	115	Gadidae	2424	352	PS121	HG1	S
8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	2424	830	PS121	HG1	S
12	100	62	2.33e-22	115	Mallotus villosus	1485	805	PS121	HG1	S
25	96.774	62	5.04e-19	104	Liparidae	1485	455	PS121	HG1	S
65	100	61	8.38e-22	113	Mallotus villosus	1485	13	PS121	HG1	S
99	100	62	2.33e-22	115	Arctozenus risso	221	52	PS121	HG1	S
10	100	62	2.33e-22	115	Lycodinae	7249	113	PS121	HG1	S
12	100	62	2.33e-22	115	Mallotus villosus	7249	3664	PS121	HG1	S
20	100	63	6.69e-23	117	Sebastes sp.	7249	384	PS121	HG1	S
65	100	61	8.38e-22	113	Mallotus villosus	7249	123	PS121	HG1	S
67	98.387	62	1.08e-20	110	Mallotus sp.	7249	133	PS121	HG1	S
8	100	64	1.92e-23	119	Reinhardtius hippoglossoides	7249	465	PS121	HG1	S
90	96.774	62	5.04e-19	104	Osmeridae	7249	76	PS121	HG1	S
20	100	63	6.69e-23	117	Sebastes sp.	7185	1296	PS121	HG2	S
31	96.774	62	5.04e-19	104	Liparidae	7185	777	PS121	HG2	S
70	95.161	62	2.35e-17	99	Liparidae	7185	126	PS121	HG2	S
83	100	62	2.33e-22	115	Amblyraja sp.	7185	24	PS121	HG2	S
10	100	62	2.33e-22	115	Lycodinae	2313	696	PS121	HG2	S
105	98.63	73	1.14e-26	130	Delphinidae	2313	47	PS121	HG2	S
26	100	62	2.33e-22	115	Gadidae	2313	87	PS121	HG2	S
34	100	64	1.92e-23	119	Leuciscidae	2313	67	PS121	HG2	S
12	100	62	2.33e-22	115	Mallotus villosus	86	17	PS121	HG2	S
36	100	61	8.1e-22	113	Cyclopterus lumpus	101	24	PS121	HG2	S

10	100	62	2.33e-22	115	Lycodinae	633	45	PS121	HG3	S
26	100	62	2.33e-22	115	Gadidae	581	19	PS121	HG3	S
34	100	64	1.92e-23	119	Leuciscidae	581	44	PS121	HG3	S
83	100	62	2.33e-22	115	Amblyraja sp.	581	32	PS121	HG3	S
10	100	62	2.33e-22	115	Lycodinae	521	54	PS121	HG3	S
12	100	62	2.33e-22	115	Mallotus villosus	68	13	PS121	HG3	S
34	100	64	1.92e-23	119	Leuciscidae	1915	49	PS121	HG5	S
24	100	63	6.69e-23	117	Salmo salar	2072	1268	PS121	HG5	S
26	100	62	2.33e-22	115	Gadidae	2072	22	PS121	HG5	S
34	100	64	1.92e-23	119	Leuciscidae	2072	43	PS121	HG5	S
12	100	62	2.33e-22	115	Mallotus villosus	233	34	PS121	HG9	S
10	100	62	2.33e-22	115	Lycodinae	200	165	PS121	HG9	S
26	100	62	2.33e-22	115	Gadidae	698	11	PS121	HG9	S
40	100	62	2.33e-22	115	Leptoclinus maculatus	698	624	PS121	HG9	S
10	100	62	2.33e-22	115	Lycodinae	166	16	PS121	HG9	S
26	100	62	2.33e-22	115	Gadidae	166	99	PS121	HG9	S
25	96.774	62	5.04e-19	104	Liparidae	422	50	PS121	HG1	S
10	100	62	2.33e-22	115	Lycodinae	3035	949	PS121	HG9	S
25	96.774	62	5.04e-19	104	Liparidae	1083	428	PS121	N3	S
26	100	62	2.33e-22	115	Gadidae	1083	189	PS121	N3	S
10	100	62	2.33e-22	115	Lycodinae	8635	17	PS121	N3	S
10	100	62	2.33e-22	115	Lycodinae	92	12	PS121	N3	S
26	100	62	2.33e-22	115	Gadidae	92	16	PS121	N3	S
10	100	62	2.33e-22	115	Lycodinae	1334	13	PS121	N3	S
14	100	62	2.33e-22	115	Lumpenidae	1334	1269	PS121	N3	S
10	100	62	2.33e-22	115	Lycodinae	2088	586	PS121	N4	S
10	100	62	2.33e-22	115	Lycodinae	260	133	PS121	N4	S
10	100	62	2.33e-22	115	Lycodinae	1914	1119	PS121	N4	S
26	100	62	2.33e-22	115	Gadidae	1914	409	PS121	N4	S
60	100	73	2.44e-28	135	Delphinidae	1914	173	PS121	N4	S
10	100	62	2.33e-22	115	Lycodinae	1905	881	PS121	N4	S
36	100	61	8.1e-22	113	Cyclopterus lumpus	1905	682	PS121	N4	S
10	100	62	2.33e-22	115	Lycodinae	622	153	PS121	S3	S
26	100	62	2.33e-22	115	Gadidae	622	11	PS121	S3	S
29	100	61	8.1e-22	113	Liparis ochotensis	622	76	PS121	S3	S
34	100	64	1.92e-23	119	Leuciscidae	254	34	PS121	S3	S
10	100	62	2.33e-22	115	Lycodinae	1824	143	PS121	S3	S
25	96.774	62	5.04e-19	104	Liparidae	175	12	PS121	S3	S
12	100	62	2.33e-22	115	Mallotus villosus	764	499	PS121	SV1	S
12	100	62	2.33e-22	115	Mallotus villosus	190	54	PS121	SV1	S
20	100	63	6.69e-23	117	Sebastes sp.	764	20	PS121	SV1	S

66	100	61	8.38e-22	113	Mallotus villosus	764	112	PS121	SV1	S	
66	100	61	8.38e-22	113	Mallotus villosus	190	18	PS121	SV1	S	
34	100	64	1.92e-23	119	Leuciscidae	9394	13	PS121	SV1	S	
12	100	62	2.33e-22	115	Mallotus villosus	52	12	PS121	SV1	S	
26	100	62	2.33e-22	115	Gadidae	128	12	PS121	SV1	S	
27	100	62	2.33e-22	115	Ammodytes sp.	1987	974	PS121	SV1	S	
14	100	62	2.33e-22	115	Lumpenidae	4352	3288	PS121	SV1	S	
10	96.774	62	5.04e-19	104	Liparidae	34	11	MSM95	N4	SW	2000
10	96.774	62	5.04e-19	104	Liparidae	36723	14643	MSM95	S3	SW	2250
10	96.774	62	5.04e-19	104	Liparidae	13263	61	MSM95	S3	SW	2250
10	96.774	62	5.04e-19	104	Liparidae	7977	380	MSM95	N4	SW	2500
10	96.774	62	5.04e-19	104	Liparidae	35669	1058	MSM95	N4	SW	1600
10	96.774	62	5.04e-19	104	Liparidae	842	210	MSM95	S3	SW	2283
10	96.774	62	5.04e-19	104	Liparidae	4878	4718	MSM95	S3	SW	2000
10	96.774	62	5.04e-19	104	Liparidae	4724	28	MSM95	HG4	SW	2250
10	96.774	62	5.04e-19	104	Liparidae	486	91	MSM95	S3	SW	2250
10	96.774	62	5.04e-19	104	Liparidae	16976	698	MSM95	HG4	SW	2000
10	96.774	62	5.04e-19	104	Liparidae	2177	100	MSM95	N4	SW	1000
10	96.774	62	5.04e-19	104	Liparidae	26373	6490	MSM95	S3	SW	2000
10	96.774	62	5.04e-19	104	Liparidae	906	54	MSM95	N4	SW	1600
10	96.774	62	5.04e-19	104	Liparidae	23406	2704	MSM95	S3	SW	2000
10	96.774	62	5.04e-19	104	Liparidae	4583	94	MSM95	HG4	SW	2420
102	98.387	62	1.16e-20	110	Clupea sp.	13263	57	MSM95	S3	SW	2250
107	100	62	2.33e-22	115	Arctozenus risso	6406	33	MSM95	HG4	SW	200
107	100	62	2.33e-22	115	Arctozenus risso	5620	18	MSM95	HG4	SW	2420
108	98.387	62	1.16e-20	110	Clupea harengus	13263	41	MSM95	S3	SW	2250
108	98.387	62	1.16e-20	110	Clupea sp.	13263	41	MSM95	S3	SW	2250
11	100	63	6.69e-23	117	Salmo sp.	67129	19624	MSM95	HG4	SW	2250
114	100	62	2.48e-22	115	Reinhardtius hippoglossoides	1825	31	MSM95	N4	SW	2000
136	100	61	8.1e-22	113	Somniosus sp.	4583	10	MSM95	HG4	SW	2420
14	100	63	6.69e-23	117	Salmo salar	36723	5922	MSM95	S3	SW	2250
14	100	63	6.69e-23	117	Salmo salar	79932	914	MSM95	HG4	SW	200
14	100	63	6.69e-23	117	Salmo salar	4583	106	MSM95	HG4	SW	2420
14	100	63	6.69e-23	117	Salmo salar	4126	26	MSM95	HG4	SW	200
14	100	63	6.69e-23	117	Salmo salar	70073	2975	MSM95	HG4	SW	2000
14	100	63	6.69e-23	117	Salmo salar	13263	22	MSM95	S3	SW	2250
17	100	61	8.38e-22	113	Mallotus villosus	26998	425	MSM95	S3	SW	1600
17	100	61	8.38e-22	113	Mallotus villosus	52342	824	MSM95	HG4	SW	2250
17	100	61	8.38e-22	113	Mallotus villosus	6672	79	MSM95	N4	SW	1300
17	100	61	8.38e-22	113	Mallotus villosus	9434	23	MSM95	S3	SW	200

17	100	61	8.38e-22	113	Mallotus villosus	10767	66	MSM95	HG4	SW	1600
17	100	61	8.38e-22	113	Mallotus villosus	5620	88	MSM95	HG4	SW	2420
17	100	61	8.38e-22	113	Mallotus villosus	36723	180	MSM95	S3	SW	2250
17	100	61	8.38e-22	113	Mallotus villosus	70073	12	MSM95	HG4	SW	2000
17	100	61	8.38e-22	113	Mallotus villosus	20414	65	MSM95	S3	SW	200
17	100	61	8.38e-22	113	Mallotus villosus	26373	55	MSM95	S3	SW	2000
17	100	61	8.38e-22	113	Mallotus villosus	35669	30	MSM95	N4	SW	1600
17	100	61	8.38e-22	113	Mallotus villosus	29172	1670	MSM95	HG4	SW	1000
17	100	61	8.38e-22	113	Mallotus villosus	75958	398	MSM95	HG4	SW	2000
17	100	61	8.38e-22	113	Mallotus villosus	2177	43	MSM95	N4	SW	1000
17	100	61	8.38e-22	113	Mallotus villosus	29757	101	MSM95	N4	SW	1000
17	100	61	8.38e-22	113	Mallotus villosus	31044	16	MSM95	HG4	SW	1000
17	100	61	8.38e-22	113	Mallotus villosus	11172	79	MSM95	N4	SW	1300
17	100	61	8.38e-22	113	Mallotus villosus	60178	603	MSM95	N4	SW	1300
17	100	61	8.38e-22	113	Mallotus villosus	33705	230	MSM95	S3	SW	2283
17	100	61	8.38e-22	113	Mallotus villosus	14707	23	MSM95	HG4	SW	50
17	100	61	8.38e-22	113	Mallotus villosus	79932	164	MSM95	HG4	SW	200
17	100	61	8.38e-22	113	Mallotus villosus	7149	151	MSM95	S3	SW	1000
17	100	61	8.38e-22	113	Mallotus villosus	7977	119	MSM95	N4	SW	2500
17	100	61	8.38e-22	113	Mallotus villosus	96159	232	MSM95	N4	SW	2250
17	100	61	8.38e-22	113	Mallotus villosus	5411	750	MSM95	S3	SW	1000
17	100	61	8.38e-22	113	Mallotus villosus	32409	1555	MSM95	N4	SW	2250
18	100	61	8.1e-22	113	Cyclopterus lumpus	4583	579	MSM95	HG4	SW	2420
18	100	61	8.1e-22	113	Cyclopterus lumpus	842	243	MSM95	S3	SW	2283
18	100	61	8.1e-22	113	Cyclopterus lumpus	296	17	MSM95	N4	SW	2705
18	100	61	8.1e-22	113	Cyclopterus lumpus	2730	544	MSM95	HG4	SW	1600
18	100	61	8.1e-22	113	Cyclopterus lumpus	563	151	MSM95	HG4	SW	2420
18	100	61	8.1e-22	113	Cyclopterus lumpus	5850	839	MSM95	N4	SW	2705
18	100	61	8.1e-22	113	Cyclopterus lumpus	36723	4167	MSM95	S3	SW	2250
18	100	61	8.1e-22	113	Cyclopterus lumpus	13263	322	MSM95	S3	SW	2250
18	100	61	8.1e-22	113	Cyclopterus lumpus	5620	132	MSM95	HG4	SW	2420
20	100	62	2.33e-22	115	Zoarcidae	842	12	MSM95	S3	SW	2283
20	100	62	2.33e-22	115	Zoarcidae	33676	52	MSM95	N4	SW	2000

20	100	62	2.33e-22	115	Zoarcidae	10767	63	MSM95	HG4	SW	1600
20	100	62	2.33e-22	115	Zoarcidae	70073	1540	MSM95	HG4	SW	2000
20	100	62	2.33e-22	115	Zoarcidae	75958	2436	MSM95	HG4	SW	2000
20	100	62	2.33e-22	115	Zoarcidae	296	20	MSM95	N4	SW	2705
20	100	62	2.33e-22	115	Zoarcidae	5620	14	MSM95	HG4	SW	2420
20	100	62	2.33e-22	115	Zoarcidae	23406	1093	MSM95	S3	SW	2000
20	100	62	2.33e-22	115	Zoarcidae	33705	167	MSM95	S3	SW	2283
20	100	62	2.33e-22	115	Zoarcidae	4371	38	MSM95	N4	SW	200
20	100	62	2.33e-22	115	Zoarcidae	5850	117	MSM95	N4	SW	2705
20	100	62	2.33e-22	115	Zoarcidae	4724	10	MSM95	HG4	SW	2250
28	100	62	2.41e-22	115	Sebastes sp.	60178	1568	MSM95	N4	SW	1300
28	100	62	2.41e-22	115	Sebastes sp.	29757	136	MSM95	N4	SW	1000
28	100	62	2.41e-22	115	Sebastes sp.	14707	295	MSM95	HG4	SW	50
28	100	62	2.41e-22	115	Sebastes sp.	75958	133	MSM95	HG4	SW	2000
28	100	62	2.41e-22	115	Sebastes sp.	2866	81	MSM95	HG4	SW	1300
33	98.438	64	8.94e-22	113	Clupea sp.	75958	28	MSM95	HG4	SW	2000
33	98.438	64	8.94e-22	113	Clupea sp.	6672	1866	MSM95	N4	SW	1300
36	100	62	2.33e-22	115	Gadidae	96159	202	MSM95	N4	SW	2250
36	100	62	2.33e-22	115	Gadidae	26373	174	MSM95	S3	SW	2000
36	100	62	2.33e-22	115	Gadidae	75958	46	MSM95	HG4	SW	2000
36	100	62	2.33e-22	115	Gadidae	70073	62	MSM95	HG4	SW	2000
36	100	62	2.33e-22	115	Gadidae	5411	64	MSM95	S3	SW	1000
36	100	62	2.33e-22	115	Gadidae	1825	31	MSM95	N4	SW	2000
36	100	62	2.33e-22	115	Gadidae	4583	68	MSM95	HG4	SW	2420
36	100	62	2.33e-22	115	Gadidae	4724	21	MSM95	HG4	SW	2250
36	100	62	2.33e-22	115	Gadidae	32409	84	MSM95	N4	SW	2250
36	100	62	2.33e-22	115	Gadidae	6406	15	MSM95	HG4	SW	200
36	100	62	2.33e-22	115	Gadidae	52342	404	MSM95	HG4	SW	2250
36	100	62	2.33e-22	115	Gadidae	1789	36	MSM95	N4	SW	2500
38	98.413	63	3.11e-21	111	Salmo sp.	70073	1644	MSM95	HG4	SW	2000
4	100	62	2.33e-22	115	Mallotus villosus	2648	53	MSM95	N4	SW	50
4	100	62	2.33e-22	115	Mallotus villosus	11172	3871	MSM95	N4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	79932	15670	MSM95	HG4	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	58	10	MSM95	S3	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	4288	180	MSM95	S3	SW	400
4	100	62	2.33e-22	115	Mallotus villosus	70073	1875	MSM95	HG4	SW	2000
4	100	62	2.33e-22	115	Mallotus villosus	4371	84	MSM95	N4	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	43398	11	MSM95	S3	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	296	12	MSM95	N4	SW	2705
4	100	62	2.33e-22	115	Mallotus villosus	32409	4910	MSM95	N4	SW	2250

4	100	62	2.33e-22	115	Mallotus villosus	69	27	MSM95	HG4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	906	14	MSM95	N4	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	29757	6342	MSM95	N4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	2866	20	MSM95	HG4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	36723	3971	MSM95	S3	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	33705	1679	MSM95	S3	SW	2283
4	100	62	2.33e-22	115	Mallotus villosus	26998	10050	MSM95	S3	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	6672	1325	MSM95	N4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	4583	473	MSM95	HG4	SW	2420
4	100	62	2.33e-22	115	Mallotus villosus	4724	259	MSM95	HG4	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	2730	89	MSM95	HG4	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	29172	7171	MSM95	HG4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	35669	945	MSM95	N4	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	31044	1904	MSM95	HG4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	7977	375	MSM95	N4	SW	2500
4	100	62	2.33e-22	115	Mallotus villosus	75958	4759	MSM95	HG4	SW	2000
4	100	62	2.33e-22	115	Mallotus villosus	4662	111	MSM95	N4	SW	50
4	100	62	2.33e-22	115	Mallotus villosus	26373	13446	MSM95	S3	SW	2000
4	100	62	2.33e-22	115	Mallotus villosus	5850	34	MSM95	N4	SW	2705
4	100	62	2.33e-22	115	Mallotus villosus	563	130	MSM95	HG4	SW	2420
4	100	62	2.33e-22	115	Mallotus villosus	486	49	MSM95	S3	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	5411	3238	MSM95	S3	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	7149	2236	MSM95	S3	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	20414	5042	MSM95	S3	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	1789	239	MSM95	N4	SW	2500
4	100	62	2.33e-22	115	Mallotus villosus	52342	4626	MSM95	HG4	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	10767	8539	MSM95	HG4	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	60	32	MSM95	HG4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	96159	19383	MSM95	N4	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	6406	66	MSM95	HG4	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	59	29	MSM95	S3	SW	2283
4	100	62	2.33e-22	115	Mallotus villosus	7771	59	MSM95	N4	SW	400

4	100	62	2.33e-22	115	Mallotus villosus	9434	709	MSM95	S3	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	123	10	MSM95	N4	SW	2705
4	100	62	2.33e-22	115	Mallotus villosus	67129	14	MSM95	HG4	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	2177	499	MSM95	N4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	4126	209	MSM95	HG4	SW	200
4	100	62	2.33e-22	115	Mallotus villosus	14707	1104	MSM95	HG4	SW	50
4	100	62	2.33e-22	115	Mallotus villosus	60178	3596	MSM95	N4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	5620	1216	MSM95	HG4	SW	2420
41	100	62	2.33e-22	115	Anarhichadidae	29172	26	MSM95	HG4	SW	1000
41	100	62	2.33e-22	115	Anarhichadidae	7977	404	MSM95	N4	SW	2500
41	100	62	2.33e-22	115	Anarhichadidae	43398	767	MSM95	S3	SW	200
44	98.387	62	1.08e-20	110	Mallotus sp.	4662	1037	MSM95	N4	SW	50
47	98.387	62	1.08e-20	110	Anarhichadidae	43398	773	MSM95	S3	SW	200
51	98.413	63	3.11e-21	111	Sebastes sp.	14707	626	MSM95	HG4	SW	50
54	100	66	1.58e-24	122	Myctophidae	9434	10	MSM95	S3	SW	200
54	100	66	1.58e-24	122	Myctophidae	4662	46	MSM95	N4	SW	50
55	98.413	63	3.11e-21	111	Salmo sp.	67129	518	MSM95	HG4	SW	2250
56	100	58	3.77e-20	108	Cyclopterus lumpus	36723	512	MSM95	S3	SW	2250
58	100	60	2.91e-21	111	Cyclopterus lumpus	5850	49	MSM95	N4	SW	2705
58	100	60	2.91e-21	111	Cyclopterus lumpus	563	10	MSM95	HG4	SW	2420
58	100	60	2.91e-21	111	Cyclopterus lumpus	4583	16	MSM95	HG4	SW	2420
58	100	60	2.91e-21	111	Cyclopterus lumpus	36723	346	MSM95	S3	SW	2250
58	100	60	2.91e-21	111	Cyclopterus lumpus	13263	15	MSM95	S3	SW	2250
59	98.387	62	1.08e-20	110	Mallotus sp.	96159	11	MSM95	N4	SW	2250
59	98.387	62	1.08e-20	110	Mallotus sp.	26373	10	MSM95	S3	SW	2000
59	98.387	62	1.08e-20	110	Mallotus sp.	31044	352	MSM95	HG4	SW	1000
63	100	61	8.38e-22	113	Mallotus villosus	4583	105	MSM95	HG4	SW	2420
63	100	61	8.38e-22	113	Mallotus villosus	563	94	MSM95	HG4	SW	2420
63	100	61	8.38e-22	113	Mallotus villosus	4724	124	MSM95	HG4	SW	2250
66	100	64	1.92e-23	119	Reinhardtius hippoglossoides	1825	299	MSM95	N4	SW	2000
7	100	64	1.92e-23	119	Clupea harengus	26998	13	MSM95	S3	SW	1600
7	100	64	1.92e-23	119	Clupea harengus	70073	608	MSM95	HG4	SW	2000
7	100	64	1.92e-23	119	Clupea harengus	1825	467	MSM95	N4	SW	2000
7	100	64	1.92e-23	119	Clupea harengus	24715	7900	MSM95	HG4	SW	400
7	100	64	1.92e-23	119	Clupea harengus	2177	42	MSM95	N4	SW	1000

7	100	64	1.92e-23	119	Clupea harengus	22357	3088	MSM95	HG4	SW	400
7	100	64	1.92e-23	119	Clupea harengus	7149	312	MSM95	S3	SW	1000
7	100	64	1.92e-23	119	Clupea harengus	4662	1736	MSM95	N4	SW	50
7	100	64	1.92e-23	119	Clupea harengus	13263	2491	MSM95	S3	SW	2250
7	100	64	1.92e-23	119	Clupea harengus	75958	31074	MSM95	HG4	SW	2000
7	100	64	1.92e-23	119	Clupea harengus	16976	4919	MSM95	HG4	SW	2000
7	100	64	1.92e-23	119	Clupea harengus	6672	1492	MSM95	N4	SW	1300
76	100	73	2.44e-28	135	Delphinidae	6406	111	MSM95	HG4	SW	200
76	100	73	2.44e-28	135	Delphinidae	2648	11	MSM95	N4	SW	50
76	100	73	2.44e-28	135	Delphinidae	4662	35	MSM95	N4	SW	50
76	100	73	2.44e-28	135	Delphinidae	20844	22	MSM95	N4	SW	2500
79	98.413	63	3.11e-21	111	Salmo sp.	67129	158	MSM95	HG4	SW	2250
8	100	63	6.69e-23	117	Sebastes sp.	7771	400	MSM95	N4	SW	400
8	100	63	6.69e-23	117	Sebastes sp.	212	70	MSM95	HG4	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	14707	9790	MSM95	HG4	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	451	362	MSM95	HG4	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	4082	291	MSM95	N4	SW	1600
8	100	63	6.69e-23	117	Sebastes sp.	2177	69	MSM95	N4	SW	1000
8	100	63	6.69e-23	117	Sebastes sp.	31044	2893	MSM95	HG4	SW	1000
8	100	63	6.69e-23	117	Sebastes sp.	29172	397	MSM95	HG4	SW	1000
8	100	63	6.69e-23	117	Sebastes sp.	4662	730	MSM95	N4	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	2648	1526	MSM95	N4	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	60178	6854	MSM95	N4	SW	1300
8	100	63	6.69e-23	117	Sebastes sp.	75958	2136	MSM95	HG4	SW	2000
8	100	63	6.69e-23	117	Sebastes sp.	943	164	MSM95	S3	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	575	332	MSM95	HG4	SW	400
8	100	63	6.69e-23	117	Sebastes sp.	2866	2538	MSM95	HG4	SW	1300
8	100	63	6.69e-23	117	Sebastes sp.	79932	311	MSM95	HG4	SW	200
8	100	63	6.69e-23	117	Sebastes sp.	7977	33	MSM95	N4	SW	2500
8	100	63	6.69e-23	117	Sebastes sp.	2426	154	MSM95	N4	SW	200
8	100	63	6.69e-23	117	Sebastes sp.	29757	11189	MSM95	N4	SW	1000
8	100	63	6.69e-23	117	Sebastes sp.	6672	381	MSM95	N4	SW	1300
8	100	63	6.69e-23	117	Sebastes sp.	4126	28	MSM95	HG4	SW	200
8	100	63	6.69e-23	117	Sebastes sp.	1044	220	MSM95	N4	SW	50
8	100	63	6.69e-23	117	Sebastes sp.	622	129	MSM95	HG4	SW	1300
85	100	62	2.33e-22	115	Micromesistius poutassou	2730	124	MSM95	HG4	SW	1600
86	100	62	2.33e-22	115	Amblyraja sp.	20844	85	MSM95	N4	SW	2500
89	98.361	61	3.77e-20	108	Cyclopterus sp.	5850	96	MSM95	N4	SW	2705
91	100	61	8.38e-22	113	Gadidae	52342	42	MSM95	HG4	SW	2250
91	100	61	8.38e-22	113	Gadidae	5411	16	MSM95	S3	SW	1000
91	100	61	8.38e-22	113	Gadidae	32409	14	MSM95	N4	SW	2250

98	100	61	8.66e-22	113	Sebastes sp.	622	14	MSM95	HG4	SW	1300
98	100	61	8.66e-22	113	Sebastes sp.	4082	21	MSM95	N4	SW	1600
98	100	61	8.66e-22	113	Sebastes sp.	1044	19	MSM95	N4	SW	50
98	100	61	8.66e-22	113	Sebastes sp.	943	12	MSM95	S3	SW	50
10	96.774	62	5.04e-19	104	Liparidae	14628	63	PS121	N4	SW	1600
10	96.774	62	5.04e-19	104	Liparidae	32275	109	PS121	S3	SW	2000
10	96.774	62	5.04e-19	104	Liparidae	4659	53	PS121	S3	SW	1300
10	96.774	62	5.04e-19	104	Liparidae	5669	22	PS121	S3	SW	2250
10	96.774	62	5.04e-19	104	Liparidae	4482	28	PS121	EG4	SW	2500
10	96.774	62	5.04e-19	104	Liparidae	1440	14	PS121	HG4	SW	1600
10	96.774	62	5.04e-19	104	Liparidae	20038	117	PS121	EG4	SW	2500
10	96.774	62	5.04e-19	104	Liparidae	31127	58	PS121	EG4	SW	1600
10	96.774	62	5.04e-19	104	Liparidae	23212	246	PS121	S3	SW	1600
10	96.774	62	5.04e-19	104	Liparidae	1762	41	PS121	S3	SW	1600
11	100	63	6.69e-23	117	Salmo sp.	405	11	PS121	HG4	SW	1300
11	100	63	6.69e-23	117	Salmo sp.	22752	27	PS121	HG4	SW	400
134	100	61	8.1e-22	113	Chelidonichthys sp.	4267	12	PS121	EG4	SW	2000
14	100	63	6.69e-23	117	Salmo salar	34580	1274	PS121	HG4	SW	250
17	100	61	8.38e-22	113	Mallotus villosus	33002	297	PS121	HG4	SW	400
17	100	61	8.38e-22	113	Mallotus villosus	5787	11	PS121	N4	SW	50
17	100	61	8.38e-22	113	Mallotus villosus	8473	36	PS121	N4	SW	400
17	100	61	8.38e-22	113	Mallotus villosus	60827	35	PS121	N4	SW	1600
17	100	61	8.38e-22	113	Mallotus villosus	34908	28	PS121	HG4	SW	2250
18	100	61	8.1e-22	113	Cyclopterus lumpus	42239	73	PS121	N4	SW	2000
18	100	61	8.1e-22	113	Cyclopterus lumpus	2421	85	PS121	S3	SW	2250
20	100	62	2.33e-22	115	Zoarcidae	4260	12	PS121	EG4	SW	1300
20	100	62	2.33e-22	115	Zoarcidae	42239	157	PS121	N4	SW	2000
20	100	62	2.33e-22	115	Zoarcidae	4482	19	PS121	EG4	SW	2500
20	100	62	2.33e-22	115	Zoarcidae	81526	66	PS121	EG4	SW	2000
20	100	62	2.33e-22	115	Zoarcidae	45037	69	PS121	EG4	SW	400
20	100	62	2.33e-22	115	Zoarcidae	32275	12	PS121	S3	SW	2000
20	100	62	2.33e-22	115	Zoarcidae	2421	18	PS121	S3	SW	2250
20	100	62	2.33e-22	115	Zoarcidae	23212	48	PS121	S3	SW	1600
20	100	62	2.33e-22	115	Zoarcidae	32522	227	PS121	EG4	SW	1300
20	100	62	2.33e-22	115	Zoarcidae	53940	27	PS121	EG4	SW	1000
36	100	62	2.33e-22	115	Gadidae	5669	12	PS121	S3	SW	2250
36	100	62	2.33e-22	115	Gadidae	28794	273	PS121	N4	SW	1600
36	100	62	2.33e-22	115	Gadidae	51573	203	PS121	HG4	SW	1600
36	100	62	2.33e-22	115	Gadidae	4482	39	PS121	EG4	SW	2500
4	100	62	2.33e-22	115	Mallotus villosus	60827	1127	PS121	N4	SW	1600

4	100	62	2.33e-22	115	Mallotus villosus	14628	160	PS121	N4	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	33002	6983	PS121	HG4	SW	400
4	100	62	2.33e-22	115	Mallotus villosus	14028	169	PS121	HG4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	28794	616	PS121	N4	SW	1600
4	100	62	2.33e-22	115	Mallotus villosus	399	12	PS121	N4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	758	18	PS121	HG4	SW	2000
4	100	62	2.33e-22	115	Mallotus villosus	8473	137	PS121	N4	SW	400
4	100	62	2.33e-22	115	Mallotus villosus	5787	19	PS121	N4	SW	50
4	100	62	2.33e-22	115	Mallotus villosus	22813	610	PS121	HG4	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	14818	107	PS121	HG4	SW	1000
4	100	62	2.33e-22	115	Mallotus villosus	34908	1219	PS121	HG4	SW	2250
4	100	62	2.33e-22	115	Mallotus villosus	6700	11	PS121	EG4	SW	1300
4	100	62	2.33e-22	115	Mallotus villosus	11926	17	PS121	N4	SW	400
4	100	62	2.33e-22	115	Mallotus villosus	378	37	PS121	S3	SW	1000
45	100	64	1.92e-23	119	Pleuronectidae	10834	805	PS121	HG4	SW	2000
45	100	64	1.92e-23	119	Pleuronectidae	104355	122	PS121	HG4	SW	1600
52	100	61	8.1e-22	113	Liparis ochotensis	28794	324	PS121	N4	SW	1600
52	100	61	8.1e-22	113	Liparis ochotensis	45037	274	PS121	EG4	SW	400
52	100	61	8.1e-22	113	Liparis ochotensis	5520	44	PS121	N4	SW	50
54	100	66	1.58e-24	122	Myctophidae	22068	177	PS121	EG4	SW	2500
54	100	66	1.58e-24	122	Myctophidae	8473	324	PS121	N4	SW	400
63	100	61	8.38e-22	113	Mallotus villosus	5787	10	PS121	N4	SW	50
64	100	62	2.33e-22	115	Ammodytes sp.	27110	339	PS121	S3	SW	400
7	100	64	1.92e-23	119	Clupea harengus	28794	17	PS121	N4	SW	1600
7	100	64	1.92e-23	119	Clupea harengus	33002	14	PS121	HG4	SW	400
7	100	64	1.92e-23	119	Clupea harengus	10374	1198	PS121	S3	SW	200
7	100	64	1.92e-23	119	Clupea harengus	12007	143	PS121	S3	SW	1000
7	100	64	1.92e-23	119	Clupea harengus	5053	38	PS121	HG4	SW	250
7	100	64	1.92e-23	119	Clupea harengus	23212	1062	PS121	S3	SW	1600
74	100	64	1.92e-23	119	Pleuronectidae	1873	11	PS121	HG4	SW	250
74	100	64	1.92e-23	119	Pleuronectidae	64956	166	PS121	EG4	SW	1000
77	96.97	66	1.19e-20	110	Carangidae	27110	188	PS121	S3	SW	400
78	100	62	2.33e-22	115	Rhodichthys regina	22068	169	PS121	EG4	SW	2500
8	100	63	6.69e-23	117	Sebastes sp.	9880	276	PS121	HG4	SW	1000
8	100	63	6.69e-23	117	Sebastes sp.	22752	164	PS121	HG4	SW	400

8	100	63	6.69e-23	117	Sebastes sp.	5053	257	PS121	HG4	SW	250
8	100	63	6.69e-23	117	Sebastes sp.	34580	18	PS121	HG4	SW	250
86	100	62	2.33e-22	115	Amblyraja sp.	22813	19	PS121	HG4	SW	2250
88	96.875	64	4.16e-20	108	Clupeidae	10374	111	PS121	S3	SW	200
91	100	61	8.38e-22	113	Gadidae	51573	10	PS121	HG4	SW	1600

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Eidesstaatliche Erklärung

Hiermit bestätige ich, Véronique J. Merten, dass ich die vorliegende Dissertation:

Pelagic deep-sea metazoan biodiversity and ecology revealed by environmental DNA analysis in combination with other censuses

bis auf die Beratung durch meine BetreuerIn in Inhalt und Form selbstständig angefertigt habe. Ich versichere, dass ich diese Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft verfasst habe. Alle benutzten Quellen und Hilfsmittel habe ich vollständig angegeben und die Zusammenarbeit mit anderen WissenschaftlerInnen habe ich kenntlich gemacht. Weder diese noch eine ähnliche Arbeit wurden an einer anderen Abteilung oder Hochschule im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht. Mir wurde kein akademischer Grad entzogen und dies ist mein erstes und einziges Promotionsverfahren.

Ich erkläre mich einverstanden, dass diese Dissertation an die Bibliothek des GEOMAR Helmholtz Zentrum für Ozeanforschung Kiel und die Universitätsbibliothek der Christian-Albrechts-Universität zu Kiel weitergeleitet wird.

Kiel, Dezember 2021

…….
Véronique J. Merten

“The deeper you go, the weirder life gets.”

- Steve Zissou, The Life Aquatics