Deep Sea Macrofauna of the Clarion-Clipperton Zone

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23-30 November 2014

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I. Executive Summary

Executive Summary

Following informal consultations between the Secretary General and exploration contractors for polymetallic nodules in January 2012 on the standardization of the biological component of environmental baseline data in exploration areas, the Authority planned a series of taxonomic exchange workshops focusing on megafauna, macrofauna, and meiofauna in contract areas.

The first of the series of workshops was convened at the Centre for Marine Biodiversity of Senckenberg Institute in Wilhelmshaven, Germany, on 10-15 June 2013. It focused on the evaluation of samples and difficulties encountered in assessing ***megafaunal*** biodiversity in the deep sea, specifically associated with polymetallic nodules deposits in the CCZ. The main taxonomic groups analysed in this workshop were: fish, holothurians, asteroids, crinoids, ophiuriods, cnidaria and protistas together with images of crustaceans, cephalopod molluscs and sponges.

This second workshop was convened at the East Sea Research Institute of the Korea Institute of Ocean Science & Technology (KIOST) in Uljin-gun, Republic of Korea, 23-30 November 2014 and focused on the evaluation of samples and difficulties experienced in assigning ***macrofaunal*** diversity (classified to species level) in the seabed of the CCZ. The main taxonomic groups analysed were: Tanidacea, amphipoda, isopoda, ostracoda, mollusca, bryozoa and polychaeta.

The objective of this second workshop was to bring together international deep-sea macrofauna experts with representatives of ISA contractors for the exploration for polymetallic nodules in the Area to facilitate the establishment of a standardized taxonomy for the baseline studies of macrofauna associated with these resources. This is to be achieved through the:

- creation of a standardized nomenclature with associated descriptions and keys, to be made available on the web for use by the contractors;
- recommendation of a standardized taxonomic identification protocol including sampling and storing methods for contractors;
- creation of a database of the locations where different species have been observed (including biogeographic variables); it was started for the megafauna workshop, and will, ultimately, create a faunal distribution atlas for the CCZ;
- provision of guidelines and procedures to be utilized by contractors, prospectors and the marine scientific researching community in applying the standardized nomenclature;
- collection of representative images for each identified species;
- creation of an atlas of the locations where different species have been observed; and
- creation of a programme of work to address any gaps in knowledge or understanding.

The workshop was attended by 12 taxonomic experts and 13 contractor representatives. It comprised a series of presentations on tanidaceans, amphipoda, isopoda, ostracoda, mollusca, bryozoa and polychaeta by the experts together with presentations by contractors on their current status of reporting macrofaunal diversity. The workshop also included three practical sessions in which samples collected by various contractors were examined by the taxonomists.

The results highlighted some of the challenges and issues facing macrofaunal taxonomy in the CCZ where biodiversity was relatively high (*Table 1*). The number of species recorded in terms of the individuals examined was high, in some cases 1:1. The number of new taxa identified from such a low number of individuals was also high and included genera and species new to science.

Therefore, to be able to assess the biodiversity in a timely and accurate way, there will need to be greater taxonomic resources. The samples were all based on morphological analyses reflecting a general absence of molecular approaches hitherto used in the baseline surveys. This absence of molecular approaches will be a constraint to producing rigorous, verifiable and robust taxonomy in the future.

Considering the low number of samples, caution must be taken in interpreting these observations, nevertheless they point to a highly diverse fauna. The low numbers pose problems in terms of both identifying and classifying the new species. To carry out molecular work it is also necessary to have more than one individual. The low numbers result from the type of sampler used. The spade box corer takes a quantitative sample of a quarter of a square metre. Because numerical abundance in the abyss is low, the probability of encountering several specimens of the same species in a sample is correspondingly small. Deployment of additional samplers, such as the epibenthic sledge, would supplement the box core samples by providing greater numbers of individual specimen to understand the diversity of the CCZ.

Several problems were encountered with the preservation of samples and treatment of material. For example, insufficient care had been taken in processing the samples once on board ship and some were poorly fixed. Some of the material was in poor condition (dehydrated, decalcified) and with parts and appendages missing and therefore could not be identified to the species level. Often individuals were fragmented or compressed which indicates that the sieving process was too vigorous. Given the cost of sampling in the abyss, more focus on sample processing is needed.

There was some mis-sorting of crustacean specimens by more than one of the contractors (e.g. mixing up isopods, tanaidaceans, amphipods and copepods). Many other phyla were represented in some mollusc samples, including brachiopods and ostracods. This suggests that there are important issues around taxonomic resolution and competencies.

There are two sets of recommendations in this Technical Study. The first, by the taxonomy experts, addresses the establishment of a DNA Barcode Library, and the second is the recommendations that came out of the workshop.

II. Presentations by Taxonomic Experts

Presentations by Taxonomic Experts

Gaps in our knowledge: What we need to know to develop sustainable management. The known unknowns…

Dr G.L.J. Paterson, Department of Life Sciences, National History Museum, United Kingdom

Dr Paterson's presentation addressed the need for a robust and accessible taxonomy to determine the patterns of distribution of biota at various scales and to identify species accurately and consistently.

He emphasized that "determining the patterns of distribution of the biota at various spatial scales, from local to area to region, requires contractors to identify species accurately and consistently". He said the abyss was the largest habitat of the planet and the least studied. Because of limited funding to undertake the basic taxonomic information needed, systematic sampling of the deep sea for macrofauna was irregular and, the specimens collected were not properly classified. This threatened the ability to assess the risk to deep sea macrofauna from mining.

Dr Paterson noted that only a few studies existed for the CCZ on macrofauna biogeographical variation. He said that it had been found that at a scale of 1000 km the polychaete diversity changes, therefore the risk of eliminating a species due to mining activities was high. Studies also showed that some species of polychaetes were wide spread, and thus had a lower risk of elimination by mining activities in the CCZ. He further noted that these conclusions were based on few samples and low taxonomic resolution. He pointed out that this situation called for strong collaboration among stakeholders in the CCZ.

Dr Paterson said that an important feature of highly diverse ecosystems is the presence of rare species. At the CCZ, the task to define rare species was gigantic. He pointed out that, to date, it had been impossible to assume that a given species was rare because it could be that the taxon was under-sampled. Again, he stressed the need for all stakeholders to collaborate in exploring the CCZ for biogeographic inferences.

A gap analysis for the CCZ biogeographic information availability (MIDAS) showed that there was little taxonomic work and few taxonomic keys for this region. The few collections of properly preserved specimens were geographically scattered and poorly referenced. In concluding, Dr Paterson noted that there was a need for an infrastructure (taxonomic vault collection) for the fauna from the CCZ to speed up the collaboration and fill the gap of taxonomic knowledge for the region.

The ecological context for the study of biodiversity of the macrofauna of the CCZ

Dr L Menot, Ifremer, France

Dr Menot said that while macrofauna was a standard group for monitoring environmental impacts, their abundance in the CCZ was extremely low because of their limited ability to move away from impacted areas. This meant that to provide significant results, many samples needed to be taken in just one environmental monitoring cruise. He suggested that although the Recommendation for Contractors issued by the Authority's Legal and Technical Commission (ISBA/19/LTC/8) highlighted the need for box core samples for quantitative studies, he thought it might be prudent to include the use of epibenthic sledges because they collected much larger numbers of macrofauna species for determining species range.

Dr Menot urged the Authority to organize a workshop on the use of statistics in environmental studies and how it could influence the design of environmental studies and monitoring impacts for contractors.

Dr Menot suggested that macrofauna may not be quite the taxa to use to assess impacts from deep-sea mining of polymetallic nodules in the deep sea of the CCZ. He based this assumption on: a) low density of individuals; b) rarity of species; and c) poor sampling. He concluded that to be able to understand the consequences of deep seabed mining, we need to understand the mechanisms for diversity maintenance that include: 1) life history traits, dispersal, population dynamics; 2) scales of heterogeneity; and 3) biological interactions.

The taxonomy and biogeography of macrofaunal Tanidaceans crustaceans with a focus on the abyssal Pacific fauna relevant to the CCZ

Dr M. Blazewicz-Paszkowycz, Department of Invertebrate Zoology & Hydrobiology, University of Lodz, Poland

Dr Blazewicz-Paszkowycz introduced *Tanidacea*, an order of crustaceans, superorder *Perarcarida*. She described Tanaids as small peracarid crustaceans that live in marine habitats over the full range of depth and which were truly benthic organisms that burrow into the upper layer of sediment constructing tubes. She said there were currently over 1,300 known nominal species of tanidaceans but that could include as many as 40,000 species. She said a recent study undertaken in the Southern Ocean - NE Pacific and Western Australia - indicated that tanaids may be an abundant and highly diverse element of abyssal benthic assemblages. From polymetallic nodule habitats in the Central Pacific, she said less than 10 species of six genera have been formally described, all of which represent the superfamily paratanaoidea.

Dr Blazewics-Paszkowycz explained that this group was widely distributed from shallow water to the deep sea and was often confused with isopoda, another crustacea. Dr Blazewicz-Paszkowycz, mentioned the difficulties in identifying these animals because they have different stages (adults, juveniles) that could be mistaken for different species, but, actually, they could be the same one at different stages of their life cycle. She stated that Tanaidacea is one of the important macrofaunal components of the CCZ. She also mentioned that molecular reverse taxonomy is an appropriate tool to provide adequate taxonomic identification of the tanaidacean found in the CCZ.

The taxonomy and biogeography of macro faunal *Amphipod Crustaceans* with a focus on the abyssal Pacific fauna relevant to the CCZ

Dr C Havermans, Operational Directorate Natural Environment, Royal Belgian Institute of Natural Sciences, Belgium

Dr Havermans presented the taxonomy and biogeography of macrofauna amphipod crustaceans. She said that the use of molecular tools as an aid in identifying this species of deep sea taxa had proven useful in light of the major challenges associated with the task. She described the efficiency of DNA barcoding using standard genetic markers by highlighting two approaches: (i) reverse taxonomy where morphological studies build upon results of the initial molecular results; and (ii) the blind approach where taxa are treated as molecular operational taxonomic units (MOTUs).

She said molecular studies, based on MOTUs, were proposed for understudied and abundant amphipod families and observed that molecular approaches should be further explored with the aim of providing a rapid and cost-effective method for large-scale biodiversity studies. She said

molecular studies of deep-sea taxa would not only facilitate rapid species assignment of hard-to-identify specimens but could also provide information crucial to better understand deep-sea ecosystems and their vulnerability to human-induced modifications.

Dr Havermans presented the taxonomic issues of another very common taxon of crustacea found in the CCZ - amphipods. These are scavengers, benthic, bento-pelagic and pelagic animals. This group, (as with many crustaceans) show sexual dimorphism (male and female differences) that also makes their identification difficult. She said that currently most of the identification was done using reverse taxonomy (DNA identification) not only for taxonomic studies but also for biogeographical patterns definitions.

The taxonomy and biogeography of macrofaunal *Amphipod* crustaceans, with a focus on the abyssal Pacific fauna relevant to the CCZ

Dr T Horton, National Oceanographic Centre, Southampton, United Kingdom

Dr Horton highlighted the taxonomy and biogeography of macrofaunal amphipod crustaceans, explaining that a recent analysis of the occurrences of gammaridean amphipods in the deep sea recorded 400 species from 54 families living at depths below 2,000m. The numbers reduced to 100 species and 31 families below 4,000m.

She said that limited studies had been conducted on the abyssal benthic amphipod fauna with the best studied region being the Northwest Pacific basin. With the current lack of knowledge of amphipod fauna in the CCZ, Dr Horton focused on amphipod taxa that were most likely encountered in the region and the resources needed to identify the taxa using standard morphology and stereo and compound microscopy. She said many references, including identification resources, were linked on the World Register of Marine Species (WoRMS), World Amphipod Database (WAD) and the World Register of Deep-Sea Species (WoRDSS) websites.

Dr Horton mentioned that there were at least eight families found only in the deep sea. Because some of the taxonomic characters are found at the mandible structure of an individual, it is quite difficult to identify them using only traditional taxonomic procedures. She concluded that DNA reverse taxonomy was a better tool for this group.

Taxonomy of macrofaunal *Isopods* of the Pacific Abyssal Fauna Relevant to the CCZ

Dr Stefanie Kaiser, Senckenberg am Meer, German Centre for Marine Biodiversity Research, Germany

Dr Kaiser described isopods as relatively rich and ubiquitous elements in marine environments and that isopod crustaceans were a popular and useful taxon to assess large-scale biodiversity and distribution patterns across marine benthic habitats. She said with the exception of a few parasitic groups, isopods lacked planktonic larval stages and brood their offspring in a ventral brooding pouch.

She said the group occupied several niches in the deep sea: scavengers, parasites, suspension feeders and predators. She said that because of their reproductive habits (brooding) their distribution was rather geographically restricted. Dr Kaiser showed that significant differences were found in high species variability composition of isopoda when comparing two sites, within the CCZ. These groups showed high site-specific abundance as well as the same species occurring in multiple sites.

She said isopods represented one of the better studied macrofaunal groups in the CCZ and was used as a model to study variation in diversity, population levels and community connectivity. Her presentation also introduced isopod taxonomy, information on sampling devices and the preservation of samples, together with preliminary findings on the diversity and distribution of isopods in the CCZ based on data from recent expeditions in the Central Equatorial Pacific.

The taxonomy and biogeography of macrofaunal Ostracod crustaceans with a focus on the abyssal benthic Pacific fauna relevant to the CCZ

Dr I Karanovic, Hanyang University, Department of Life Science, College of Natural Sciences, Seoul, Republic of Korea

Dr Karanovic's presentation focused on the taxonomy and biogeography of macrofaunal ostracod crustaceans that inhabit aquatic environments from freshwater puddles to abyssal ocean depths. She said the main ostracod characteristic was the bivalve, dorsally-hinged calcified shell, of which there were over 65,000 fossil species and 8,000 living species described so far.

She described ostracods as small animals with body length from 9.2mm-3cm and characterized by a simple body with eight appendages with the shell being an important taxonomic character. Dr Karanovic said deep sea ostracod faunas were relatively well studied in the North Atlantic and the Mediterranean and, to a lesser extent, in the Arctic and Western Pacific Oceans. She noted that deep sea ostracods belong to 37 families with approximately 200 genera and about 500 species and that although cosmopolitan distribution had been proposed for many deep sea ostracods, more recent studies contradicted that. She suggested that apparent cosmopolitanism was just an artifact of incomplete taxonomic knowledge.

Dr Karanovic presented a group that is normally considered as belonging to another artificial category - meiofauna. She pointed out that ostracods can reach various centimetres in size in the deep sea, and, therefore, was included sometimes as macrofauna. Ostracods have also been studied extensively on environmental paleoreconstruction and climate changes. Dr Karanovic mentioned that many of the taxonomists were, indeed, paleontologists first.

The taxonomy of ostracods is also difficult and based mainly on the hard shell they exhibit. Most of the ostracod from the deep sea are of the podocopid type with a predominance of the order Cytherocopina. They were found in low abundance and rarely targeted as the group being sampled. Today, there are some 3,681 records of ostracods from the deep sea from 700 locations, but yet to be described as being from the CCZ.

Abyssal Pacific mollusca with particular emphasis on the Clarion-Clipperton Zone

Mr P Valentich-Scott, Santa Barbara Museum of Natural History, United States of America

Dr Valentich-Scott described abyssal mollusks as being moderately sampled and poorly studied. He said the *HMS Challenger* expedition in the 19th century had yielded many mollusk specimens from depths greater than 2,000m. At the turn of the 20th century, the *Albatross* expedition greatly expanded Pacific abyssal sampling with WH Dall publishing numerous accounts of the Mollusca of this expedition. He said frequent French South Pacific expeditions, from the late 20th century to the present, have produced 27 volumes of Tropical Deep Sea Benthos monographs, including extensive treatments of molluscan families. The accounts have been expanded in the 21st century through extensive monographic treatments.

He reported that two books covering bivalves from Arctic Alaska to northern Peru were published in 2002 and 2012 but that, in both volumes, the depths of the CCZ were sparsely covered. He concluded that many new species of molluscs would be collected in the CCZ and he hoped the workshop would assist in delineating many of the new taxa, as well as previously unknown species distributions in the region.

Dr Valentich-Scott mentioned that one of the problems was that most of the samples collected were fixed with formalin for too long, thus the shells of these small animals dissolve. He suggested that the fixing time must be short and the animals preserved in alcohol thereafter.

The taxonomy and biogeography of macrofaunal Bryozoa, with focus on the abyssal benthic Pacific fauna relevant to the CCZ

Dr D Gordon, Principal Scientist, National Institute of Water & Atmospheric Research, New Zealand

Dr Gordon's presentation summarized bryozoa as a phylum of colonial invertebrates with about 6,000 described living species and 15,000 fossil species. He said bryozoa were distributed from the intertidal zone to a depth of more than 8,000m, with colonies ranging in size from a single individual (zooid) to massive, coral-like growths about 1m in diameter. In shallow water and at continental-shelf depths, bryozoans can form part of the rock wall and shelf assemblages.

He described bryozoa as having three living orders in two classes: Ctenostomata and Cheilostomata (Gymnolaemata) and Cyclostomata (Stenolaemata) with morphologies that included encrusting, both flexibly and rigidly erect, free living, conical and shell-boring forms.

He said the only bryozoans recorded in the vicinity of the CCZ so far were three species of the Cheilostomata family, the Bifaxariidae.

Dr Gordon pointed out that this was a group of colonial animals, some of them calcified and some of them not. He noted that colonies ranged from the individual size (centimeter scale) to more than 1 m in diameter. He noted that there were at least three species living at the CCZ and that some of these colonies were rooted in the soft sediments of the deep sea, but many of them used secondary substrates such as rocks, nodules, and crusts. He concluded that their taxonomy was based on zooid polymorphs (feeding, avicularium, reproductive and kenozoid) characterization

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The taxonomy and biogeography of macrofaunal *Annelids* with focus on the abyssal benthic Pacific fauna relevant to the CCZ

Dr Helena Wiklund, Natural History Museum, Senckenberg am Meer, Frankfurt, Germany

Dr Wiklund explained that polychaetes were an important group in marine habitats, and were often one of the most abundant groups in benthic samples from all depths. Despite all that, there were still not many records of described polychaeta species from the abyssal Pacific region. She said the reports of the *Challenger* Expedition, carried out by trawlers, described mainly large animals or animals inhabiting large tubes. Later expedition sampling used box corers which allowed the collection of smaller infaunal species. Among those samples, the most dominant polychaete families were Spionidae, Paraonidae, Cirratulidae, Syllidae, Sabellidae and Acrocirridae. She added that during the Kaplan Project, samples were also collected for molecular analyses and yielded cryptic species among polychaetes as well as shorter than expected distribution ranges for many of the species.

Dr Wiklund, mentioned that this is probably the most abundant taxa of macrofuana from the deep sea although poorly studied at the CCZ. Utilizing an image of an annelida, she said that the annelida had been described using traditional procedures for identification. However, reverse taxonomy (DNA technique) is increasingly required. She concluded that these were soft-bodied animals and, thus, had to be carefully handled. They should be sorted under chilled water, exposed to a short fixation time, if needed, but soon after preserved in ethanol.

Advances in molecular methodologies and the application and interpretation of molecular methodologies for macrofauna classification: Their relevance to environmental assessment and monitoring

Dr Thomas Dahlgren, Uni Research, Norway

Dr Dahlgren emphasized the importance of baseline studies of macrofaunal composition and species ranges to understanding the possible impact of future exploitation of deep sea mineral resources. He said data was needed to describe impacted areas in terms of species lists (fauna classification) and to use the selected species to understand their impact resilience by their ability to recolonize from refuges such as the planned Areas of Particular Environmental Interest (APEIs). He said that while some biodiversity data was available for ecosystems at deep sea hydrothermal vents (massive sulphides) and deep sea mounts (manganese crusts), very little was known of the biodiversity at abyssal fields. He said the lack of species records from the CCZ had persisted despite the large number of scientific cruises to the central east Pacific Ocean during the last 40 years.

Dr Dahlgren said that even with good quality samples that were now being accumulated from the CCZ, with excellent morphology and associated DNA sequence data, major challenges remained in achieving species lists for the specific area that was studied. He pointed out that the data was required to assess species range sizes and population connectivity. He said producing species lists would require a database where the analysed DNA or specific track pattern (eDNA) was matched to a species name but that much basic research still remained to be done before the latter method could be used.

Dr Dahlgren presented the state-of-the-art advances in molecular methods and their applications/ interpretation for macrofauna classification. He said that the big gap of knowledge on deep sea taxonomy, in particular at the CCZ, is that science is far from describing the species of macrofauna inhabiting the CCZ. He stated that this was demonstrated with rarefaction curves. He also mentioned that, although there have been more than 200 cruises to the CCZ, the output of information on diversity had been very slow using traditional taxonomy procedures, and that the sampling, was as yet, neither pragmatic nor systematic. He emphasized that one thing was certain, and that was that there was no lack of sampling effort. Dr Dahlgren reiterated that taxonomic challenges were real. He pointed out that the polychaetes records for the CCZ were in too poor a state to be used as reference and their geographical allocation was scarce and inaccurate. He observed that the main challenge was the new and appropriate sampling efforts as well as DNA sampling of individual tissues. He continued that the use of DNA taxonomy introduced another problem, that is, DNA bar coding for the fauna of the CCZ was poor (with no appropriate DNA sequence libraries). The advantages of DNA taxonomy were enormous. He said that among those, a certain identification of an organism, storage of its DNA sequence for further reference to assign similar individuals to the same code (species), storage information for future analyses (phylogenic, gene flow, connectivity, species range, population structure, cryptic species) were required. DNA techniques could also be used to fast assess bulk sample identification (eDNA) using a robust DNA barcode library as well as for a single sample from a single specimen (reverse taxonomy).

Developing molecular pipelines to provide samples for barcoding and other analyses

Dr Helena Wiklund, Senckenberg Research Institute and Natural History Museum, Frankfurt, Germany

Dr Wiklund said sampling the deep seabed was both costly and time consuming, and often yielded very few specimens of each species due to patchy distribution which meant that every specimen was incredibly valuable and needed to be carefully documented. She said barcoding the animals could give some information about distribution and change if samples were taken at different localities and at different times. She noted that knowledge about the life history of these animals was needed before any predictions could be made.

She said it was necessary to handle the animals carefully in order to draw any conclusions about them. Onboard vessel sampling and sieving was crucial for obtaining good DNA samples. She suggested using cold water (4-8°C) for sieving/removing the sediment as it was very important to keep the specimens cool. Live-sorting the samples on the ship was invaluable for individual preservation. She said specimens should be photographed live in order to accurately document colour, presence of eyes or appendages; features which were valuable for species descriptions. Photographed specimens should be fixed in ethanol and stored at low temperature for preservation and for DNA extractions. She said sequencing could also help with identification of reference sequences already published in the GenBank (www.ncbi.nlm.nih.gov). It could also facilitate the analysis of distribution, connectivity, phylogenetic and species descriptions. All material should be deposited in a collection and made available to scientists.

The pros and cons of DNA barcoding: Preliminary results from recent cruises to the CCZ

Dr A Janssen, S Kaiser[1], K Meißner[2], N Brenke[1], L Menot[3], U Raschka[1], P Martínez Arbizu.[1]
[1]DZMB, German Centre for Marine Biodiversity Research, Wilhelmshaven, Germany.
[2] DZMB, German Centre for Marine Biodiversity Research, Hamburg, Germany.
[3] Institut français de recherche pour l'exploitation de la mer, Ifremer, Brest, France.

Dr Janssen said abyssal benthic communities were known to be extremely diverse. She noted that with many species represented by single specimens, it was virtually impossible to describe all the species by a traditional morphological approach. In keeping with the plans to begin mining in the next one or two decades, there was a need to characterize faunal diversity, abundance and distribution in order to predict the effects of mining on deep-sea organisms. She said the approach of reverse taxonomy had the potential to greatly speed up the identification process to assess diversity and distribution ranges.

She continued that the most fruitful advantage of DNA taxonomy was that suitable programmes could be conducted fast and automated to construct MOTUs. Their study had identified 380 polychaete MOTUs and 178 isopod MOTUs out of 1,605 specimens. An additional advantage was the ease in comparing DNA sequences. Genetic barcodes could be uploaded to a reference database (GenBank) and named according to an existing phylogenetic framework. Dr Janssen said DNA sequences could only assign MOTUs to sequences of known species listing in a database but not annotate them to a species name if the identity was unknown.

She concluded that the reverse taxonomic approach appeared to be robust and successful in their study. However, when considering the scenario of 12 different contractors, using taxonomic standardization, to assess the mostly undescribed, biodiversity of benthic communities in areas within the CCZ, the work would not only be a challenge but would present an opportunity for joint efforts and unparalleled collections. She said the use of molecular barcodes could be shared among contractors, published in online repositories and become a standard procedure.

Conceptual and logical design of a deep sea environmental database for the Area: Geology, oceanography and ecology

Dr G. Paterson[1], Dr L. Menot[2] and Dr S. Mulsow[3]
[1] Department of Life Sciences, Natural History Museum, London SW7 5BD, UK
[2] Ifremer, REM-EEP-LEP, ZI de la Pointe du Diable, CS 10070, 29280 Plouzane France
[3] International Seabed Authority

Dr Mulsow presented the work done in collaboration with Dr Paterson and Dr Menot. He described the status of the Authority's work in trying to establish a database for all the information recorded and reported on by contractors, especially data related to environmental studies to create a baseline definition of the habitat of the CCZ. He observed that there was not enough useful information within the Authority and that the little information it had was used to feed an open source database (Ocean Database View™). The information available enabled some statistical use of the database. He requested taxonomists to present their input for the conceptual design of mandatory variables related to molecular taxonomy that should be included in the database.

The value of collections – Lessons from the oil & gas industry

Dr Tammy Horton, National Oceanography Centre, Southampton, United Kingdom

Dr Horton presented experiences from the UK Oil & Gas Industry and the different models used by the Oil & Gas Industry with their environmental impact assessments (EIA). The UK model was based on external consultancies. She said a major concern was quality control on taxonomy and that lessons learnt from the oil and gas industry were that after an environmental study was done and the EIA report approved, species were not stored for future reference and were lost to mankind. She recommended that in the case of deep sea mining, a dual-level model could be used to identify and classify the animals. Initial classification should be conducted by a trained identifier and the final identification and reporting done by an expert taxonomist. She concluded by saying that specimens should be properly stored for reference and that all ancillary information and metadata of each voucher species should be shared through an appropriate database, i.e. the ISA database.

III. Presentations by Contractors

Presentations by Contractors

Interoceanmetal Joint Organization: Status of Macrofaunal Studies Carried Out by IOM

Dr Valcana Stoyanova

Dr Valcana Stoyanova informed participants that IOM had collected samples between 1988 and 2014 from 1,036 stations using box corers. She said that since the signing of its contract in 2001, macrofaunal studies had taken place in 2009 and in 2014. In 2009, 11 stations were sampled randomly (1 station per 360km^2) with raw data collected from 44 samples. In 2014, 16 stations were explored (1 station per 250km^2) resulting in 48 samples. A total of 71 macrofaunal specimens were collected for genetic studies. With regard to epibenthic samples, Dr Stoyanova said that the craters of removed nodules were siphoned off and then, with the washing residue, added to the 0-3cm samples. Samples were stored in four per cent buffered formalin for five days and transferred to 80 per cent alcohol with Rose-Bengal dye. Samples for genetic analysis were stored in 96 per cent ethanol at 4°C. She said that the samples were stored at IOM headquarters with sorting done by environmental consultants. No epibenthic sledge was used because the sampling was primarily geological. Only meiofauna was studied.

Dr Stoyanova concluded that IOM was open to cooperative research.

Dr Stoyanova described the work done by Interoceanmetal Joint Organization. The sampling campaigns started in 1988. However, she pointed out that it was only in cruises during 2009 and 2014, that the contractor collected samples for fauna (macrofauna included). In 2009, 11 stations were taken randomly in block H11 (ca. 4000 km^2). The raw data from the resulting 44 samples were sent to the Secretariat. In April 2014, 16 stations were collected in block H22 (4000 km^2) resulting in a total of 48 samples. Another 46 samples (from 46 stations) originated from the rinsing of nodules. Seventy-one individuals were collected off the nodules for genetic studies. In 2014, one half of each one of the 27 box core samples was collected for macrofauna analysis. The macrofauna samples of 2009 and 2014 are stored at IOM's headquarters. Environmental consultants do sorting. Most of the taxonomic work for these samples is still pending.

Yuzhmorgeologiya: Macrofauna investigation on Russian exploration areas of polymetallic nodules

Dr Viacheslav Melnik

Dr Melnik informed participants that sampling in the eastern polygon of the exploration area took place from west to east in 2006-2009, 2011 and 2013. A total of 196 box core samples have been collected resulting in 8,760 macrofauna individuals consisting of 52 per cent polychaetes, 34 per cent crustacean and seven per cent bivalve among 18 taxa (with the rest < 1%). The collected meiofauna amounted to 26,230 individuals (61% nematodes). Of the macrofauna, 4,574 individuals were polychaetes (Problems of destruction during rinsing; used literature was *Fauchald* (1977) from the Internet), 1,423 individuals were tanaidacea, 1,391 individuals were isopods (determined to genus-level without difficulties [except *Mannopsidae*] using O.Kusakin (1979-2003) in Russian), 616 individuals were bivalves, and 214 individuals were gastropods.

Animals associated with nodules resulted in 218 samples of epifauna with 1,198 individuals of which 278 individuals were bryozoans.

Dr Melnik said Yuzhmorgeologiya would like to see a special workshop dedicated to the epifauna of nodules.

Government of the Republic of Korea: Sampling and taxonomy of macrobenthos for KODOS

1Dr Ok Hwan Yu, KIOST, Korea
2Craig Smith et al, University of Hawaii at Moana

Dr Yu of KIOST informed participants that the Government of the Republic of Korea had undertaken its macrofaunal studies in stages: stage 1 (1998 - 2004 and 2006) using a small box corer sieved at 500 um, and 2009 with a medium box corer (0.25 m^2), whereas during stage 2, (2011 - 2014), samples from the reference site KOMOI, and in the benthic impact experiment site, were sieved to 250 um. When comparing results of 2012 with 2013, 45 species in 11 taxa were found, which increased to 96 species in 21 taxa with two new species of *Neotanais* discovered. The results were compared with the Kaplan study which found that a higher polychaete family diversity was recorded in 2011-2012 at the Kaplan sites. Dr Yu described the effect of the box-corer's bow wave as being problematic.

China Ocean Mineral Resources Research and Development Association: Introduction to biological baseline surveying in COMRA's contract area in the CCZ

Dr Zhang Dongsheng

Dr Zhang Dongsheng informed participants that COMRA had undertaken nine cruises between 1997 and 2014 and collected 47 samples of which 28 have been analysed and the data from 25 samples have been transferred to the ISA Secretariat. In 2013, three samples were also collected in the APEIs. A 0.25 m^2 box corer with a camera (in real time) was used and samples were sieved to 250 µm. The samples were dyed with Rose-Bengal for use under the dissection microscope and fixed in formalin and stored in ethanol. He said that macrofaunal results are available for the years 1998, 1999, 2001, 2003 and 2013. In 1999, only low densities were found, possibly due to El Niño effects from 1998. He also said that densities were generally higher in the eastern section of the exploration area compared to the western part. The 16 taxa identified included polychaeta (>50%), isopoda, tanaidacea, and bivalves. Only five species were encountered in both study areas.

Dr Zhang Dongsheng also stated that COMRA perceived the lack of taxonomists as a general problem which is why it had organized a special training course covering *polychaetes* and *ophiuroidea* for nine trainees in Hong Kong in 2014. A second workshop was scheduled to take place in 2015. The contractor recommended the use of a multicorer with a TV camera for the study of meiofauna but acknowledged that problems existed with its sample collection and processing methods.

Deep Ocean Resources Development Co. Ltd.: Status of Japanese Activities on Biological Survey of Macrofauna in the CCFZ

Dr Masatsugu Okazaki

Dr Okazaki informed participants that field studies had taken place in the Japan Deep-sea Impact Experiment Area (JET) in 1991-1996 and 2011-2012; and in the High Abundance Area (HAA) in 2012-2013. He said that the three HAA stations (20-25 km apart) were sampled for megafauna, macrofauna and meiofauna. During 1991-1996, 23 samples had been collected, with 8 during 2011-2013. He also said that DORD's smallest sieving mesh size was only recently changed from 300 µm to 250 µm, and its samples were stored in 10 per cent neutralized formalin (all dyed red). He noted that in 1991-1993 and 2011-2013, only 10 high-level taxa had been recorded.

Dr Okazaki said that DORD reported issues with the fact that identification only took place at the higher taxonomic level. Furthermore, the change of sieve mesh size made comparisons virtually impossible. He said sample sizes were too small, and the contractor was uncertain as to what to do with large meiofauna (>250 µm). He described future challenges with (a) following ISBA/19/LTC/8 and (b) obtaining data from JET and HAA to classify macrofauna into lower-level taxa because of the lack of taxonomic experts in Japan.

Institut français de recherche pour l'exploitation de la mer: Environmental Studies carried out by Ifremer in the Clarion-Clipperton Fracture Zone

Dr Florence Pradillon

Dr Pradillon described Ifremer's work on macrofauna associated with polymetallic nodules in its exploration area. She said that Ifremer had collected macrofaunal data during two cruises, "*Nodinaut*" in 2004 and "*Bionod*" in 2012. She explained that the box-corer used sampled an area of 0.25 m^2 and the multicorer a diameter of 10 cm. Box-cores were sieved with 1000 µm, 500 µm and 300 µm mesh sizes for macrofauna, and multicores were sieved with 40 µm mesh size for meiofauna. Epibenthic sledge samples were sieved through 300 µm. Dr Pradillon said that the samples of 10 box-cores were stored in formalin and of nine box-cores (Kaplan Project) are stored in alcohol. Additional samples were obtained from 12 multicores and 11 Nautile dives. In 2012, an additional nine quantitative and four qualitative box cores as well as five epibenthic sledge samples were collected. Dr Pradillon informed participants that in 2004, 902 specimens had been collected and included 553 polychaetes identified to the family level; 124 tanidaceans and 73 isopods identified to genus level and 12 gastropods identified to species level. She said that all of the samples were stored at the Natural History Museum of Paris. She continued that in 2012, a total of 12,743 individuals had been collected with epibenthic sledges, whereas ten box-cores resulted in 904 individuals with 463 isopods separated into 59 morphospecies.

Dr Pradillon acknowledged that information on animal distributions and on environmental drivers was desperately needed and that the differences in isopod density were larger in habitats with nodules than without (non-metric multi-dimensional scaling test showing no significant difference at family-level).

Government of India: Marine Benthos of the Indian EEZ

Dr Hashim Manjebrayakath, Ministry of Earth Sciences (MOES)

The contractor's presentation focused on the EEZ of India and was not directly relevant to either the Indian claim area in the Indian Ocean or the CCZ.

Federal Institute for Geosciences and Natural Resources of the Federal Republic of Germany: Macrofauna in the German Mn-nodule license area of the CCZ

Mr Uwe Raschka

Mr. Raschka made a presentation on macrofauna in the German licence area. He said that BGR had undertaken four cruises in its area in 2010 (with RV "*Sonne*"), 2012 (with RV *"L'Atalante*"; the "*Bionod*" cruise), 2013 and 2014 (with RV "*Kilo Moana*") during which an epibenthic sledge (EBS) was trawled over 2,000 m and 0.25 m^2 box-cores collected. All samples were sieved through 300 µm with pre-cooled water and stored in 96 per cent ethanol. In 2010, the 9,254 individuals from

the EBS included 1,388 polychaetes (15.0%), whereas the 5,606 individuals out of the box-cores included approximately 1,500 polychaetes (26.6%). BGR reported that it expected to undertake two project cruises in March-June 2015.

The contractor concluded that the study was the first step of inventory in the CCZ which had a highly diverse deep-sea ecosystem with a high number of rare species. Although the sample sizes were still too small to assess connectivity, there was also significant positive correlation of genetic and geographical distance.

Nauru Ocean Resources Inc.: ISA Workshop on Macrofauna

The presentation by NORI was sent electronically. The contractor explained that the company worked in its two eastern exploration areas in 2012 and in its two western areas in 2013. Biological specimens were only recovered while sampling for nodules. Thirty-six faunal specimens (27 megafaunal and nine macrofaunal) were measured, photographed and frozen on board. Taxonomical specialists for ophiuroidea, crinoids, foraminifera and fish were consulted, whereas the arthropods (crustacean), molluscs and porifera have not yet been reviewed by taxonomists.

Future plans of the contractor include dedicated environmental research campaigns (possibly in collaboration with other contractors) as well as the development of an EIA. The contractor was seeking guidance on the minimum standards for sampling (with and without remotely operated vehicles (ROV)), on the level of taxonomic identification necessary, on the standards for storing voucher specimens, and on the identity of taxonomic experts. Further, the contractor suggested the consideration of a 'taxonomic clearing house' as well as the development of guidelines for habitat mapping. The contractor advised it supported collaboration among contractors and asked who would perform the region-wide strategic EIA.

Tonga Offshore Mining Ltd.: TOML Environment Studies in the EEZ and Reflections on the EIA Project

Renee Grogan

Ms Grogan informed participants that TOML, which had only recently signed an exploration contract with the ISA, had undertaken one cruise in 2013 which resulted in the loss of its box-corer. The contractor's major concern, currently, was to find a way to make taxonomical use of geological cruises and/or to make use of old data.

Global Sea Mineral Resources NV: Status of activities on biological baseline surveying in the CCFZ

Mr Jacques Paynjon, Dr Ellen Pape and Ann Vanreusel, Ghent University, Belgium

Global Sea Mineral Resources NV (GSR), like TOML, had only recently signed its exploration contract with the ISA. Mr Jacques Paynjon informed participants that GSR had undertaken its first cruise in 2014. The result was the collection of ten box-cores collected for macrofauna, meiofauna, bacterial cell counts and DNA. He reported that the company's plans include the creation of a DNA sequence reference database to replicate samples. The contractor sought guidance in regard to what could be done with macrofauna-sized nematodes and copepods and was interested in ISA's support for a genetic biodiversity workshop.

United Kingdom Seabed Resources Ltd: UKSR environmental baseline programme: Abyssal benthic biological baseline studies (ABYSSLINE)

The presentation by UKSR was sent electronically. The contractor explained that its environmental baseline programme was called ABYSSLINE. For its first cruise in 2013, an international team of scientists from the UK, Norway, Germany and the USA was contracted. The exploration followed a stratified random study design and investigated the first 30x30 km study area using 12 box-cores, ten mega-cores, four baited traps, one baited camera, three respirometers and sediment traps as well as five epi-benthic sledges and a ROV for that cruise, over 4.5 km.

The presentation informed participants that in total, 178 megafauna morphotypes had been videotaped and categorized into nine phyla and that according to box-core samples, macrofaunal biodiversity appeared to be moderate to high with 74 high-level taxa (family or coarser) among 1,254 individuals collected. The presentation stated that foraminifera were identified as key organisms across all size classes as well as important epifauna and that ROV collections contained almost 90 per cent copepods. DNA was extracted from 1,263 crustaceans and that microbe populations on sediment and on nodules were genetically distinct from each other. The presentation reported 89 per cent of the surface biomass was dominated by bacteria.

Participants were informed that UKSR intends to hold a joint cruise with a new contractor, Ocean Minerals Singapore, in 2015 to sample two 30x30 km blocks, one in each claim area.

Japan Oil, Gas and Metals National Corporation: The outline of an environmental survey (sediment sampling for macrofauna)

Dr Teruyoshi Narita

Dr Narita reported that Japan Oil, Gas and Metals National Coporation (JOGMEC) was established in 2004, and had conducted 27 cruises between 2008 and 2014 as part of its baseline survey of polymetallic sulphide deposits in the Pacific Ocean. He advised that Japan had signed a contract for the exploration for cobalt-rich ferromanganese crusts (CRC) in the Area on 27 January 2014 and provided a schematic image of JOGMEC's baseline survey. Dr Narita explained that samples were first stored in buffered formalin with Rose Bengal and then transferred into pure ethanol. Taxonomic results indicated the presence of seven phyla with seven classes, 23 orders and 24 families for the polymetallic sulphides area. With regard to the CRC area, multicores collected on six seamounts since 1997 found six phyla with five to eight classes in buffered formalin with Rose Bengal. Dr Narita concluded that a comparison of the two minerals showed that the sulphides contained mostly polychaetes and crustaceans, whereas CRC contained sipunculids and polychaetes.

IV. Establishing a DNA Barcode Library

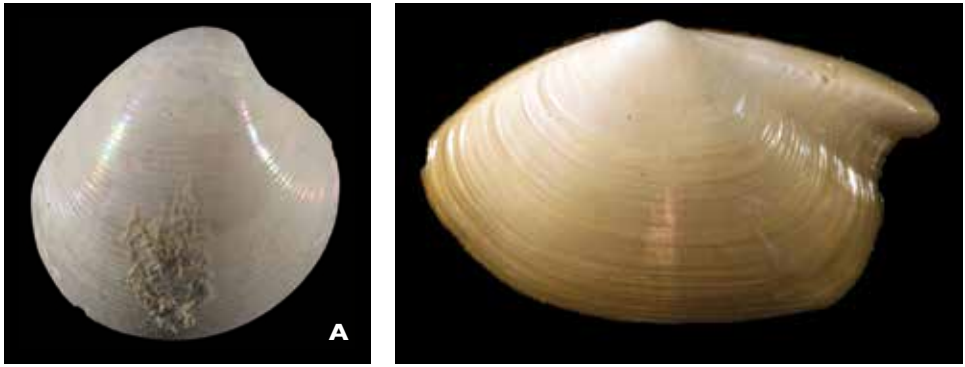

Figure 1: A: *Vesicomya galathea*, collected in Bahía de Panamá, 5°09'48"N, 81°41'12"W, 3900-4000 m. B: *Bathyspinula filatova* (B). Both species from the deep sea, they are fragile and not easy to sample. Photo credit: Paul Valentich-Scott, Santa Barbara Museum of Natural History

Figure 2: Unidentified amphipods from the deep sea collected at the Mid-Atlantic Ridge, 2500m. Courtesy T. Horton

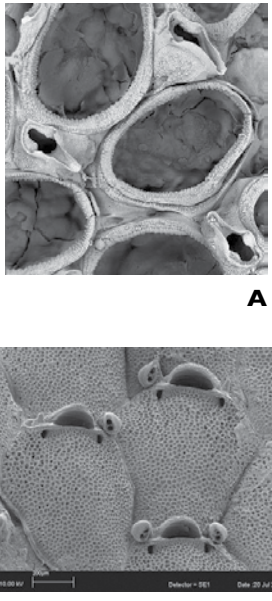

Figure 3: Lophophorate animals that live in large colonies, commonly known as bryozoans. Quite abundant in the deep sea. It contains soft and hard parts that provide taxonomic characters for their identification. SEM picture shows (A) a specimen of *Otomicropora otus* . The bottom SEM shows (B) a specimen of *Mangana magnesia* (B). Courtesy of D. Gordon.

ESTABLISHING A DNA BARCODE LIBRARY

Taxonomic challenges in the Area

There are several challenges associated with the taxonomy of macrofaunal deep-sea taxa in general and for the CCZ, in particular. Benthic communities are extremely diverse and many species that occur in samples such as singletons, species descriptions of deep-sea taxa are often incomplete, with many other species (up to 90% or more) new to science. Problems may also be encountered with regard to the correct evaluation of intraspecific variability, polymorphism, distinct life history stages and sexual dimorphism. Additionally, specimens are often damaged during sampling when essential parts needed for identification are lost (e.g. appendages or heads normally used to count abundances of taxa). In this context, the use of molecular tools to facilitate the morphological identification (also referred to in the literature as "reverse taxonomy" or "DNA taxonomy") is indispensable. Furthermore, there is evidence that the wide occurrence of cryptic species (species with a similar morphology but genetically distinct) in deep-sea taxa, underlines the need for molecular studies.

An integrative approach for baseline studies: DNA and morphology

Despite a rather long history of deep-sea sampling, including over 100 cruises to the CCZ during the last 40 years, the deep sea remains the habitat with the highest proportion of undescribed species. To speed up the necessary taxonomical work, 12 experts insisted on the use of an integrative (i.e. a molecular-morphological) approach for a consistent taxonomy in baseline studies for assessing the biodiversity of the CCZ and future environmental impact assessments. This implies the use of molecular tools in the preliminary phase. This allows for rough phylogenetic identification, or pre-sorting of the specimens, in genetic clusters, with subsequent morphological studies building on these results, hereafter referred to as "reverse taxonomy". For this approach, DNA sequences of the mitochondrial protein-coding gene cytochrome c oxidase I ('Folmer fragment', COI), or any other genetic marker with a good taxon sampling, available in GenBank, are used to determine MOTUs, based on a similarity threshold value characterizing the distinct genetic clusters. These DNA sequences can be submitted to a BLAST search of a sequence database (e.g. GenBank), which will allow a rough, initial identification. However, this identification of the sequence leads to accurate results only in cases where sequences of closely-related taxa are available. The initial diversity assessment allows comparison between samples and facilitates the subsequent morphological analyses of the identified MOTUs and the tentative phylogenetic assignment of the taxon at hand. With the advancement of these integrative studies, the obtained DNA sequences of macrofaunal taxa of the CCZ will feed a growing sequence database, which in the longer term will allow a direct and accurate species identification through these "barcodes".

Dr Thomas Dahlgren of Uni Research, Norway, introduced the advances in molecular methods and their interpretation for macrofaunal classification in the context of assessment and monitoring studies. He emphasized the importance of taxonomy as a parameter to measure the degree of population connectivity, variation in biodiversity and community structure across the CCZ.

Determining whether a specimen represents a particular species based solely on morphology is often difficult for deep-sea taxa since morphological descriptions can be incomplete, with holotypes in poor condition or unavailable, type localities not precisely defined and DNA sequence data absent. More pragmatic species descriptions are needed, where molecular data in combination with morphology are used to describe taxa. Molecular studies also provide information on species diversity and community structure, on the scale of species ranges and hence, their resilience to

disturbance, and the population structure. Cryptic species are common in shallow waters and have also been shown to occur in a wide range of deep-sea taxa including arthropods and annelids and the only way to discover cryptic species and describe them is by use of molecular data.

The scale of connectivity is an important factor for conservation and scales of genetic structure at around 150 km call for an ambitious sampling effort in the CCZ. A weak genetic structure (low F_{ST}) is also an important factor to be considered for biodiversity conservation.[1] Results also show that mtDNA (a general genetic marker available for all possible taxa), which populates wide samples of DNA barcode genes, can be used as a proper estimate for the analyses of population genetic structure and connectivity. When faunal lists, with associated DNA sequence data, have been established for the CCZ, eDNA sampling may represent an interesting method for macrofauna biodiversity assessment studies. However, much remains to be learnt about the nature of macrofauna (and megafauna) eDNA before the method can be recommended for use on a regular basis.

Dr Helena Wiklund of the Natural History Museum, London, UK, made recommendations for improving sampling and preservation techniques to optimize the preservation of the high number of rare animals for morphological and molecular studies. It was suggested that during on-board processing of the samples, time on deck should be minimized and careful sieving, with chilled seawater, should be undertaken. Specimens should ideally be live-sorted under a stereo microscope and then photographed to document the initial and best state in which the animal was found. Specimens are preserved in 80-95 per cent ethanol (depending if bulk-fixed or individual specimens) and kept chilled or frozen for storage. For each specimen, a tissue sample is taken after further and more detailed photographing. DNA is then extracted and COI amplified, optionally, together with other genes suitable for phylogenetic analyses. These sequences can be used for several purposes:

(i). species identification;

(ii). distribution and connectivity analyses;

(iii). phylogenetic analyses; and

(iv). species descriptions.

The proposed protocol is summarized in *Figure 1*.

1 FST or the fixation index is a measure of population differentiation due to genetic structure. Developed as a special case of Wright's F-statistics, it is one of the most commonly used statistics in population statistics.

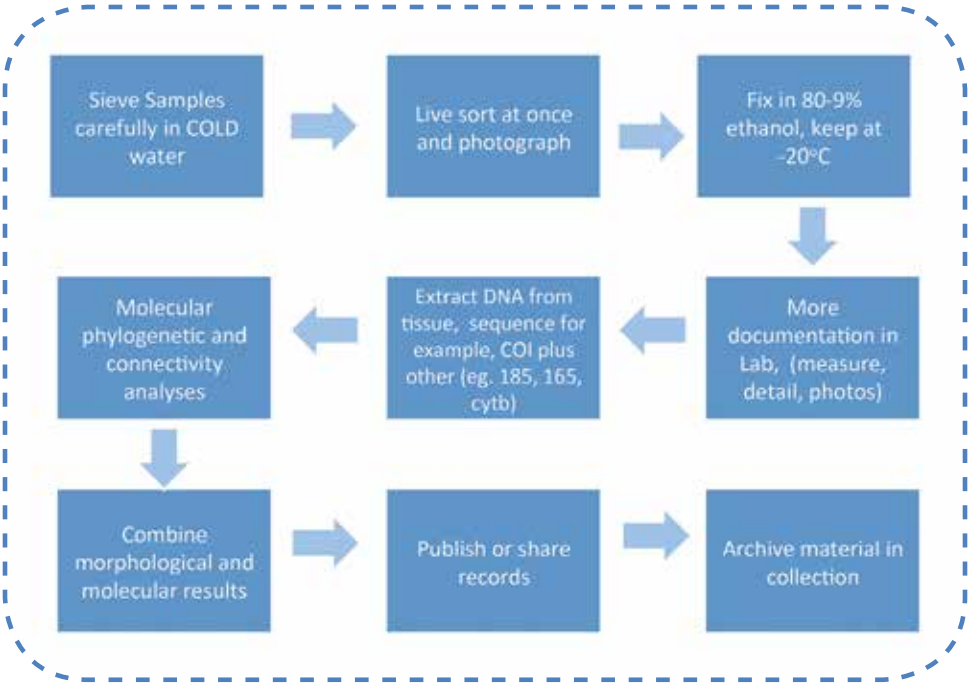

Figure 1: Flowchart summary of a proposed protocol applicable for integrative molecular and morphological studies

Dr Annika Janssen, of the German Centre for Marine Biodiversity Research, showed the results of a DNA barcoding study on polychaetes and isopods coming from recent cruises to the CCZ in the German and French licence areas. She highlighted the fact that nodule exploration was expected to start in the near future and thus, a rapid characterization of the nodule fauna is required. The reverse taxonomy approach was applied to test its efficiency to overcome the challenges for deep-sea macrofaunal taxonomy. DNA was extracted using the Chelex® method for both polychaetes and isopods collected and preserved in 96 per cent ethanol. The barcode marker COI was amplified using universal primers and subsequently outsourced for sequencing. MOTUs were identified based on a defined similarity threshold. For both polychaetes and isopods, the number of shared MOTUs between the two claim areas, situated 1,300 km apart, was very low (12% and 2%, respectively). Within the German licence area, the number of shared MOTUs were 27 per cent for polychaetes and 13 per cent for isopods between the area designated for mining with the one to be preserved, situated only 60 km apart. After morphological examination, it appeared that some species were composed of several genetic lineages, indicating that cryptic speciation is also relatively common for these taxa. Nonetheless, the sequencing success rate appeared to be quite low, only of 40-50 per cent for both groups. This study confirms that barcodes are critical for a robust assessment of the biodiversity and distribution patterns of CCZ macrofauna. Furthermore, they can be published in existing online repositories and easily exchanged between contractors.

Dr Charlotte Havermans, of the Royal Belgian Institute of Natural Sciences, introduced the combination of genetic and morphological studies on deep-sea amphipods. The use of DNA barcoding has been presented under two different approaches: (i) reverse taxonomy, where morphological studies build upon the results of the initial molecular results; and (ii) a "blind" taxonomic approach where taxa are treated as MOTUs.

The efficiency of the DNA barcoding method, based on the distribution of intra- vs. interspecific divergences, was tested for both shallow-water and deep-sea amphipods. Results showed a bimodal distribution of these two types of divergences as well as the presence of a barcoding gap, indicating the accuracy of the method for species identification. Furthermore, this approach also contributed to a better understanding of amphipod species' distributional ranges, testing whether species are genetically homogenous or not over large geographic and bathymetric ranges, and showed that deep-sea amphipod diversity is largely underestimated. Due to the significant number of barcoding amphipod studies, the use of threshold values to determine species as MOTUs becomes more efficient, hence the "blind" taxonomic approach on certain understudied deep-sea amphipod families could provide a baseline for large-scale comparative biodiversity studies. Finally, molecular studies do not only serve for rapid species assignments of specimens hard to identify, but also provide crucial information on the vulnerability of deep-sea regions to human-induced modification, ranging from genetic connectivity to general deep-sea biogeography.

Dr Magdalena Błażewicz-Paszkowycz of the University of Lodz, Poland, demonstrated results of genetic studies on Atlantic deep-sea tanaidaceans. She explained that precaution must be taken for an improved DNA extract quality and sequencing success rate of smaller-sized specimens, by the use of a "cool chain" for sampling. To do so, (epibenthic sledge) samples brought on deck should be washed with precooled seawater (4°C max.) or treated in a room at 4°C, preserved in precooled ethanol (-20°C) and stored at -20°C. Ethanol should be changed after 24 hours. During the first 24 hours after fixation, the container with the sample should be gently rotated (in order to avoid freezing of the water leaking from the fixed animals). Samples should be subsequently sorted on ice and eventually preserved in precooled ethanol at -20°C.

These precautions significantly improved the results of the molecular analyses. Dr Magdalena Błażewicz-Paszkowycz said COI sequences can be extremely useful for matching the different morphs of taxa or when the number of morphological characters is low.

V. Recommendations for the establishment of a DNA barcode library

Figure 4: Polychaetes from the deep sea. This group of macrofauna is one the most abundant although small in size, therefore difficult to sample appropriately. The top picture shows a specimen of (A) Polynoidea sp, collected in the south Pacific Ocean and (B) a picture of an specimen of *Ophiotryocha scutellus*, both collected at the deep sea. Images C and D, are annelids representatives of the polychaete fauna in the UKSRL claim area and collected from 4200 m depth and studied and photographed live onboard R/V Melville. Currently being identified using DNA taxonomy. Courtesy of D. Glover, T. Dahlgren and H. Wiklund.

Recommendations for the establishment of a DNA barcode library

Conserving the link between the voucher specimen and the barcode. The link between a voucher specimen and a DNA sequence is a crucial element for the proposed integrative studies. A voucher specimen is created by assigning a unique reference number to the specimen from which the tissue is taken. This is done through the steps carried out in the laboratory: DNA extraction, amplifications, purification and sequencing. Molecular voucher specimens as well as DNA extracts should be conserved in recognized long-term conservation facilities, such as national history museums.

Treatment and preservation of samples for molecular studies. Ideally, samples should be sorted live and preserved in 80-96 per cent ethanol. Specimens should be photographed before further handling. It is important for specimens to be photographed live as significant features for several taxa, such as presence of eyes and colour patterns, may disappear when the specimen is fixed. Examples include polychaetes. DNA extractions can be carried out on board the exploration vessel or at the home institute and should be stored at -20°C, preferably -80°C, for longer-term storage. Samples are preferably stored at -20°C until DNA has been extracted.

Establishing consistent methodologies and protocols for a standardized integrative taxonomic approach. Based on previous studies, standardized protocols for each taxon shall be established in order to ensure an optimal processing of the samples. Detailed information will be given regarding the method and tissue quantity used for DNA extraction, the primers used for COI amplification and optimal amplification parameters.

Increasing the speed and improving the quality of integrative studies through data sharing among contractors. The establishment of a solid and profitable DNA barcode database will only be possible if all contractors submit their obtained DNA sequences to a public repository, such as GenBank, or a common database in BOLD (http://www.boldsystems.org/). Accession numbers obtained after exploration cruises should be reported allowing analyses by experts for species identification and connectivity studies, as well as for subsequent morphological analyses. Upon accurate morphological identification of each of these specimens, this database will grow and become increasingly useful for comparative biodiversity studies and impact assessments. Hence, it is to the advantage of all contractors to provide this information after each study.

Protocols for molecular methods for macrofaunal taxonomy in areas beyond national jurisdiction

1. Standardized sampling methods for DNA studies

Fauna collected with different sampling gears (boxcorer, megacorer, epibenthic sledge or ROV) is sieved using chilled seawater and kept alive in seawater in the fridge at 4-8°C until further processing. All fauna is photographed alive while kept on ice, and then preserved in 80-96 per cent molecular grade ethanol. Samples are stored at -20°C. For mollusks, it is important to open a hole in the shell (preferably in the anterior end so as not to destroy important morphological characters) before preservation to allow the ethanol to penetrate the tissue.

The procedure for obtaining DNA sequences from sampled specimens will be detailed in the next section. This procedure includes the steps of DNA extraction and amplification. After a quality

check, PCR products can be sent to companies providing a sequencing service.[2] Hence, PCR purification and DNA sequencing protocols are not included, but can be obtained upon request.

2. Tissue sampling and DNA extraction

Photographing

Animals are photographed in detail and measured before extraction.

Tissue sampling

A bit of tissue for extraction, at least approximately 0.5 mm x 0.5 mm, is taken from each animal, saving enough material to keep for vouchers. For several taxa, specific body parts should be removed in order to preserve as much of the taxonomically important characteristics of the animal as possible, so that it can be used for further species identification and, potentially, species descriptions.

This semi-destructive method of tissue sampling is specified for each taxon in Table 1. In the case of crustaceans possessing a robust carapace, it might be necessary to crush the carapace in order to ensure the penetration of the digesting enzymes into the tissue.[3]

2 Polymerase Chain Reaction (PCR) is a relatively simple and inexpensive tool that can be used to focus on a segment of DNA and copy it billions of times over. PCR is used every day to diagnose diseases, identify bacteria, match criminals to crime scenes, and in many other ways (Source: University of Utah, learngenetics.utah.edu/content/labs/pcr)

3 A carapace is a dorsal (upper) section of the exoskeleton or shell in a number of animal groups, including arthropods such as crustaceans and arachnids as well as vertebrates such as turtles and tortoises.

Table 1. Specific guidelines for each deep-sea taxon studied: the tissue to be sampled for DNA extraction, the method used and the primers for PCR. Specific primers are detailed in Table 2.

	Tissue used for extraction	DNA extraction	Primers for COI	Primers for 16S
Amphipoda	1 (or more) pleopod(s)*	Spin-column method	Folmer	16Sar–16Sbr AMPH1–AMPH2
Isopoda	1 (or more) pereopod(s), preferably P2-4*, from the same side	Chelex® or spin-column method	Folmer	/
Tanaidacea	One cheliped, or 1 (or more) pereopods preferably P3 and P5	Chelex® or spin-column method	jgLCO – jgHCO, LCO1490 – Nancy	16S SF – 16S SR
Polychaeta	1 -3 parapodia or 1-2 segments (0.5 mm x 0.5 mm)	Chelex® or spin-column method	polyLCO – polyHCO; LCO Folmer – COIE	Ann16SF–Ann16SR 16Sa – 16Sb
Bivalvia	Preferably adductor muscle,* not from the foot	spin-column method	Folmer; polyLCO – polyHCO	Ann16SF–Ann16SR; 16Sa – 16Sb
Gastropoda	Any tissue	spin-column method	Folmer; polyLCO – polyHCO	Ann16SF–Ann16SR; 16Sa – 16Sb
Aplacophora	Tissue from the mid body,* front and back ends should remain intact	spin-column method	Folmer; polyLCO – polyHCO	Ann16SF–Ann16SR; 16Sa – 16Sb
Scaphopoda	Tissue from the mid body,* front and back ends should remain intact	spin-column method	Folmer; polyLCO – polyHCO	Ann16SF–Ann16SR; 16Sa – 16Sb
Brachiopoda	Any tissue	spin-column method	Folmer	Ann16SF–16SbrH
Cnidaria	Polyps or bits of tissue	spin-column method	Folmer	Ann16SF–16SbrH
Bryozoa	Any tissue	spin-column method	Folmer	Ann16SF–16SbrH
Porifera	Bits of tissue from anywhere	spin-column method	polyLCO – polyHCO	/
Echinodermata	Small tissue	spin-column method	Folmer	16SarL–16SbrH; Ann16SF–16SbrH

Isolation of DNA

DNA can be extracted using several methods, such as (among others), (i) the Chelex®method; (ii) spin-column based methods using extraction kits; and (iii) the phenol-chloroform method. The first represents a quick and cheap method to process a large number of specimens and, additionally, provides an adjustable quantity of DNA. However, DNA is not purified prior to PCR. This may lead to poor preservation of DNA and, thus, is less suitable for long-term storage, whereas the second allows for longer-term storage of DNA samples in the provided elution buffer. If DNA is required from mucous tissue (e.g. from molluscs) or if none of the methods above has been successful the phenol-chloroform method may be used (Winnepenninckx et al. 1993).

Conservation of DNA extracts

DNA extracts should be stored at -20°C, preferably -80°C to ensure usage of the samples after a long period.

For each deep-sea taxon that has been studied with molecular tools, particular methods and taxon-specific guidelines, which can help the contractors to carry out this work faster and more efficiently, will be provided in this document.. Table 1 provides detailed information on the tissue used for DNA extraction, the extraction method and the primers used for amplification for each of the taxa studied so far.

The Chelex® extraction method (method by Dr A. Janssen for isopods/polychaetes): Chelex® 100 BioRad was used to extract DNA from tissue samples, according to Walsh et al. (1991). The DNA was extracted according to the following protocol:

(a) tissue was dissected from the organism and then washed in distilled water several times;

(b) tissue was transferred into 30 µl of Chelex (adjustable, depending on the size of the tissue) in 0.2 ml PCR tubes;

(c) this was followed by incubation in a thermocycler: for 60 min at 56°C, followed by boiling for 10min at 99°C;

(d) the sample was centrifuged for 30 seconds at 6000 rpm; and

(e) the supernatant was used as template for amplification.

Method using extraction kits for tissue samples. Protocol by the manufacturer and consumables are all provided in the kit.

3. DNA amplification

The PCR for the "Folmer fragment" of the gene cytochrome c oxidase subunit I (COI) is carried out using a range of primers. The ones best suited to the respective animal group are used.
The universal primers recommended for amplifying this fragment are LCO1490 and HCO2198 (Folmer et al. 1994) and they work for most animal groups. Other genes (e.g. 16S, 18S rDNA) can be used in the instances where:

(i) COI is not informative for species delimitation in certain taxa;

(ii) an extensive optimization of the protocol is required;

(iii) COI amplification encounters problems; or

(iv) other genes have been more frequently used than COI for a particular taxon, allowing a better comparison.

A primer list for COI and other genes is provided in *Table 2*; the same primers are used for sequencing.

Table 2. List of primer pairs from 5' to 3' end that have been tested for deep-sea taxa for amplification and sequencing COI, 16S and 18S rDNA. The specified primers for COI are used for amplifying the "Folmer" fragment, i.e. the region used for DNA barcoding approaches. The "Folmer" primers (LCO1490 and HCO2198) are universal primers and are being used for most animal groups. COI-E was designed for oligochaetes and has been used with success together with LCO1490 for polychaetes. Primers polyLCO and polyHCO were designed for polychaetes. The primer pair 16Sar – 16Sbr represent universal primers used for amplifying 16S in most animal groups. 16SbrH is a variant on 16Sbr and can be used with 16Sar. AMPH1 – AMPH2 were designed for amphipods and are internal to the universal primers; Ann16SF – Ann16SR were designed for oligochaetes but are also successfully applied on polychaetes and several other invertebrate groups. 16Sa and 16Sb are primers used for (bivalve) mollusks. 16S SF – 16S SR have been tested and approved both for isopods and tanaidaceans.

Gene	Primer name	Primer sequence	Reference
COI	LCO1490(F)	GGTCAACAAATCATAAAGATATTGG	**Folmer** et al. 1994
	HCO2198(R)	TAAACTTCAGGGTGACCAAAAAATCA	**Folmer** et al. 1994
	COI-E(R)	TATACTTCTGGGTGTCCGAAGAATCA	Bely & Wray 2004
	polyLCO(F)	GAYTATWTTCAACAAATCATAAAGATATTGG	Carr et al. 2011
	polyHCO(R)	TAMACTTCWGGGTGACCAAARAATCA	Carr et al. 2011
	Nancy	CCCGGTAAAATTAAAATATAAACTTC	Simon 1994
	jg LCO	TITCIACIAAYCAYAARGAYATTGG	Geller et al. 2013
	jg HCO	TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. 2013
16S	16Sar	CGCCTGTTTATCAAAAACAT	Palumbi et al. 1991
	16Sbr	CCGGTTTGAACTCAGATCATG	Palumbi et al. 1991
	16SbrH	CCGGTCTGAACTCAGATCACGT	Palumbi et al. 1996
	AMPH1	GACGACAAGACCCTAAAAGC	France & Kocher 1996
	AMPH2	CGCTGTTATCCCTAAAGTA	France & Kocher 1996
	Ann16SF	GCGGTATCCTGACCGTRCWAAGGTA	Sjölin et al. 2005
	Ann 16SR	TCCTAAGCCAACATCGAGGTGCCAA	Sjölin et al. 2005
	16Sa(F)	CGCCTGTTTATCAAAAACAT	Xiong & Kocher 1991
	16Sb(R)	CTCCGGTTTGAACTCAGATCA	Xiong & Kocher 1991
	16S SF	GACCGTGCTAAGGTAGCATAATC	Riehl et al. 2014
	16S SR	CCGGTCTGAACTCAAATCGTC	Tsang et al. 2009

The 25 µl PCR reactions comprise 0.02U/µl Taq DNA polymerase, 0.2mM dNTPs, 0.5 µM of forward and reverse primers, 1× PCR-buffer and 1 µl (about 30ng) of template DNA. PCR

conditions are often based on the following scheme: an initial denaturation at 94°C (or 95°C for 16S) for 2-3 minutes, followed by a number of cycles (30-40) consisting of

(i) 20-40 seconds at 94 (95)°C for 20 seconds,

(ii) 20-40 seconds at 42°C (45-50°C; annealing T), and

(iii) 60 seconds at 65-72°C (extension) and then a final elongation step at 65-72°C for 10-15 minutes, before cooling down to 4°C. Storage of PCR products can be done in a fridge as long as the products are processed for quality control and sequencing in a few days. If not, PCR products should be frozen at 20°C.

Gel electrophoresis and Sanger Sequencing[4]

PCR-products that produced clear light bands after electrophoresis on 1-2 per cent agarose gel can be sent to sequencing companies, using the same set of primers as used for the PCR. Before the resulting sequences are further analysed, electropherograms shall be checked for ambiguous base calls and sequences translated into amino acids shall be checked for stop codons. This quality control is necessary to avoid inclusion of pseudogenes in the analyses, leading to false identifications.

4 Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and protein) and their fragments, based on their size and charge; Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in-vitro DNA replication.

VI. Workshop Recommendations

WORKSHOP RECOMMENDATIONS

The following recommendations were formulated from the discussions among taxonomic experts and contractor representatives and are grouped under four subheadings: a) sampling and preservation; b) taxonomic resolution; c) technical co-operation; and d) data access and availability.

Sampling and Preservation

(a) The workshop recommends that contractors undertake biologically-focused sampling cruises and that mixed discipline cruises allocate sufficient ship berth space for biological teams. Sufficient wire time must be allocated to be able to: extract enough samples for biology by defining an area of research - both the scientific aims and spatial extent; take samples randomly in the research area as a preliminary survey; and, analyse the samples and assess the species cumulative curve. This would make the estimation of the number of samples required to provide adequate assessment of the number of species possible. Repeated sampling to overcome any shortfall would also need to be done.

(b) The workshop strongly recommends that ISA organize and convene a workshop on Strategic Environmental Assessment (SEA), the Environmental Impact Assessment (EIA) process, and its application in the CCZ and to bring together the appropriate experts as soon as was practical.

(c) The workshop recommends that box core samples for biology should not be divided into subsamples for other disciplines.

(d) Contractors were urged to use the LTC guidelines in the processing of box core samples and that if they use any additional sample subdivision or processing steps, this be reported in the annual report to the ISA.

(e) Contractors were urged to take samples for molecular taxonomic analyses to support greater accuracy in biodiversity and ecological assessments.

(f) Contractors were urged to follow the most appropriate 'cold-chain' protocol to support molecular samples

(g) Contractors were urged to deposit all identified material (including molecular samples) in an internationally-recognized museum or collections facility. The material should be freely accessible for study and be able to be sent on loan to researchers. The need for material to be archived in an appropriate collections facility should be specified in the contractor's plan of work and the contractor should identify the facility in which their material will be deposited. The transfer of taxonomic material to the collections facility will have to be financed by contractors and should also be detailed in the plan of work.

(h) The workshop notes that the epibenthic sledge device should be included as part of the sampling for baseline studies.

Taxonomic resolution

Specimens must be identified to species level and follow the World Registry of Marine Species (www.marinespecies.org).

Technical cooperation

(a) The participants recommended that a technical cooperation framework should be established to support:

(i) Training

(ii) Visiting-scientist programmes

(iii) Joint industry/academic partnerships

(iv) Taxonomic consistency and quality control

(v) The participants strongly urge the ISA to develop a technical cooperation framework to enable the building of support which will be essential to advancing our understanding of the risk and impacts of mining in the CCZ.

(vi) The participants urge the ISA to establish a mechanism which would support contractors seeking taxonomic expertise – a form of taxonomic clearing house mechanism.

(vii) The participants recommend that the Authority develop a series of inter-calibration workshops to bring together contractors with groups of taxonomists to review and assess collections made in different contract areas.

(viii) The Authority should use the provided list of deep-sea taxonomists to create an expert panel to assist them in the assessment and validation of species level assessments as part of the annual data returns.

(ix) Encourage contractors to collaborate among themselves and with relevant academic partners to address gaps and provide consistency, particularly in taxonomic identification.

Data access and availability

(a) The workshop fully supports the Authority's plans to develop the existing database. We urge researchers and contractors to work with the Authority to provide expertise in developing the taxonomic modules.

(b) Taxonomists working on material from the CCZ were urged to publish their data in recognized scientific journals as soon as was practical.

(c) Participants were encouraged to facilitate the publication of special volumes in appropriate scientific journals, such as ZooKeys (*http://www.pensoft.net/journals/zookeys/*) and Zootaxa (*http://www.mapress.com/zootaxa/*), to enable taxonomists to describe the fauna of the CCZ as soon as sufficient material is available.

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