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Toward a reliable assessment of potential ecological impacts of deep-sea polymetallic nodule mining on abyssal infauna

Lidia Lins [1,2]* Daniela Zeppilli [3] Lénaïck Menot,[3] Loïc N. Michel,[3] Paulo Bonifácio,[3] Miriam Brandt,[4] Ellen Pape,[1] Sven Rossel [2] Katja Uhlenkott,[2] Lara Macheriotou,[1] Tania Nara Bezerra,[1] Nuria Sánchez,[3] Joan M. Alfaro-Lucas [3] Pedro Martínez Arbizu,[2] Stefanie Kaiser,[5] Chisato Murakami,[6] Ann Vanreusel[1]

[1]Marine Biology Research Group, Ghent University, Ghent, Belgium
[2]German Centre for Marine Biodiversity Research (DZMB), Senckenberg am Meer, Wilhelmshaven, Germany
[3]IFREMER, Centre de Bretagne, REM/EEP/LEP, ZI de la pointe du diable, Plouzané, France
[4]MARBEC, Univ. Montpellier, Ifremer, CNRS, IRD, Sète, France
[5]Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Łódź, Poland
[6]Deep Ocean Resources Development Co., Ltd, Tokyo, Japan

Abstract

The increasing demand for metals is pushing forward the progress of deep-sea mining industry. The abyss between the Clarion and Clipperton Fracture Zones (CCFZ), a region holding a higher concentration of minerals than land deposits, is the most targeted area for the exploration of polymetallic nodules worldwide, which may likely disturb the seafloor across large areas and over many years. Effects from nodule extraction cause acute biodiversity loss of organisms inhabiting sediments and polymetallic nodules. Attention to deep-sea ecosystems and their services has to be considered before mining starts but the lack of basic scientific knowledge on the methodologies for the ecological surveys of fauna in the context of deep-sea mining impacts is still scarce. We review the methodology to sample, process and investigate metazoan infauna both inhabiting sediments and nodules dwelling on these polymetallic-nodule areas. We suggest effective procedures for sampling designs, devices and methods involving gear types, sediment processing, morphological and genetic identification including metabarcoding and proteomic fingerprinting, the assessment of biomass, functional traits, fatty acids, and stable isotope studies within the CCFZ based on both first-hand experiences and literature. We recommend multi- and boxcorers for the quantitative assessments of meio- and macrofauna, respectively. The assessment of biodiversity at species level should be focused and/or the combination of morphological with metabarcoding or proteomic fingerprinting techniques. We highlight that biomass, functional traits, and trophic markers may provide critical insights for biodiversity assessments and how statistical modeling facilitates predicting patterns spatially across point-source data and is essential for conservation management.

The growing demand for metals and dwindling terrestrial mineral reserves is driving the exploration for marine mineral resources located at great depths. Covering more than 70% of Earth's surface, the deep sea (below 200 m water depth) is one of the least known realms on Earth, yet it contains a unique reservoir of mineral deposits, such as polymetallic nodules (Boschen et al. 2013; Haldar 2013; Thurber et al. 2014; Thompson et al. 2018).

These deep-sea polymetallic (or so-called manganese) nodules are ferromanganese oxide deposits accreted around a nucleus of diverse types, such as volcanic fragments or biological remains (Cronan 2000). They occur in areas where environmental conditions are stable and sedimentation rates are very low. Polymetallic nodules cover vast areas of the abyssal seafloor (Cronan 2000). Besides areas in the Central Indian Ocean Basin, South West and Central Indian Ridge, and Mid-Atlantic Ridge, the Clarion–Clipperton Fracture Zone (CCFZ) for instance is a targeted area for deep-sea mining. The CCFZ is a large area with 6 million km^2 laying between 4000 and 5500 m depth within the central Pacific Ocean with polymetallic nodule fields occurring as patches (Wedding et al. 2015; Kuhn et al. 2017; Glover et al. 2018).

*Correspondence: lidia.linspereira@ugent.be

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Mining operations will inevitably cause the loss of the majority of the fauna using the nodules as a habitat or inhabiting the upper layers of the sediments (Ahnert and Schriever 2001; Borowski 2001; Miljutin et al. 2011; Vanreusel et al. 2016). Jumars (1981) summarized the impacts on the benthic fauna derived from mining activities as follows: (1) direct mechanical impacts along the track of the nodule collector where the sediment and associated fauna are crushed or dispersed in a plume; the nodules are removed and the sediment is compacted by the nodule collector, (2) burial of benthic fauna by the sediment plume, and (3) alteration of food resources and changes in biochemical conditions caused by the removal of the top sediment layers. Other expected changes from mining exploitation are a reduction of habitat heterogeneity, alongside modification and destruction of the habitat through metal mobilization and sediment resuspension, which will alter the structure of the highly diverse benthic communities for decades and probably even millennia (Oebius et al. 2001; Koschinsky and Hein 2003; Janssen et al. 2015; Zeppilli et al. 2015; Jones et al. 2017; de Jonge et al. 2020).

Most of the polymetallic nodule reservoirs lie in areas beyond national jurisdiction (the seabed which lies beyond the limits of the continental shelf). These mineral resources are thus common heritage of mankind as stated by the United Nations Convention on the Law of the Sea (UNCLOS), which has established the International Seabed Authority (ISA) to regulate mining activities. The ISA is currently developing and adopting rules, regulations and procedures for the exploration and commercial exploitation of these unique deep-sea reservoirs (Lodge et al. 2014). In the CCFZ, one of the main challenges related to the environmental management of deep-sea mining is the lack of data and basic scientific knowledge targeted for mining (Glover et al. 2018; Smith et al. 2020). In deep oceans, the vast majority of species generally remain undiscovered and/or undescribed (Bouchet et al. 2016) and polymetallic nodule fields are no exception (Miljutina et al. 2010; Glover et al. 2018; Pasotti et al. 2020). According to Glover et al. (2018), an initial focus on integrative taxonomy (morphology and DNA) is needed to provide the necessary data including natural history, connectivity, value, and function of species to further implement ecosystem monitoring services that are needed prior to mining activities.

The ISA has already produced a set of documents and recommendations for the CCFZ for managing exploration and exploitation of its non-living resources (Lodge et al. 2014). These recommendations on how to assess potential environmental impacts have been issued to contractors, and include guidelines on the collection of environmental baseline data in addition to the exploration of marine minerals (ISBA 2020). However, information on methodological guidelines for ecological surveys of abyssal fauna in the context of deep-sea mining impacts is still scattered (A. Glover et al. 2016), even though such information is important for scientists, contractors, and decision-makers (Smith et al. 2020).

In the present paper, we propose a specific and detailed methodology to investigate metazoan infauna (meio- and macrofauna) inhabiting polymetallic-nodule province. We hereby focus on first-hand experience and literature from the CCFZ, but many of the methods are generally applicable to other nodule-rich areas and deep-sea sediment habitats. We review and suggest efficient procedures for sampling design, devices and methods including gears, sediment slicing and sieving, morphological and genetic identification, isotope and biomass studies, assessment of functional traits, as well as biodiversity assessment using metabarcoding and proteomic fingerprinting. By offering a baseline for ecological surveying of abyssal infauna, being an essential ecological variable, we aim to contribute with the production of accurate data, which can be used as a measurement of ecosystem health and in deep-sea mining risk assessments.

The CCFZ nodule province and its associated fauna

The CCFZ lies in abyssal depths of about 4000–5500 m water depth and is mostly located within mesotrophic waters with a POC flux of about 0.5–1.5 g C m^{-2} yr. It is characterized as an oligotrophic area with a gradient of food input to the seafloor reflecting the E-W and S-N decrease in surface primary productivity gradients (Hannides and Smith 2003; Smith and Demopoulos 2003; Vanreusel et al. 2016). The vast water column overlying the CCFZ consists of different water masses, including an oxygen minimum zone (OMZ, 100–1000 m depth range in the Pacific) (Hannides and Smith 2003). Sedimentation rates are low, ranging from 1.15 to less than 0.2 cm $kyear^{-1}$ (Volz et al. 2018). Sediments in the CCFZ consist of a relatively thin coverage (50–200 m) of finely-grained pelagic remains, mainly of biogenic origin, overlaying hard basalts (Kuhn et al. 2017). The seafloor is characterized by a succession of abyssal hills, seamounts, and horst and graben structures oriented N-S (Craig 1979) and E-W (for the BGR, the German exploration area), which were developed originally along the axis of fast spreading centers (Macdonald et al. 1996; Glover et al. 2016; Kuhn et al. 2017; Volz et al. 2018; Simon-Lledó et al. 2019). Most of the polymetallic nodules in the CCFZ can be found on the sediment surface, but some may also be buried into the sediment (Seibold and Berger 1993; Hein and Petersen 2013). The average density of polymetallic nodules in the CCFZ is 15 kg m^{-2}, occasionally reaching 75 kg m^{-2} (Hein and Petersen 2013). The presence of polymetallic nodules increase habitat heterogeneity, thus forming a unique habitat within the abyss and a reservoir of undescribed species (e.g., Glover et al. 2002; Miljutin and Miljutina 2009; Paterson et al. 2016; Bonifacio and Menot 2019; Ramiro-Sánchez et al. 2019; Christodoulou et al. 2020, 2019; Patel et al. 2020).

Among the different benthic size classes, the meiofauna, mostly composed of nematodes and copepods, but also of tardigrades, gastrotrichs, and kinorhynchs, is an important benthic compartment within the CCFZ due to their high densities

(~50 and 550 ind. 10 cm^{-2}) (Pape et al. 2017; Hauquier et al. 2019; Uhlenkott et al. 2021), and their important role in benthic food webs and ecosystem functioning (Zeppilli et al. 2015). It is important to highlight that although protozoans such as foraminiferans, can be also major components of meiofauna, they require different methods for extraction and here we only consider metazoans. Even though polymetallic nodule density have been suggested to negatively affect the abundance of meiofauna (Miljutina et al. 2010; Uhlenkott et al. 2020), their presence also offers a specific habitat for some unique meiofauna taxa, which dwell on the nodule surfaces and occupy their crevices and internal cavities (Thiel et al. 1993; Veillette et al. 2007*a*, 2007*b*). Furthermore, the taxonomic composition of nematode and tardigrade assemblages inhabiting the surface and crevices of nodules differ from those in the surrounding soft sediments, and some species are even exclusive to nodule crevices (Bussau 1993; Thiel et al. 1993; Bussau et al. 1995).

Macrobenthic communities within the CCFZ are mostly composed of polychaetes and crustaceans (mainly tanaidaceans and isopods), although mollusks, sipunculids, echiurans, and echinoderms are also encountered (Hessler and Jumars 1974; De Smet et al. 2017; Wilson 2017; Pasotti et al. 2020). In this manner, nodule areas of the CCFZ are not different from abyssal sediments elsewhere—at least at higher taxon level. Polychaetes and crustaceans together can represent up to 79% of the absolute sampled organisms (Hessler and Jumars 1974; Wilson 2017). More than 500 macrofaunal morphospecies, including polychaetes, isopods, tanaidaceans, and ophiuroids have been documented in different studies of the CCFZ (Paterson et al. 1998; Glover et al. 2002; Wilson 2017; Bonifácio et al. 2019; Janssen et al. 2019, 2015; Błażewicz et al. 2019*b*; Christodoulou et al. 2020), but very few have been formally described (Riehl et al. 2014; Paterson et al. 2016; Bonifacio and Menot 2019; Jakiel et al. 2019; Wiklund et al. 2019; Chim and Tong 2020; Riehl and De Smet 2020).

The structure and composition of polychaete and tanaidacean assemblages reported for the eastern CCFZ is mostly controlled by organic carbon fluxes to the seafloor (Bonifácio et al. 2019; Błażewicz et al. 2019*b*). Investigating benthic communities of both western and eastern CCFZ, Wilson (2017) found contrasting productivity-diversity relationships for polychaetes, tanaidaceans, and isopods. While both polychaetes and tanaidaceans showed highest diversity at the highest productivity site, this trend was reversed for Isopoda (Wilson 2017). At the local scale, nodule density also influences the composition of polychaete assemblages (Bonifácio et al. 2019) and was reported to have either a positive (Yu et al. 2018) or no (Pasotti et al. 2020) influence on macrofaunal densities.

Size limits for meio- and macrofauna

The meio- and macrofauna are two groups that differ in terms of size structure and taxonomy (Warwick et al. 2006; Somerfield et al. 2018), although the separation between them may be blurred at the boundaries. Most taxonomic groups can contribute to both size fractions. The meiofauna size class may include larvae and juveniles of macrobenthic organisms also called "temporary meiofauna" (Giere 2009). Similarly, the macrofauna size class may contain large nematode and copepod species even though these are typically meiofauna taxa (Gunton et al. 2017). Nevertheless, both groups are thought to perform different functions within the benthic ecosystem (Somerfield et al. 2018). For example, studies suggest that meio- and macrofauna-sized nematode individuals represent two distinct assemblages. While the former is considered to exhibit a mostly interstitial lifestyle, large nematodes, generally possess a burrowing lifestyle (Sharma et al. 2011; Sharma and Bluhm 2011).

The choice of size limits for meio- and macrofauna studies and their consequences are continuously under discussion (Warwick 2014). The lower size limit of the meiofauna tended to decrease over the years, i.e., 74 μm (Wigley and McIntyre 1964), 62/65 μm (Thiel 1966; Snider et al. 1984), 50 μm (Dinet 1973), 40/42 μm (Thiel 1971; Renaud-Mornant and Gourbault 1990; Miljutina et al. 2010). Today, a lower size limit of 32 μm, separating meiofauna from the so-called nanofauna (mainly consisting of flagellates, ciliates, and yeasts), is commonly accepted for deep-sea meiofauna (Zeppilli et al. 2015). While 32 μm is now the conventional lower limit for the meiofauna, there is a discussion regarding its upper limit. Mare (1942) suggested 2 mm as upper limit for meiofauna organisms but since then, 1 mm has been commonly used (Giere 2009). This upper size limit of the meiofauna does not match with the lower size limit of the macrofauna. In the deep sea, the lower size limit for macrofauna is normally set to 250 or 300 μm (e.g., Hessler and Jumars 1974; Dinet et al. 1985; De Smet et al. 2017; Montagna et al. 2017; Yu et al. 2018). This overlap in size limits is due to an overlap in the size distribution of the two groups where the adults of the larger meiofaunal species are in the macrofaunal size-range while juveniles of macrofauna are in the meiofaunal size range. In order to be inclusive of the potential size range of meiofaunal taxa, we recommend here to not use an upper sieve when processing meiofauna samples. This would be consistent with the method used for collecting macrofauna, which has no upper sieve size limits.

The lower sieve size for the macrofauna has also been a matter of debate among the pioneers of quantitative studies of the deep-sea macrofauna. Hessler and Jumars (1974) used a sieve with a 297 μm screen (1.75-phi units); Gage (1997) used a 420 μm screen and Dinet et al. (1985) used a 250 μm screen. In any case, with such a small sieve size compared to the screen mesh used to sample coastal macrofauna (i.e., > 1 mm), a large number of nematodes and copepods have also been retained in deep-sea macrofauna samples. Hessler and Jumars (1974) defined the macrofauna sensu *lato* as all metazoans retained on the 297 μm sieve, and the macrofauna

sensu stricto as all metazoans excluding the nematodes and copepods. For biological reasons, considering that nematode and copepod counts in macrofauna samples are underestimated, and for practical reasons, because sorting meiofaunal taxa in macrofauna samples can be quite time consuming, most studies on the deep-sea macrofauna to date focused on macrofauna sensu stricto. In its very first recommendations for the assessment of the possible environmental impacts arising from exploration for polymetallic nodules, the ISA recommended the use of a 250 μm mesh screen to sample the macrofauna (ISBA 2002). However, meanwhile, the scientific community has reached a consensus of using a ca 300 μm mesh size. This "300 μm" mesh size has been used in most sampling programmes conducted by contractors, including UK Seabed Resources (UKSR), Bundesanstalt für Geowissenschaften und Rohstoffe (BGR), Global Sea Mineral Resources (GSR), L'Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer), as well as CCFZ expeditions in the framework of the JPI Oceans pilot action "Ecological aspects of deep-sea mining" (De Smet et al. 2017; Pasotti et al. 2020). Thus, for comparability between studies we recommend the use of a 300 μm sieve for macrofauna. In a nutshell, (Fig. 1), meiofauna is defined here as all metazoans retained on a sieve with a mesh size of 32 μm; macrofauna sensu *lato* is defined as all metazoans retained on a 300 μm sieve, while macrofauna sensu stricto excludes meiofaunal taxa.

Sampling gears

Besides the size limits, whenever considering differences in sampling meio- and macrofauna, one should also take into consideration the sampled surface area (sampler) used for each group (Fig. 1). For deep-sea quantitative assessments of meio- and macrofaunal organisms, the most frequently used sampling gears are the multi- and boxcorers, respectively (Montagna et al. 2017). A multicorer (MUC) is designed to collect small sediment samples (surface area sampled by one core is typically around 78.5 cm^2) and it is generally used for the investigation of meiofauna. Sampling a larger amount of sediment, the boxcorer (Hessler and Jumars 1974) is a quantitative sampler (typically 0.25 m^2) which is generally used to sample macrofauna. The epibenthic sledge (EBS) is a qualitative sampler that is more accurately sampling the epifauna and yields larger sample sizes, which is useful for taxonomic inventories (Brandt and Schnack 1999; Brenke 2005; Kaiser and Brenke 2016). According to Montagna et al. (2017) MUC samples provide better estimations of abundance and taxon richness for both meio- and macrofauna. Meiofauna being sampled from subcores, the authors found little difference between MUCs and boxcorers for meiofauna metrics, MUCs providing the advantage of preserving vertical distribution patterns. However, boxcorers were found to capture more macrofauna diversity, as they sample larger surfaces. The major advantage of a MUC over a boxcorer is its hydraulic dumping system that allows the core to gently penetrate the sediment thus limiting the bow wave effect that is flushing the sediment surface and its fauna (Jóźwiak et al. 2020). The deployment of the boxcorer at low speed when it approaches the seafloor, as described by Hessler and Jumars (1974), may however limit this bow wave effect. Moreover, in abyssal systems such as those where polymetallic nodules lie, sedimentation rates and thus faunal standing stocks are on the lowest oceanic range. For this reason, the area of a boxcore (0.25 m^2) is the minimal size required to accurately sample the sparse abyssal macrofauna. In addition to MUC sampling, pushcores operated by a remotely operated vehicle (ROV) can be used to perform targeted sampling of meiofauna.

The EBS (Brenke 2005) has also been widely used for macrofaunal sampling in the CCFZ. Although the EBS is more suitable for sampling mobile epi- and suprabenthic fauna, suspended infauna organisms are also collected. The device is mostly used as a qualitative sampler due to its limitations in assessing richness and abundance (Lins and Brandt 2020). When sampling with the EBS, however, considerably more specimens are obtained for genetic analysis than with the boxcorer. In low density environments, such as the CCFZ, the likelihood of sampling specimens of the same species is low. Therefore, the use of different types of gears, such as EBS and boxcorer, can complement each other in the huge task of understanding diversity patterns.

In the context of polymetallic nodule exploration and future exploitation, the presence of nodules is an important factor to keep in mind during sampling operations. The nodules can hamper the opening of some gears and affect the volume of the sediment that can be sampled in each boxcorer and MUC, and it is fundamental to record both the nodule number and volume for later comparison between samples. For MUC vertical slicing, the presence of nodules can hamper the slicing process and consequently the accuracy of fauna density estimation, and result in a lower sediment volume available for the analysis of environmental variables.

Sampling design and replication

Given the remoteness of the CCFZ (closest distance to the nearest mainland harbor is about 1500 km) and its great water depth (between 4 and 5 km), benthic sampling is very costly which is the reason mostly expeditions have concentrated efforts in eastern area. It is evident that both industrial as well as academic stakeholders are often confronted with constraints on sampling design and number of replicate samples available to characterize the benthic fauna of an area (Table 1). Furthermore, limited ship time is not only a result of the high costs. Interdisciplinary research is essential to perform comprehensive deep-sea biodiversity surveys. Indeed, in addition to the characterization of benthic biota, knowledge of local water column features and biogeochemical characteristics are also crucial to allow inferring robust ecological

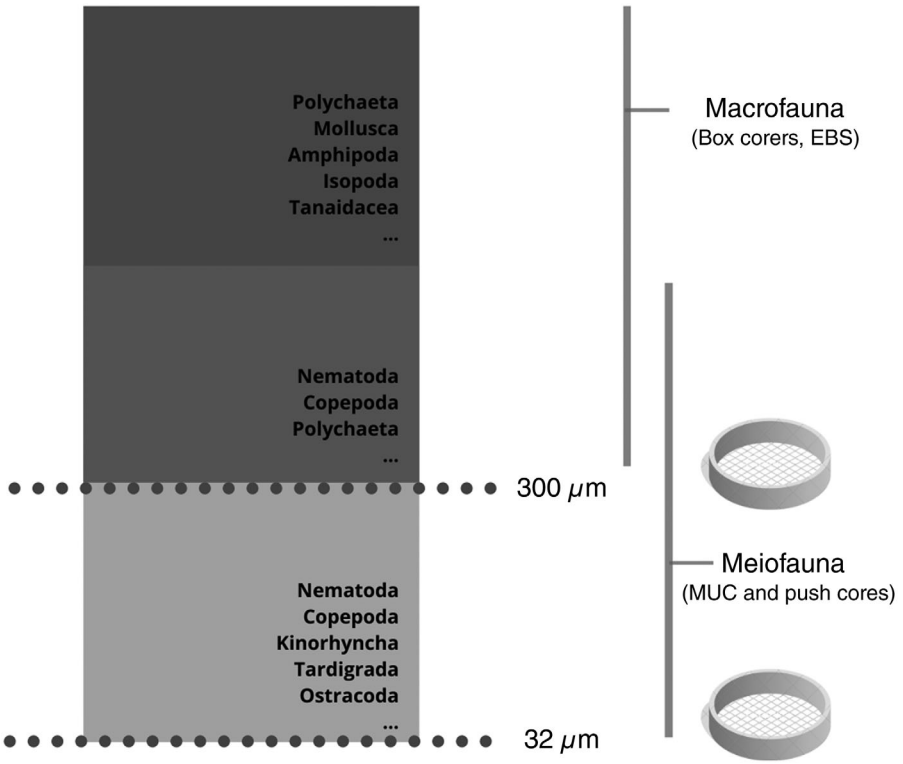

Fig 1. Size limit representations for both meio- and macrofauna based on sieve mesh size and sampling gears. Dark gray colors represent taxa only belonging to the macrofauna, middle gray shows the overlap between meio- and macrofauna groups and light gray exhibit the taxa only belonging to the meiofauna. EBS = epibenthic sledge; MUC = multicorer.

patterns. Consequently, in the CCFZ, an interdisciplinary cruise of 60 d, which is in general the maximum capacity for a vessel to stay continuously at sea, will spend on average 10 d for transit from and to the mainland, which leaves restricted time for coring (4 h each for the deployment of MUC and boxcorer), trawling (> 6–7.5 h per trawl of a dredge and EBS), water column sampling and profiling (2 h per profile of a Niskin carrousel with CTD), lander deployment (2 h for deployment and recovery of time lapse cameras, baited traps, biogeochemical sediment profilers), and plankton sampling (3 h for one haul of a single plankton net) (Table 1). A conservative estimate of a full series of single deployments of the above tools requires 40 h in perfect weather conditions, without gear failures and excluding transit time between stations. A more realistic estimate therefore is two full days (48 h), incorporating the likely extra time. In other words, a 60-day cruise in the CCFZ allows for only 25 deployments of each of the different gear types.

Table 1. Average deployment speed and time per deployment for the most commonly used gears in the CCFZ area. N/A = not applicable.

Gear	Mean deployment speed	Average time per deployment/haul
Multicorer (MUC)	~1 m s^{-1}	4 h
Boxcorer (BC)	~1 m s^{-1}	4 h
Epibenthic sledge (EBS)	~0.5 m s^{-1}	6–7.5 h
Lander	~1 m s^{-1}	2 h
Water column sampling and profiling	N/A	2 h
Plankton net	N/A	3 h

Therefore, the dilemma with which each of these cruises are confronted with is the trade-off between the number of replicate deployments and the number of stations (sites, habitats) to sample. More stations means a larger coverage of different locations (or habitats) that can be characterized for their biota and environment, but also fewer deployments per station and therefore less replicates, and thus lower statistical power (Kain et al. 2015). Even though using individual cores from the same MUC deployment as replicates (sometimes called parallel samples) can sometimes show a higher spatial variance in comparison with cores from independent deployments (Montagna et al. 2017), this strategy is statistically not ideal, since these cores are not collected independently. They are considered pseudo-replicates and may therefore lead to false conclusions of densities and biodiversity (Colegrave and

Ruxton 2018). If pseudo-replicates have to be used, this information should be clearly stated in the methods, and results should be interpreted with caution.

The low densities of benthic biota (meiofauna about 50–500 ind/10 cm^2 and macrofauna about 100–200 ind m^{-2}) in the CCFZ (Hessler and Jumars 1974; Yu et al. 2018; Uhlenkott et al. 2020), in combination with high species richness, results in the requirement for large sample sizes, usually not allowing to subsample boxcores or cores from a MUC. This stresses the need to increase replication in order to collect sufficient specimens to fully characterize the biodiversity of the present fauna (De Smet et al. 2017; Pape et al. 2017). A third factor to incorporate in an appropriate sampling design is the degree of seafloor patchiness and thus the scale at which (micro-) habitats and associated fauna vary (Vanreusel et al. 2016). In case of the CCFZ, both the seabed coverage of nodules, as well as the topography through the presence of ridges and troughs, may vary at scales of tens to hundreds of meters (Simon-Lledó et al. 2019). A profound mapping of the seafloor in terms of substrate, bathymetry and slope is therefore a prerequisite to select the location and size of specific, potentially representative sampling stations. This is especially important for spatial investigations of fauna based on distribution modeling, which has been applied for meio- and macrofauna on different scales in the CCFZ (Kuhn et al. 2020; Uhlenkott et al. 2020, 2021).

Rarefaction curves for meio- and macrofauna from previous studies have shown how a limited number of replicates significantly underestimate biodiversity present in an area, while the estimated number of replicates required for a reliable biodiversity assessment remains a challenge (De Smet et al. 2017; Pape et al. 2017; Wilson 2017; Smith et al. 2020). Low organismal densities complicate the precise and accurate sampling of the abyssal benthos (Hessler and Jumars 1974; Andrew and Mapstone 1988), especially when it comes to assessing the impact of human activities, such as nodule mining (Jumars 1981). Indeed, environmental impact assessments (EIA) need quantitative metrics and statistics to either accept or reject the null hypothesis that there is no impact (Niner et al. 2018).

The classical way of assessing an impact is to perform an ANOVA on data obtained after to a Before-After/Control-Impact (BACI) sampling design (Underwood and Chapman 2003). In order to illustrate the influence of low densities in the framework of an EIA, we computed the statistical power of a BACI ANOVA as a function of a range of mean abundance per sample (Fig. S1). In the context of an EIA, the statistical power provides a measure of the ability of an analysis to correctly conclude that there is an impact (i.e., the a priori probability to correctly reject the null hypothesis when the null hypothesis is false). In our simulations, we considered three levels of impacts (90%, 50%, and 25% change in abundance after the disturbance) and five levels of replication (3, 5, 10, 20, and 50 replicate samples in both the control and impact areas). The desired power of an analysis is classically set at 80%. With such a threshold, the 3–5 replicate samples routinely collected in the CCFZ can confidently detect only a 90% change in abundance of the most abundant taxa, while detecting a 25% change would require over 50 replicate samples (Fig. S1). A similar power analysis computed on the average number of species or genera per core suggested that taxonomic richness could be more powerful than abundance to detect an impact, yet still requires significantly higher replication than is usually performed (Fig. 2). For example, at the Ifremer license area, considering the average number of nematode species and standard deviation (57.7 ± 13.7 species per core; Miljutina et al. 2010), 10 replicate samples would detect a 50% change in species richness due to mining. At Domes A (north Equatorial Pacific), where the average number of polychaete species per box-core is lower (11.8 ± 4.8 species per box core, Wilson (2017)), at least 20 replicate samples would be needed to confidently detect an impact of 50% change in species richness.

These examples illustrate the need to carefully choose the biological metrics that will provide accurate and precise indicators for the monitoring of mining impacts. The effort invested in environmental baseline studies and pilot-scale mining operations should thus be scaled to provide the most reliable and cost-effective sampling design and indicators.

Core processing on board

Once on board, the boxcorer and MUC cores have to be inspected, photographed, and described before sediment slicing and fixation (Fig. 3). The cores need to be checked for nodule presence and coverage, potential disturbances of the sediment–water interface (e.g., clear or turbid water), appearance of the sediment (e.g., color, texture, firmness, redox layer depth) if the sediment surface is horizontal or skewed (this can complicate slicing afterwards), and presence of sessile fauna (photos taken individually) (Fig. 3). Nodules, sediment samples, and fauna have to be temporarily stored in filtered cold (2–4°C) seawater to avoid DNA degradation, as the temperature differences between bottom (~ 2°C) and surface water (~ 28°C) in the CCFZ are large.

Reporting the size, weight, and volume of individual nodules is important to estimate the volume of sediment and consequently the density of taxa obtained. Moreover, organisms attached to the nodules should be counted (only heads) and stored appropriately (a snippet sample for DNA is transferred to a 2 mL tube with cold 96% ethanol and the individual is fixed in separate tube). In meiofauna studies, nodules are crushed and centrifuged (for instance, by using Ludox) to extract attached or crevice meiofauna.

For preserving individuals for proteomic fingerprinting, tests on shallow-water harpacticoid copepods showed that either 98% denatured (with 1% Butanone and/or MEK) or 96% denatured ethanol are appropriate for fixation of sediment samples. Mass spectra of tested specimens did not show any impact of different ethanol concentrations on spectra

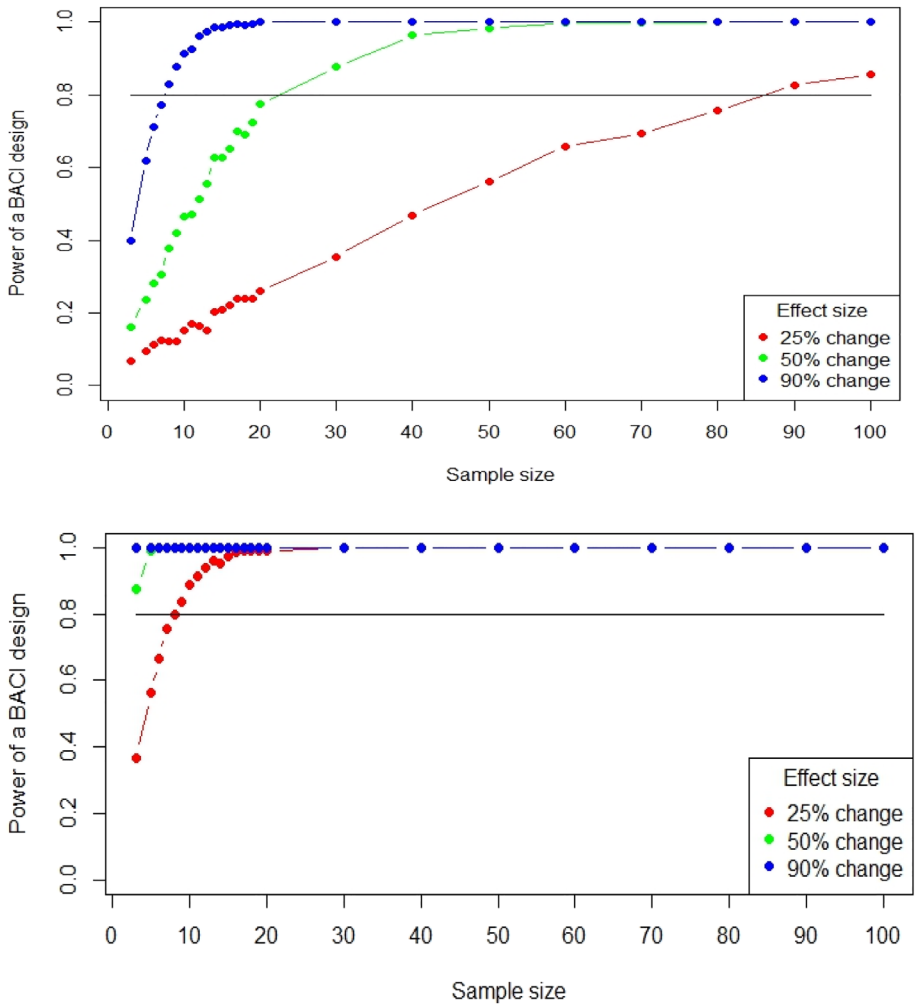

Fig 2. Top—Statistical power of a BACI ANOVA to detect a change in the mean number of macrofauna species, assuming that number of species has a normal distribution of mean = 11.8 and SD = 4.8 as at Domes A, north Equatorial Pacific (Wilson 2017). Bottom—Statistical power of a BACI ANOVA to detect a change in the mean number of nematode genera, assuming that the number of genera has a normal distribution of mean = 47.2 and SD = 5.8 as at the BGR, the German exploration area in the CCFZ (Hauquier et al. 2018).

compositions (Sven Rossel, personal observation). To avoid a negative impact of storage, sediment samples should be fixed in high concentration (> 96%) ethanol and stored at −25°C (Rossel and Arbizu 2018) and sorted on ice. Until further processing, samples need to be stored in small amounts (~ 2 mL) of ethanol in microcentrifuge tubes.

Sediment slicing

Before slicing, the overlying water is siphoned off through a sieve to sample the re-suspended fauna, and this water residue is fixed and follow up the pick up the nodules if present. More than 85% of macro- and meiofauna abundance is found in the first 5 cm of sediment and these organisms are assumed to feed on the labile organic content located at the sediment–water interface (Spiess et al. 1987; Smith and Demopoulos 2003). The superficial sediments in the CCFZ can be distinguished from deeper sediments by its difference in compactness, in 3, 5, or 10 cm thick with 95% of the fauna found in the top 0–3 cm (Cosson et al. 1997). The ISA recommends contractors to sample vertically down to 10 cm (ISBA 2020). In order to ensure comparability across CCFZ studies and historical data, the boxcorer is subdivided into 0–3, 3–5, 5–10 cm horizons (Table 2), despite the ISA recommendations of boxcorer slicing into layers of 0–2, 2–5, and 5–10 cm. MUC cores are sliced every 1 cm down to 5 cm when no nodules are present (Martínez Arbizu and Haeckel 2015). If nodules are present while slicing a core, the bulk sample of the top 5 cm is used and the sediment around the nodule should be sampled in detail (Table 2; Fig. 3). After that, the nodules are rinsed over a 32 μm sieve to separate the sediment from the nodule and include it in the sediment sample.

The meiofauna samples collected with the MUC are generally only sieved and sorted upon arrival at respective research institutions. To extract the meiofauna, the sediment is usually

Fig 3. Snapshots from a multicorer (MUC) sampling and nodule inspection. (**a**) MUC arrives back on board showing different sediment heights; (**b**) core superficial layer skewed due to the presence of a nodule; (**c**) core slicing hampered by the presence of a nodule found in the middle of a MUC core; (**d**) undisturbed sediment surface exhibiting a small nodule. Photos taken by Tania Nara Bezerra during the 2017 environmental baseline campaign in the GSR license area.

centrifuged according to the differential flotation method (Heip et al. 1985), which can be unreliable when executed on a moving vessel. For macrofauna, the sieving of the first boxcorer layer (0–3 cm) is performed on a 300 μm sieve in a cold room to avoid DNA degradation if genetic analyses are intended and the organisms are best live-sorted immediately (Glover et al. 2016). The other layers are fixed in 96% denaturated ethanol (EtOH and stored at −20°C before processing. For the macrofauna live-sorting on board, samples should be kept cold over an "ice bed" and all metazoans assigned to easily identifiable taxonomic groups under the stereomicroscope. After sorting, the individuals are stored at −20°C in ethanol which is exchanged after 24–48 h to remove remaining water and for better DNA preservation. Polychaetes are generally stored in cold 80% EtOH, and nematodes in DESS, and both are stored at 4°C (Yoder et al. 2006). The use of DESS has been successful in preserving not only the DNA, but also to maintain the shape of nematodes for further morphological taxonomical analyses. Nevertheless, its efficacy in preserving DNA tissue on a long-term basis is still questioned, and therefore it is advisable to process these samples as soon as possible. The sieving of the 3–5 and 5–10 cm box corer layers can be done with (filtered) seawater at room temperature and fixation is usually in 4% buffered formalin if morphological identification is a priority or in cold 96% ethanol if both morphological and genetic identifications are aimed for.

Morphological and genetic identification

Species are the basic units in biology, biodiversity, and ecology (Claridge et al. 1997). Species identifications are thus required to assess, monitor, and preserve biodiversity. Even though, as mentioned previously, deep-sea benthic standing stocks are low, sediments including those in the nodule-bearing abyss harbor high meio- and macrofaunal biodiversity (Glover et al. 2002; Lambshead and Boucher 2003; Smith et al. 2008; Pavithran et al. 2009; Ramirez-Llodra et al. 2010). Currently, most ecological studies conducted in abyssal nodule areas only provide genus or family level resolution (e.g., De Smet et al. 2017; Hauquier et al. 2019) or even higher taxonomic levels (Ingole et al. 2001). Identification of deep-sea benthic species is difficult and time-consuming because of the required taxonomic expertise, which is generally scant for the deep sea, and because most deep-sea species are still undescribed (e.g., Glover et al. 2002; Christodoulou et al. 2019). This leads to papers reporting either morphospecies (e.g., Species A, B, etc.) or "operational taxonomic units (OTUs)" in the case of genetic identification, which cannot be compared with other studies except if they have the same taxonomist, thereby preventing the assessment on a regional scale using data from different studies. Furthermore, several studies have already documented the existence of cryptic (i.e., genetically distinct but morphologically identical taxa) macro- and meiofaunal species in the CCFZ (Smith et al. 2008; Janssen et al. 2015), leading to incorrect identifications and an underestimation of diversity. Sharing pictorial identification keys among scientists and contractors would certainly aid the issue of the current lack of taxonomical standardization in the CCFZ (Glover et al. 2016).

Another way to circumvent identification issues and allow taxonomic standardization is to combine morphological identification with DNA barcoding of individual specimens as an integral part of taxonomy for the discovery and cataloguing of biodiversity (Janssen et al. 2015; Glover et al. 2016). Molecular identification of species relies on marker genes (barcodes) that can be used to identify thresholds of intra- vs. interspecific divergence (Hebert et al. 2003). Barcode genes show greater interspecific genetic variation than intraspecific variation (Hebert et al. 2003), and therefore should display a gap

Table 2. Corer sieving and fauna sorting overview. EtOH = ethanol; DESS = a solution containing dimethyl sulphoxide, disodium EDTA, and saturated NaCl.

Sampling device	Horizon	Purpose	Group	Taxon	Sieve size	Further treatment	Intended analysis	Fixative	Storage
Multicorer (MUC)	0–5 cm or 0–1, 1–2, 2–3, 3–4, 4–5 cm	Quantitative	Meiofauna	All	32 μm	Differential	Morphology	Formaldehyde (4%)	RT
				Nematoda		Flotation	Morphology/genetics	DESS (20%)	4°C
				All		Centrifugation	Morphology/genetics/proteomics	EtOH (96%)	−20°C
Boxcorer (BC)	0–3, 3–5, 5–10 cm	Quantitative	Macrofauna	All	300 μm	Live sorting in cold seawater	Morphology/genetics/proteomics	EtOH (96%)	−20°C
	0–3 cm	Quantitative		Nematoda	300 μm		Morphology/genetics	DESS (20%)	4°C
Epibenthic sledge (EBS)	Supranet	Qualitative	Epi-macrofauna	All	300 μm		Morphology/genetics/proteomics	EtOH (96%)	−20°C
Epibenthic sledge (EBS)	Epinet	Qualitative	In-macrofauna	All	300 μm		Morphology/genetics/proteomics	EtOH (96%)	−20°C

between the distributions of intra- and interspecific sequence variation (Meyer and Paulay 2005). Although a 3–4% threshold is generally accepted as sufficient gene disparity to characterize different species, there is no uniform divergence threshold for species delineation across phyla (Meyer and Paulay 2005). There is no uniform divergence threshold for species delineation across phyla (Meyer and Paulay 2005). Indeed, even within a single animal order there can be large differences in this threshold value between families and genera (Tempestini et al. 2018). In addition, amplification success of numerous barcode genes heavily varies among taxonomic groups. Both these caveats explain the lack of truly universal barcode genes. Hence, a combination of traditional morphological species identification with DNA-barcoding remains highly important (Janssen et al. 2015).

This integrative taxonomy approach (i.e., the combination of morphological and DNA taxonomy) requires appropriate sampling and processing techniques. Glover et al. (2016) presented an "end-to-end" methodology for the taxonomical study of macro- and megafauna in the CCFZ. It included protocols for laboratory imaging and sampling, DNA extraction and sequencing, DNA analysis, and data and sample management pipelines. General recommendations for contractors are given by ISA for macrofauna (ISA 2015) and for nematodes, the dominant meiofaunal phylum (ISA, 2013).

Ideally, the morphological (high-resolution photographs and measurements) and genetic data obtained for all sampled taxa should be stored in a reference database. When publishing genetic data in peer-reviewed journals, sequences should be submitted to any of the three International Nucleotide Sequence Databases (GenBank, ENA, or DDBJ) and/or to BOLD (http://www.boldsystems.org/). When facing low barcode amplification or sequencing success, a potential strategy could be to focus on a group that can be sequenced rather successfully (e.g., the genus *Halalaimus* for the Nematoda; *Paranarthrurella* and *Armatognathia* for the Tanaidacea) (Pape et al. 2017; Błażewicz et al. 2019*a*).

The crucial point during PCR amplification is the attachment of the primer to the template DNA, so trying to find a conservative nucleotide sequence among a closely related group of specimens (e.g., families or genera) should facilitate the PCR, but at the same time this can decrease the accuracy of the data.

The mitochondrial COI (cytochrome c oxidase subunit I) offers the best species-level resolution in most taxa except for ctenophores, sponges, nematodes, and some benthic cnidarians (corals and anemones), for which COI is either difficult to amplify or not resolutive enough (Blaxter et al. 2005; Bucklin et al. 2016). However, PCR amplification success of COI in, for example, nematodes, polychaetes, and isopods is rather low and taxon-specific. Thus, besides COI, other barcode genes are typically used for deep-sea meio- and macrofauna, as well as for microorganisms, such as 28S, 18S, and 16S (Riehl et al. 2014; Mercado-Salas et al. 2019; Khodami et al. 2020).

To conclude, we suggest that focusing on key groups, which are scientifically interesting in an ecological, behavioral, or evolutionary perspective for instance, and for which DNA amplification has been successful, can be a good start to increase comparability in a greater scale across locations within the CCFZ, and to consequently assess biodiversity and future environmental impacts.

Genomic fingerprinting by bulk or environmental DNA metabarcoding

High-throughput sequencing (HTS) technologies now allow producing millions of DNA sequences from individual samples. HTS can be applied to barcode gene analysis, a process called metabarcoding, allowing the description of biodiversity in samples that are complex community mixtures.

Metabarcoding can be performed from bulk DNA extracted from a collection of organisms (e.g., flotation-extracted meiofauna), approach termed (bulk) DNA metabarcoding, or from environmental samples where DNA is extracted directly from air, water, or sediment samples, termed environmental DNA (eDNA) metabarcoding. Metabarcoding is especially useful in remote and hard-to-access areas, or when targeting organisms of small size and exhibiting cryptic diversity (Goodwin et al. 2017). Studies mainly differ in (1) the type of barcode gene used, (2) the precision of the taxonomic identification they allow, considering their marker gene and the reference databases available, and (3) the level of degradation of the DNA extract, determining the length of the barcode region that can be used (Taberlet et al. 2012). Given that the majority of reference sequences deposited in public databases such as GenBank and Silva (https://www.arb-silva.de/) for meiofauna taxa are ribosomal (V1–V2 region of the 18S small ribosomal subunit), one would preferentially target this locus in order to capitalize on the collective output of past barcoding work. However, because ribosomal RNA (rRNA) genes are under less selective pressure than mitochondrial genes, they provide lower taxonomic resolution and therefore rarely allow accurate taxonomic identification beyond genus level. It is thus advisable to adopt a multigene approach in metabarcoding studies, combining ribosomal loci for increased taxonomic coverage, with COI, for species-level assignments.

Sediment sampling and core slicing should be performed as detailed previously; however, additional precaution should be taken in order to avoid contamination issues. Chirurgical masks should be used during slicing, and lab coat/cold uniform, and two pairs of gloves should be worn, allowing to easily change gloves between samples or when dirty. In addition, all tools used for slicing and/or subsampling should be decontaminated between samples (e.g., rinse off tools with water, decontaminate using 1–3% bleach or equivalent commercial solution, rinse with ultrapure water, and leave in a bath of ultrapure water for additional rinsing). As this decontamination procedure is lengthy, it is recommended to have all tools in double so that one set can be used while the other one is decontaminated.

The choice of substrate for DNA extraction is important for metabarcoding studies as this will significantly affect retrieved taxonomic compositions (Deiner et al. 2015; Koziol et al. 2019). Most importantly, aboveground water, even if sampled in large volumes, is not suited for the detection of benthic biodiversity (Antich et al. 2020; Brandt et al. 2021*a*). In addition, choosing between bulk DNA or eDNA primarily depends on the target taxa and has to be carefully planned prior sampling. DNA extraction from bulk samples collected by, e.g., sieving or elutriation allows the recovery of more metazoan diversity (Brannock and Halanych 2015), especially if DNA is extracted from size-sorted samples (Aylagas et al. 2016; Elbrecht et al. 2017). However, direct DNA extraction from large volumes of sediments (≥ 10 g) has been found to be comprehensive for eukaryotes, and more appropriate when microbial compartments are also of interest (Dopheide et al. 2019; Brandt et al. 2021*a*). For sediment eDNA metabarcoding, implementing sample homogenization and extraction replicates of small sediment volumes has been suggested as an efficient way to account for patchy species distributions and as a cost-effective alternative to increased extraction volumes (Lanzén et al. 2017). Overall, DNA extraction methods should be consistent among samples to allow comparability, and at least three PCR reactions (technical replicates) should be performed per sample to smooth the intra-sample variance and reduce PCR biases (Ficetola et al. 2015; Alberdi et al. 2018; Dickie et al. 2018; Dopheide et al. 2019; Macheriotou et al. 2020).

DNA amplicon libraries can be prepared in numerous ways, all generally involving the addition of platform-specific adapters and sample-specific indexes, DNA purification, and pooling of libraries at equal concentration for sequencing. Following HTS, typically conducted on Illumina platforms, the user is confronted with tens to hundreds of millions of raw sequences that need to be bioinformatically processed to produce a list of putative taxa. The bioinformatic analysis of metabarcoding data has evolved a great deal in recent years with a plethora of algorithms having been developed for each processing step. Bik et al. (2012) provide an overview of bioinformatic processing steps and the tools and pipelines available, but many others were developed in the last years such as USEARCH (Edgar 2010), VSEARCH (Rognes et al. 2016), OBITOOLS (Boyer et al. 2016), DADA2 (Callahan et al. 2016), FROGS (Escudié et al. 2018), or the web-application SLIM (Dufresne et al. 2019). First, bioinformatic processing usually includes various quality-filtering steps, where primers, sample tags, and sequencing adaptors are removed from raw sequences. These are then trimmed to remove low-quality ends and quality-filtered (based on nucleotide quality-scores or error rates). Next, a key choice one has to make is to define the molecular entity that will serve as a proxy for species in the dataset; this can result from either

clustering or denoising sequences, resulting in OTUs or amplicon sequence variants (ASVs), respectively (Macheriotou et al. 2020, 2018). These two options represent distinct strategies for countering errors generated during sequencing; the former does so by grouping (clustering) processed sequences within a user-defined similarity threshold, while the latter applies a data-based and quality-aware correcting algorithm to the sequences. Correction algorithms are increasingly popular as they effectively remove sequencing errors and increase reproducibility, conceptual simplicity, and streamlined processing (Callahan et al. 2017). However, recent literature suggests that both molecular entities are complementary rather than mutually exclusive, ASVs allowing describing diversity at the intraspecies level, OTUs at the interspecies level (Turon et al. 2020; Brandt et al. 2021*b*). Spurious molecular units are a serious issue in metabarcoding, and even after stringent quality-filtering steps (incl. chimera removal, and tag-switching filters), many OTU/ASV table entries are singletons (i.e., have total abundance of 1), or comprise clusters with low sequence ("read") counts. Small counts are more likely to be spurious, especially singletons, either because the OTU/ASV itself is spurious (e.g., an undetected chimera, a pseudogene), or because of tag switching. It is thus current practice to remove singletons and filter molecular clusters based on their relative abundance per sample or in the total dataset (Wangensteen and Turon 2016). These minimal abundance filters have to be chosen with caution as they significantly affect qualitative detection measures. To avoid arbitrary filtering based on relative abundance, Froslev et al. (2017) have developed an alternative curation algorithm, LULU, which filters OTUs/ASVs based on their identity and co-occurrence rates to more abundant OTUs/ASVs.

Although ecological patterns can be investigated without taxonomic identities, species names are useful for inferring biological traits or ecosystem function, as behind each name, there is a phenotype, an ecological role, and a geographic distribution. Therefore, once ASVs/OTUs have been generated, one can identify their Linnaean identity through taxonomic assignment by which each ASV/OTU sequence is compared to a reference sequence of known taxa. The comparison can be tree-, alignment-, phylogeny-, or probability-based, the choice of which can affect the resulting taxonomy (Holovachov et al. 2017). In this respect, the Ribosomal Database Project's (RDP) naïve Bayesian classifier has been the suggested methodology for 16S ribosomal OTUs (Navas-Molina et al. 2013) providing up to 89% genus level accuracy (Wang et al. 2007). However, a recent study comparing taxonomy prediction algorithms on 16S rRNA and ITS sequences found that alignment-based methods (e.g., BLAST) allowed similar accuracy than probabilistic methods, although the latter have the advantage of providing a confidence level for each taxonomic rank (Edgar 2018). The limitations in taxonomic assignment quality are therefore mostly due to the limited amount of data available rather than algorithms, and this is especially true for understudied ecosystems such as the deep-sea. Arbitrarily large and uneven databases containing sequences that have not been truncated to the target sequence length, can decrease the number of accurate taxonomic assignments (Werner et al. 2012; Ritari et al. 2015; Edgar 2018; Macheriotou et al. 2018), and recent work shows that taxonomic assignments based on public databases remain poor for deep-sea metabarcoding data generated with "universal" primers (Brandt et al. 2021*b*). For this reason, the development of smaller, taxon-specific and ecosystem-specific reference databases will be necessary to obtain reliable taxonomic information from metabarcoding studies performed in poorly characterized areas such as the CCFZ.

Proteomic fingerprinting by MALDI-TOF MS

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an emerging species identification tool for biodiversity research from the field of microbiology. The method is widely used for the identification of bacteria, fungi, and viruses (La Scola et al. 2010; Chalupová et al. 2014; Singhal et al. 2015). However, numerous pilot studies have shown its applicability for differentiation of metazoans ranging from meiofauna and planktonic species (Bode et al. 2017; Rossel and Arbizu 2018) to insects (Maasz et al. 2017) and fish (Mazzeo and Siciliano 2016). Identification relies on differentiation based on a so-called species-specific proteomic fingerprint (Fig. 4). Therefore, sizes of the proteins expressed in an organism are measured with mass spectrometry and resulting peak patterns are used to differentiate animals at species level. However, not only presence and absence of protein mass peaks are used for identification, but also the intensities (abundance of a protein in the sample) of the respective peaks play a role. Based on mass peak abundances and intensities, some studies on metazoan identification were able to show differentiation of cryptic species (Bode et al. 2017), ontogenetic stages (Laakmann et al. 2013), and even different sexes (Maasz et al. 2017). The advantages of this method in comparison to DNA barcoding of the mitochondrial CO1-gene lie in the lower costs per specimen and the shorter time needed for preparation, measurement and analysis of the data (Rossel et al. 2019).

In order to extract and embed peptides and proteins from microscopic animals for measurement, α-cyano-4-hydroxycinnamic acid (HCCA) as a saturated solution in 50% acetonitrile, 47.5% LC–MS grade water, and 2.5% trifluoroacetic acid is used. Depending on size, 2–5 μL of HCCA are applied to a specimen in a microcentrifuge tube after complete evaporation of ethanol (Volta et al. 2012; Bode et al. 2017; Rossel and Martínez Arbizu 2018). After 5 min of incubation, the protein solution is transferred for measurement to a target plate. After co-crystallization of peptides, proteins and matrix solution the target plate can be measured. Usually, protein sizes for identification of

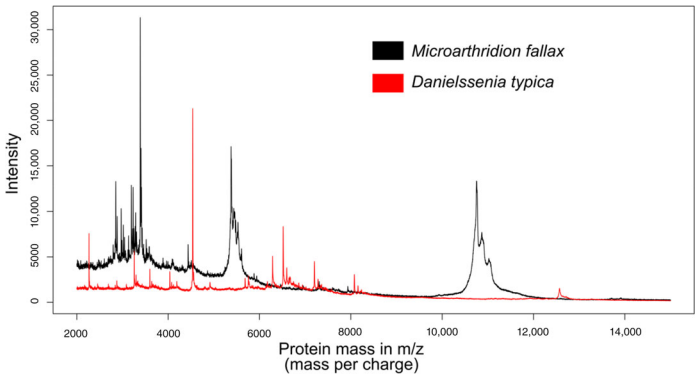

Fig 4. Mass spectra of *Microarthridion fallax* and *Danielssenia typica*, two harpacticoid copepod species from the meiofauna of the North Sea. Clear differences between the mass spectra of the two species can be distinguished.

metazoans are measured within a range from 2000 to 20,000 Da (Laakmann et al. 2013; Mazzeo and Siciliano 2016; Rossel and Arbizu 2018). To avoid misidentifications based on poor signal quality, a data processing pipeline to recognize mass spectra of minor quality should be applied (Palarea-Albaladejo et al. 2018).

Different data analysis strategies have been tested so far with good results for species identification. Often factory delivered software is used, returning identifications and identification probabilities based on provided or self-generated libraries (Yssouf et al. 2014). These are sometimes accompanied by cluster analyses (Dieme et al. 2014), principal component analysis (PCA) (Hynek et al. 2018), or solely based on cluster analyses (Bode et al. 2017; Kaiser et al. 2018). However, it is arguable if employing clustering or ordination methods alone makes it possible to recognize singletons or species boundaries in general (Collins and Cruickshank 2013; Rossel and Martínez Arbizu 2018). Hence, for library-based identifications apart from factory delivered software, it is advisable to use supervised methods such as random forest (RF) with a post hoc test for false positive identification recognition (Rossel and Martínez Arbizu 2018). The post hoc test also allows for working with incomplete libraries, as species new to the library are automatically recognized as such (Rossel and Martínez Arbizu 2018). Hence, libraries can be built up from only a few samples of a deployment and still be appropriate for species identifications (Rossel et al. 2019). Also, DNA sequences and MALDI-TOF libraries can be simultaneously generated by splitting specimens (if their size allows this) into multiple pieces using some for genomic and some for proteomic fingerprinting (Rossel and Arbizu 2018; Rossel et al. 2019; Rossel and Martínez Arbizu 2019). If no libraries for a region are available at all, automatic biodiversity estimation based on a clustering analysis and automatic cluster recognition can be performed (Rossel and Martínez Arbizu 2020) to allow biodiversity comparisons of different investigated areas. A combination of both approaches can be used for areas with an incomplete reference library. During the first step, specimens can be identified using RF, followed by an analysis of the remaining specimens using the clustering approach to generate species data across stations.

Until present, only few studies provided accessible data, since so far no data depository for mass spectra data (comparable to GenBank or BOLD for DNA sequences) is widely used. However, data can be stored either processed or unprocessed at the Dryad data depository (https://datadryad.org/). Because of the variability in measured protein sizes, data has to be processed conjoined to ensure protein sizes to be put into homologous bins for analysis. Hence, it is advisable to store unprocessed data alongside processed data matrices.

Ecological tracers: Stable isotopes/functional traits/biomass

Traditional measurements of diversity usually involve species identities and their abundances, and are on the core of ecological monitoring and impact assessments (e.g., Svensson et al. 2012). However, they only represent two of the multiple facets of biodiversity, and other measurements may provide complementary and critical information about different aspects of species, assemblages and ecosystems (e.g., McGill et al. 2006). Measurements such as species biomass, fatty acids, stable isotopes and functional traits are increasingly used in deep-sea ecology research, and their application in the assessment of ecological impacts of deep-sea mining may provide critical insights and help answering current and future key-questions (e.g., Quattrini et al. 2017; Ashford et al. 2018; Alfaro-Lucas et al. 2020). Moreover, these additional measurements can be combined with biodiversity data to widen the scope of biodiversity assessments.

Biomass

Biomass may inform about energy transfer between species and groups and may be used to "weight" functional and isotopic metrics. Also, changes in environmental variables can be mirrored by changes in biomass. It can provide insights about disturbances and changes in community structure (Leduc et al. 2014*a*; Lins et al. 2015; Román et al. 2019). Other characteristics may also be derived from biomass, such as respiration rate, which can give an indication of metabolic intensity in a community (Heip et al. 1985; Baguley et al. 2008), as well as community contribution to decomposition and mineralization of organic matter.

Nematode biomass is commonly calculated indirectly through the volumetric method according to Andrassy's formula:

$$G = a^2 \cdot \left(\frac{b}{1.6}\right) \cdot 106$$

with G = wet weight in μg, a = maximum body diameter (μm), and b = total length (μm) (Andrássy 1956). Biomass in

terms of μg C ind^{-1} can be calculated as 12.4% of G (Jensen, 1987). Biomass values can be used to estimate nematode respiration rates (Resp) according to de Bovée and Labat (1993):

$$\mathrm{Resp}\,(T) = 0.0449 \cdot \mathrm{DWT}.0.8544 \cdot e^{\left(\frac{\ln Q10}{10}(T-20)\right)}$$

where DWT is individual body mass (DWT), Q10 = 2 and T = Temperature at the seabed (°C): Resp (T) = 0.0449 DWT × 0.8544 exp (lnQ10/10) × (T − 20) (de Bovée and Labat 1993). This respiration formula is preferred instead of Shirayama (1992) respiration values measured for deep-sea nematodes at 2–4°C, because the latter suggests no temperature dependence of respiration rates in the deep sea, while de Bovée and Labat (1993) incorporated temperature in their formula. Direct in situ or ex situ respiration measurements on deep-sea meiofauna have not yet been successfully done, as the animals do not survive the pressure difference when recovered to the surface, and no device has yet been built for in situ measurements. Recent in situ metabolic rates measured for the megafauna (Holothuroidea) revealed no differences between shallow-water and bathyal individuals, but rates were considerably lower for abyssal organisms (Brown et al. 2018). These results can give an indication of potentially lower metabolic rates for abyssal meio- and macrofauna, but currently these measurements are still lacking.

Functional traits

In contrast to species diversity, "functional diversity generally involves understanding communities and ecosystems based on what organisms do, rather than on their evolutionary history" (Petchey and Gaston 2006). It is an alternative to producing more ecologically meaningful statements about the role of environmental and biological drivers of diversity, community structure, the assembly of communities and to better understand the linkage between community and ecosystem ecology (McGill et al. 2006; Weiher et al. 2011; Mittelbach 2012). Functional diversity is defined as the value and range of functional traits of organisms, i.e., organism features that reflect responses to the environment (response traits), effects on ecosystem functioning (effect traits) or a mixture of both (Díaz and Cabido 2001). Traits (e.g., size, mobility, feeding strategy, etc.) may be obtained from direct measurements of individuals and/or from bibliography. Some trait databases exist where scientists may retrieve information, e.g., polytraits (database on biological traits of polychaetes, http://polytraits.lifewatchgreece.eu/) (Faulwetter et al. 2014), sFDVent (a global trait database for deep-sea hydrothermal vent fauna, https://datadryad.org/stash/dataset/doi:10.5061/dryad.cn2rv96) (Chapman et al. 2019), and WoRMS (WoRMS Editorial Board 2020) (http://www.marinespecies.org/). Multiple analysis frameworks and indices are readily available, allowing the analyses of categorical and continuous traits (Villéger et al. 2008; Laliberte and Legendre 2010; Mouchet et al. 2010). Contrary to trophic markers (*see* below), trait-based methods are non-destructive, and can be applied regardless of the available number of specimens or biomass. They have been successfully applied to deep-sea meio- and macrofauna in a number of environments (e.g., Kalogeropoulou et al. 2015). However, they require more knowledge about multiple aspects of studied species' biology.

For example, functional diversity of nematode assemblages has been assessed based on buccal morphology as defined by Wieser (1953): selective deposit feeders, non-selective deposit feeders, epistratum feeders, and predators, complemented with the notion of "scavengers" (Jensen 1987). This classification can be used to calculate a trophic diversity index (Heip et al. 1985; Pape et al. 2013). However, feeding processes are complex (e.g., ontogenetic differences in feeding behavior, etc.), and eco-morphological classifications cannot capture all of this complexity.

Trophic markers: Fatty acids and stable isotopes

Trophic markers are usually biochemical parameters that are measured in a consumer's tissues and provide indirect, time-integrated information about its feeding habits ("you are what you eat" concept; Dalsgaard et al. 2003; Middelburg 2014). Use of trophic markers can help to infer trophic niches, track carbon and/or nitrogen flow in deep-sea fauna (Ginger et al. 2001; Duros et al. 2011; Würzberg et al. 2011; Veit-Köhler et al. 2013) and improve our understanding of the functional role of organisms within ecosystems (Fonseca et al. 2018). Two of the most widespread trophic markers used are fatty acids and stable isotope ratios.

Fatty acids markers can, to some extent, help to identify food items of deep-sea consumers (e.g., microalgae, zooplankton, bacteria; Kelly and Scheibling 2012). Recently, fatty acid profiles have shown that some meio- and macrofauna, such as nematodes, polychaetes and isopods (Leduc 2009; Würzberg et al. 2011; Guilini et al. 2013; Leduc et al. 2014*b*) can be selective feeders with taxon-specific preferences. Moreover, the degree of unsaturation (the number of double bonds) of fatty acids can also be used as a proxy for how degraded food items are (Dalsgaard et al. 2003). Well-preserved food items indeed retain much of their polyunsaturated fatty acids (PUFAs). Conversely, those compounds are typically absent from degraded material, and refractory organic matter contains mostly saturated fatty acids (Dalsgaard et al. 2003). Most components of macro- and meiofauna have been shown to be dependent on "labile" organic matter as a food source (Ginger et al. 2001; Würzberg et al. 2011). Those compounds can also be used as food source indicators, as they are "essential" fatty acids, i.e., compounds for which possibilities of synthesis and/or bioconversion by metazoans are limited (Dalsgaard et al. 2003). Specific fatty acid compounds, groups, or ratios thereof commonly used as biomarkers include the 16 : 1ω7/16 : 0 ratio for which values close to or higher than 1 suggest a diatom-based diet. Similarly, a 22 : 6ω3 (*DHA*) to 20 : 5ω3 (*EPA*) close to 1, can indicate reliance on a

dinoflagellate diet (Dalsgaard et al. 2003). High levels of 20 : 4 ω6 fatty acids in macrofauna could be caused by preferential feeding on foraminiferans (Kharlamenko et al. 2015). The use of fatty acids as trophic markers has been thoroughly reviewed by Dalsgaard et al. (2003), Kelly and Scheibling (2012), and Couturier et al. (2020).

However, it is important to interpret these data with caution, as no fatty acid marker can be unambiguously assigned to a potential food item. Moreover, physiology of deep-sea organisms is poorly known, and bioconversion of fatty acid compounds could occur in natural environments. Biosynthesis of PUFAs from other fatty acids can complicate interpretation of fatty acid concentrations in organisms (Dalsgaard et al. 2003; Leduc et al. 2014*a*). Furthermore, PUFAs can be affected by temperature, with lower temperatures increasing the level of unsaturation (Dalsgaard et al. 2003).

The use of stable isotopes in ecology relies on the fact that an organism's isotopic composition is a proportional mix of its food sources' isotopic compositions ("you are what you eat"; Middelburg 2014). Stable isotope analyses have accordingly been used to study the organic matter sources used by organisms in ecosystems (Boschker and Middelburg 2002; Veit-Köhler et al. 2013). Moreover, isotopic ratios of different elements can provide different, yet complementary, information. Carbon isotopic ratios ($^{13}C/^{12}C$) can be used to identify baseline items supporting animal populations and track their influence throughout the food web (Middelburg 2014). Nitrogen isotopic ratios ($^{15}N/^{14}N$) can serve the same purpose, or be used as an indicator of trophic position (Middelburg 2014). Sulfur isotopic ratios ($^{34}S/^{32}S$) can help to differentiate different energy pathways such as photo- and chemosynthetic inputs to a consumer's diet (Vetter and Fry 1998). A frequent goal of stable isotope studies is to identify and quantify dependence of a given consumer on potential food items. However, this requires extensive sampling of organisms, but also of their food sources, which is often hard to achieve in deep-sea ecosystems. Alternatively, other methods can be less demanding regarding input data, and allow to generate insights regarding resource use and niche partitioning in animal populations or communities (Layman et al. 2007; Jackson et al. 2011).

Like fatty acids, stable isotope ratios are imperfect trophic markers. However, joint use of stable isotopes and fatty acids is typically very efficient, generating insights that could not have been reached with any of the methods alone, as each method can compensate for the other's caveats (Leduc et al. 2015; Mascart et al. 2018; Mordukhovich et al. 2018). Both techniques require to extract meiofaunal organisms from the sample, and require a threshold amount of biomass. Due to limited meiofauna biomass, this often forces investigators to pool many organisms in one single sample to reach sufficient biomass (Veit-Köhler et al. 2013). Such "bulk" analyses represent the signal for a whole assemblage, and not for separate species. However, in some instances, species-specific analyses can be performed, resulting in richer ecological information. This is notably the case when meiofauna is very abundant, when communities are heavily dominated by a few taxa, or when dealing with large species such as nematodes *Deontostoma tridentum*, *Metaphanoderma* sp., *Curvolaimus* sp., *Enoploides* sp., *Metoncholaimus* sp., or *Oncholaimus* sp. (Leduc et al. 2015; Lins et al. 2015; Mordukhovich et al. 2018; Zeppilli et al. 2019). In the CCFZ, however, the low abundances and biomass might complicate feasibility of such analyses.

In order to perform fatty acid analyses, samples have to be stored at low temperatures (−80°C) prior to freeze-drying and weighing before analysis (Würzberg et al. 2011). Lipid extraction procedures vary for meiofauna (Guilini et al. 2011; Lins et al. 2015) and macrofauna (Würzberg et al. 2011). Complete sampling and preparation advice can be found in Couturier et al. (2020). For stable isotope analysis, samples should be stored frozen (−20°C or lower), oven-dried, or freeze-dried. In both cases, chemical preservatives (formalin, ethanol) should be avoided as they can may alter samples and influence measurements (Bosley and Wainright 1999; Carabel et al. 2009; Couturier et al. 2020; Le Bourg et al. 2020).

Distribution modeling: From observations to predictions

To conserve and protect areas proposed for deep-sea mining, it is not only important to have comprehensive knowledge of their taxa (Glover et al. 2018), but also to ascertain their ecological patterns and distributions. In conservation management, distribution or habitat models are a common tool to predict patterns spatially across point-source data, i.e., the investigated positions (Guisan and Thuiller 2005). Commonly, a modeling algorithm is applied to first describe the observed pattern in the most appropriate way and, to subsequently predict the distribution at positions not yet investigated. Several algorithms that can be used to this end are generalized linear models (GLM), most commonly used in marine studies, but other approaches such as artificial neural networks or tree-based methods have also been applied (Elith and Leathwick 2009).

In the CCFZ, simple spatial interpolation has been used in the proposal of marine protected areas (MPAs) (Wedding et al. 2013) that have been adopted in a modified version by the ISA in 2012 to designate Areas of Particular Environmental Interest (APEIs) (Wedding et al. 2015). This approach already included a continuous spatial dataset on invertebrate macrofauna abundance, but the framework was mainly based on abiotic variables such as nitrogen flux, nodule abundance, and water depth (Wedding et al. 2013).

Kuhn et al. (2020) applied RF regression to predict the continuous spatial distribution of meiofauna and macrofauna-sized epifauna abundances in the German exploration area (BGR). On high taxonomic level, the abundance of polymetallic nodules is an important predictor, influencing the different size classes of organisms in different ways and should

be considered in models. For instance, meiofauna dwelling within the sediments was predicted to be less abundant in areas with high coverage of polymetallic nodules, whereas epifauna is predicted to be most abundant in areas with high small-nodule coverage (Kuhn et al. 2020).

Furthermore, a combination of distribution modeling and clustering has been suggested as a framework for assisting the design of impact and preservation reference zones in the CCFZ area based on bathymetry and backscatter value as predictor variables (Uhlenkott et al. 2020). Due to the fact, that the deep sea remains under-sampled (Ramirez-Llodra et al. 2010) even distribution models based on predictor variables that are far from ideal can help to identify traits and patterns in the deep-sea plains (Uhlenkott et al. 2021). But RF regressions were also used to investigate differences in the distribution of meiofauna abundance over several years (Uhlenkott et al. 2021). Another advantage of distribution modeling can be the identification of new and possibly interesting sampling spots in an objective manner (Uhlenkott et al. 2020).

Conclusions and perspectives

- We recommend multi- and boxcorers for the quantitative assessments of meio- and macrofauna, respectively.
- We suggest not to use an upper sieve for meiofauna extraction, except to remove larger inorganic particles a 1 mm sieve can be used. In any case, there is no match of the upper size limit of the meiofauna (> 1 mm) with the lower size limit of the macrofauna (300 μm).
- For a correct impact assessment study, biodiversity data is required at the species level. Abundant and species-rich groups provide relevant information at genus level for monitoring purposes. A combination with metabarcoding allows to identify biodiversity shifts.
- Metabarcoding allows for the description of biodiversity based on DNA directly extracted from environmental samples or bulk samples containing organisms or organism remains and thus, it may be an especially useful approach for remote and hard-to-access areas, such as nodule fields, and for small organisms. As taxonomic databases are still very incomplete regarding deep-sea taxa, metabarcoding studies should integrate taxonomy and barcoding in order to fill database gaps. While taxonomic identification is useful for inferring ecological state, biotic indices can also be derived from taxonomy-free machine learning approaches.
- Proteomic fingerprinting may also be a promising avenue and can be a faster and cheaper alternative to metabarcoding.
- The measurement of aspects of diversity other than species and their abundances including biomass, functional traits, and trophic markers may provide critical insights that can help to answer current and future key questions and can be combined with more classical biodiversity assessments.
- Statistical modeling facilitates predicting patterns spatially across point-source data and it is necessary for designing future sampling efforts, and predict species distributions ranges and thus, conservation management.
- Global and coordinated efforts between scientists, public institutions, private companies and contractors in general, are needed to feed open repositories with biodiversity data (morphological, genetic, and functional) in order to generate the needed data and address the key questions involving deep-sea mining impact assessments.

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