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Opinion paper

A primer for use of genetic tools in selecting and testing the suitability of set-aside sites protected from deep-sea seafloor massive sulfide mining activities



Rachel E. Boschen^{a, b, *}, Patrick C. Collins^c, Verena Tunnicliffe^d, Jens Carlsson^c,
Jonathan P.A. Gardner^b, Jonathan Lowe^e, Ann McCrone^f, Anna Metaxas^g,
Frederic Sinniger^{h, i}, Alison Swadling^j

^a National Institute of Water and Atmospheric Research, Private Bag 14901, Wellington, New Zealand

^b School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington 6140, New Zealand

^c Area 52 Research Group, School of Biology & Environment Science and Earth Institute, University College Dublin, Belfield, Dublin, Ireland

^d Department of Biology & School of Earth and Ocean Sciences, University of Victoria, British Columbia V8W 2Y2, Canada

^e Nautilus Minerals Inc., PO Box 1213, Milton, Queensland, Australia

^f WWF-New Zealand, PO Box 6237, Wellington 6041, New Zealand

^g Department of Oceanography, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H4R2, Canada

^h Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima, Yokosuka, Kanagawa, Japan

ⁱ Tropical Biosphere Research Center, University of the Ryukyus, 2422 Sesoko, Motobu, Okinawa, Japan

^j Deep Sea Minerals Project, Secretariat of the Pacific Community, Suva, Fiji

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ABSTRACT

Seafloor massive sulfide (SMS) mining will likely occur at hydrothermal systems in the near future. Alongside their mineral wealth, SMS deposits also have considerable biological value. Active SMS deposits host endemic hydrothermal vent communities, whilst inactive deposits support communities of deep water corals and other suspension feeders. Mining activities are expected to remove all large organisms and suitable habitat in the immediate area, making vent endemic organisms particularly at risk from habitat loss and localised extinction. As part of environmental management strategies designed to mitigate the effects of mining, areas of seabed need to be protected to preserve biodiversity that is lost at the mine site and to preserve communities that support connectivity among populations of vent animals in the surrounding region. These “set-aside” areas need to be biologically similar to the mine site and be suitably connected, mostly by transport of larvae, to neighbouring sites to ensure exchange of genetic material among remaining populations. Establishing suitable set-asides can be a formidable task for environmental managers, however the application of genetic approaches can aid set-aside identification, suitability assessment and monitoring. There are many genetic tools available, including analysis of mitochondrial DNA (mtDNA) sequences (e.g. *COI* or other suitable mtDNA genes) and appropriate nuclear DNA markers (e.g. microsatellites, single nucleotide polymorphisms), environmental DNA (eDNA) techniques and microbial metagenomics. When used in concert with traditional biological survey techniques, these tools can help to identify species, assess the genetic connectivity among populations and assess the diversity of communities. How these techniques can be applied to set-aside decision making is discussed and recommendations are made for the genetic characteristics of set-aside sites. A checklist for environmental regulators forms a guide to aid decision making on the suitability of set-aside design and assessment using genetic tools. This non-technical primer document represents the views of participants in the VentBase 2014 workshop.

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1. Introduction

Deep-sea mining is rapidly becoming a reality, with deposits of

* Corresponding author. Department of Biology & School of Earth and Ocean Sciences, University of Victoria, British Columbia V8W 2Y2, Canada.

E-mail address: reboschen@uvic.ca (R.E. Boschen).

seafloor massive sulfides (SMS), polymetallic nodules and cobalt-rich crusts currently of commercial interest. In the case of SMS mining, exploitation is expected to occur in the southwest Pacific before 2020 (Baker and Beaudoin, 2013). SMS deposits are formed through the rapid precipitation of minerals as hot, sulfide-rich hydrothermal fluids are cooled by ambient seawater at seafloor vents, often creating chimney structures on the seafloor. The resulting deposits are often rich in gold, silver, and base metals, such as lead, copper and zinc (Krasnov et al., 1995). Globally there are 165 recorded SMS deposits (Hannington et al., 2011) that exist across a range of depths and geographical locations (reviewed by Boschen et al., 2013).

The hydrothermal environment that forms deposits also supports unique chemosynthetic communities that are reliant on the hydrothermal activity at these deposits to survive (reviewed by Van Dover, 2000). Where hydrothermal activity has ceased, relict (inactive) deposits are colonised by diverse communities characterised by slow growing sessile suspension-feeders (Galkin, 1997; Collins et al., 2012; Boschen et al., 2015a). The communities inhabiting both inactive and active deposits are vulnerable to disturbance, with mining activities expected to remove all large organisms and their habitat in the immediate exploitation area (Van Dover, 2011). One of the mitigation strategies is to preserve genetic diversity within the region by providing “set-aside” areas with similar physical and biological characteristics to the mine site that are designated as no-impact zones (Coffey Natural Systems, 2008; International Seabed Authority, 2010, Collins et al., 2012). To be effective, these set-aside sites need to support communities with taxonomic composition, abundance and diversity similar to the mine site. The populations of species at the set-aside site also need to have genetic properties similar to those found at the mine site and to be connected to other populations in the region as part of a coherent network, with high connectivity among sites (International Seabed Authority, 2011; Van Dover et al., 2012). With mining cessation, it is possible that the altered habitat may also sustain some recolonization from these set-aside areas, although this will depend upon the scale and nature of habitat regeneration.

However, assessing the suitability of a set-aside site or the connectivity within a network of sites is a considerable challenge to environmental managers. To assist this assessment, there are a number of techniques available, of which genetic tools are a subset. These tools can be used to assess the diversity of communities at sites and use the natural genetic variability of individuals and populations to assess the genetic structure of, and the connectivity among, neighbouring populations. This information can be used to determine if potential set-asides have similar biodiversity to the mine site, to identify populations that are potentially more vulnerable to mining disturbance and to identify populations that are sufficiently diverse and well connected to help maintain regional genetic diversity or to facilitate the recovery of mined sites. As such, genetic tools can be used to help identify suitable set-aside sites and assess the connectivity among sites within a network. An example of such an approach was developed to support the proposal for a network of areas of particular environmental interest set aside in the Clarion-Clipperton polymetallic nodule region in the north Pacific (Smith et al., 2008).

The aim of this document is to provide best practice recommendations for using current genetic tools to select and assess the suitability of either individual or a network of set-aside sites in the context of potential future mining of SMS deposits. The document includes a brief overview of communities inhabiting SMS deposits and the distribution of hydrothermal vent fauna; introduces the concept of population and genetic connectivity within vent systems; discusses the concept of the set-aside; provides an overview of the genetic tools currently available for set-aside assessment;

and outlines how genetic tools can be used during the stages of set-aside selection, assessment and long term monitoring. We also provide a checklist for regulators and environmental managers regarding the suitability of a proposed set-aside in terms of genetic connectivity. This document stems from discussions at the VentBase 2014 meeting at the National Institute of Water and Atmospheric Research, New Zealand. This workshop followed on from VentBase 2012, which produced a similar guideline document on Environmental Impact Assessment development for SMS mining (Collins et al., 2013a). VentBase was established as a forum where academic, commercial, governmental and non-governmental stakeholders can develop a consensus regarding the management of exploitation in the deep-sea, specifically the mining of SMS deposits. A primary goal of VentBase is the production of best-practice documents that can inform stakeholders and highlight the most up-to-date science in order to underpin effective management (Collins et al., 2013b; <http://www.indeep-project.org/ventbase>).

2. Biology of SMS deposits and the distribution of vent fauna

Biological communities of macrofauna (animals < 2 and > 0.5 cm) at SMS deposits fall into three broad categories: (1) vent endemic hydrothermal communities dependent on a chemosynthetic food web associated with active venting of hydrothermal fluids; (2) a halo/peripheral community usually at a short distance from active venting; and (3) the fauna of inactive SMS deposits, where venting has ceased. In the last two habitats, opportunistic ‘background’ fauna that typically characterize other deep-sea habitats may congregate to take advantage of additional food, such as bacterial mat dislodged from the vents (Erickson et al., 2009).

Biological communities associated with hydrothermally inactive SMS deposits harbour many taxa similar to those encrusting hard substrata in the deep sea (Galkin, 1997; Collins et al., 2012), although there are a limited number of studies. Levels of endemism among taxa on these deposits are poorly described but a specialised fauna adapted to the weathered sulfide environment (Van Dover, 2007, 2011) may exist, and a recent study identified faunal assemblages that appear to be unique to inactive SMS deposits (Boschen et al., 2015a). These organisms are typically sessile, slow-growing suspension feeders (Galkin, 1997; Collins et al., 2012; Boschen et al., 2015a) and would likely take decades to recover from mining disturbance, if they recover at all (Van Dover, 2011; Boschen et al., 2013).

The vent fauna inhabiting hydrothermally active areas exists in close proximity to hydrothermal flow, because it is reliant on the primary production of chemosynthetic bacteria that use reduced substances in the vent fluids for energy (reviewed by Van Dover, 2000). Vent communities typically contain relatively few species but individual abundance and overall biomass can be large (Grassle, 1985). Vent animals typically have rapid growth rates, enabling them to mature rapidly and to colonise new vent habitat via larval dispersal (Lutz et al., 1994). Although vent communities undergo natural disturbance, such as habitat loss through changes in hydrothermal or volcanic activity (Lutz et al., 1994; Tunnicliffe et al., 1997), perturbation from mining activities could pose an additional stressor, with the potential for cumulative impacts to negatively affect vent species (Van Dover, 2011). As the vast majority of vent species cannot survive away from hydrothermal activity, vent communities should be considered to be at high risk from anthropogenic activities, such as deep-sea mining and drilling, which are expected to remove hydrothermal habitat and to change remaining areas (Van Dover, 2011, 2014; Nakajima et al., 2015).

On a global scale, vent communities differ across oceans and regions, known as biogeographic provinces. There are many

biogeographical models for vent fauna (Mironov et al., 1998; Tunnicliffe et al., 1998; Van Dover et al., 2002; Bachraty et al., 2009; Moalic et al., 2012), with a recent review suggesting eleven biogeographic provinces (Rogers et al., 2012). The existence of provinces means there may be different vent communities inhabiting potential mine sites in different regions. For example, vent communities in the Central Southwest Pacific province are dominated by gastropods, mussels and tubeworms (Sen et al., 2014), communities in the Mid-Atlantic Ridge province are dominated by shrimp and mussels (Murton et al., 1995) and the East Scotia Ridge province is dominated by Kiwa crabs and stalked barnacles (Rogers et al., 2012).

Hydrothermal vents have a patchy distribution on mid-ocean ridges, island arcs, back-arc basins and seamounts (Hannington et al., 2011; Boschen et al., 2013). Within a vent field, the spatial distribution of organisms is directly related to the chemical composition of the vent fluid and the distance from the fluid source. Zonation of species can occur at scales of hundreds of metres from the vent source as a result of spatial changes in the influence of venting (Sudarikov and Galkin 1995), with patches of hydrothermally active habitat often interspersed with inactive SMS areas, or located within large inactive areas. At other locations (e.g. vents on the Juan de Fuca Ridge), smaller patches of species of different successional stages and environmental tolerances can be interspersed on a single chimney (Sarrazin et al., 1997). Transitions between distributional patterns can occur at time scales from months to years to decades, and are regulated by changes in the intensity of fluid flux and in the chemical composition of the fluid. For non-motile species, changes in vent fauna distributional patterns occur at the death of existing individuals and colonization by larvae. When the dominant species are mobile, individuals can adjust their location in response to changes in the environment and directly influence distribution patterns (Sen et al., 2014).

Although the fauna in inactive SMS deposits is less characterized compared to those at active deposits, the vent fauna are considered to be at greater risk from habitat loss and localised extinction (Van Dover, 2011, 2014). In order to assess the potential impact of seabed mining on communities at SMS deposits, environmental managers need information on the distribution and connectivity of faunal populations to determine the vulnerability of regional connectivity networks to disruption, and to assess the potential for recolonization at the affected site. In particular, they require information about the connectivity of vent-endemic populations, which are particularly at risk from SMS mining activities that alter fluid flows. Hydrothermal habitat is patchy, so that habitat change or loss poses a considerable threat to the persistence of vent species that are often endemic to the biogeographic region.

3. Population connectivity

Population connectivity describes how individuals or groups of individuals from the same species are able to move between populations and, in the case of genetic connectivity, the extent they are able to exchange genes. Vent habitat is patchy, and sometimes ephemeral, due to changes in the source of hydrothermal flow. Thus, for spatially fragmented populations of vent fauna distributed over individual vents or vent fields, maintaining connectivity among populations could be complex. The persistence and maintenance of these populations is determined by the balance between the loss of individuals and the provision of new recruits to the population, which can either be supplied by the resident population or neighbouring populations. Ultimately the nature of genetic exchange among these populations will determine the persistence of healthy populations and the recovery of extirpated ones.

Although some vent species are mobile as adults (fish, crabs)

and may disperse between vents, most vent-endemic animals are sessile as adults. For these organisms, the larva is the motile dispersal phase that enables connectivity among sites. These early life stages are released into the water column where they may spend prolonged periods, enabling dispersal over considerable distances. For example, larvae of the vent tubeworm *Riftia pachyptila* can drift in the water column for 38 days (Marsh et al., 2001), whilst *Bathymodiolus* mussel larvae can spend a year as plankton (Arellano and Young, 2009). The distance travelled by these larvae will depend on a variety of factors, such as larval behaviour and ocean currents (Hilário et al., 2015).

Most larvae are small (<0.5 mm), passive particles moved by water currents (White et al., 2010). Current directions may vary with both height above the seafloor and over time, thus larval dispersal patterns can change with depth and season. For example, current reversals at 9°N along the East Pacific Rise (EPR) restrict the dispersal of *R. pachyptila* to 100 km south and 47 km north along the ridge axis (Marsh et al., 2001). With other flow regimes, the same species may travel much further: current regimes at 13°N EPR are thought to extend the dispersal distance of *R. pachyptila* to 245 km (Marsh et al., 2001). Larval behaviour can also influence dispersal, as larvae that can alter their buoyancy or develop increased swimming capacity can enter different flow regimes and undergo different dispersal trajectories (Mullineaux et al., 2005; Adams et al., 2012).

Following dispersal, for larvae to recruit to the population they need to find suitable habitat on which to settle, survive to adulthood and reproduce. However, not all individuals in a vent field have equal opportunity to contribute to the next generation; many rarely or never reproduce because conditions for maturation to adulthood are too poor (Mullineaux et al., 2005; Tunnicliffe et al., 2014). Although the persistence of vent populations is generally regulated by local larval supply from populations within the same vent field (Metaxas, 2004; Mullineaux et al., 2005), some extirpated populations may only recover through colonization from distant unaffected populations. Recovery rates will depend on distance from the potential source populations, and the composition of the colonists will depend on the larval community composition at the time of colonization (Marcus et al., 2009; Metaxas and Kelly, 2010; Mullineaux et al., 2010).

In areas where seabed mining occurs, it is likely that all fauna inhabiting the SMS deposit will be removed and the habitat highly modified. Biological recovery of this area can only occur if the appropriate habitat conditions exist for recruits (e.g., substratum, fluid flow, suspended sediment) and if recruits are available from populations outside the impacted area. To determine which populations may be able to provide recruits to a proposed mining site, environmental managers need to know the connectivity among populations in the wider region before mining activity commences. However, colonization rates of most species are not known, and population connectivity estimates are complicated by a paucity of information about population size, reproductive biology, larval duration and ocean currents (Hilário et al., 2015). One option available to environmental managers to start elucidating patterns in population connectivity is the use of genetic tools in genetic connectivity assessments.

4. Genetic connectivity

Genetic connectivity relates to the exchange of genetic material among populations. Those populations that exchange more genetic material between them are more genetically similar and are considered to be more connected (Waples and Gaggiotti, 2006). As most of the exchange of genetic material in vent populations occurs through larval dispersal we may consider larvae as 'packages of

genes'. Movement of genetic material between populations is known as gene flow.

4.1. Population connectivity can be measured through gene flow

The patchy occurrence of vent habitat means vent species are often divided into spatially discrete populations that are usually connected by gene flow, although in some cases vent populations can be self-sustaining. Gene flow among populations tends to be homogenising by preventing genetic differentiation of populations (Kelly and Palumbi, 2010; White et al., 2010). Because recruits reflect the genetic composition of the adult population at their site of origin, we can use this genetic signal at recipient sites to quantify the magnitude and direction of movement of larvae between sites (Apte and Gardner, 2002; Wei et al., 2013) and deduce patterns in genetic connectivity among populations.

4.2. Genetic variability in populations

Natural selection allows populations to adapt continuously to the local environment as it favours certain genetic variants within populations. The numerous factors that influence population connectivity introduce variability in their genetic composition. Genetic variation is important because loss of genetic variability can eradicate unique local adaptations and may also hamper the capability of a population to adapt to environmental stresses, thereby reducing its resilience. Similarly, rapid reductions in population sizes (contractions) can reduce the genetic variability of populations and negatively affect population viability and adaptability (Allendorf et al., 2013).

The age structure and genetic composition of a population may vary over time and can reflect the age of the vent site, how well connected that site is to other sites (affecting influx of individuals), and events that affect growth and mortality. In relatively short-lived vent systems where vent site life-spans may be measured in decades, such as those found on volcanically dominated mid-ocean ridges, gene flow can be highly variable in time and space (Matabos et al., 2008). Other vent sites are extant for thousands of years, especially those accumulating massive sulfide deposits (Jamieson et al., 2013). However within these sites, individual vent outlets may open and close, with related changes in gene flow among populations. The genetic variability among different age groups within the population (cohorts) can also provide information on patterns of connectivity among populations, including unusual occurrences such as mass spawning events triggered at specific sites.

4.3. Effective population size

The effective population size is the number of individuals within a population that contributes offspring to the next generation (Neel et al., 2013). Effective population size differs from population size as not all individuals contribute offspring. Although one adult individual may be capable of producing hundreds of thousands, even millions, of offspring, in many populations only a small number of adults contribute to future generations in each reproductive pulse (Hedgecock et al., 2007). Thus, only a small proportion of individuals within a vent population may actually pass on genes to subsequent generations during any specific breeding event. This effect, due primarily to random or unpredictable events associated with habitat patchiness, environmental variability and fertilisation success, and very high levels of larval mortality, contributes to variability in the number and genetic composition of larvae from any one site from one generation to the next (Hedgecock, 1994; Hedgecock and Pudovkin, 2011). The result is temporal variability

in the number and genetic composition of recruits arriving at a site.

Effective population size is an important consideration in conservation genetics and the management of marine populations (Allendorf et al., 2013). An additional concept in marine ecology that describes groups of populations where individuals rarely interbreed but are connected by dispersing larvae is the metapopulation, also sometimes called the "patch model" (Gaines et al., 2007). For a metapopulation to be sustained in a region where mining has occurred, the network of connectivity must be maintained. Also, there need to be sufficient individuals to contribute to the next generation within and among vent fields, possibly also seeding habitat recovering at the mined site.

4.4. Models of genetic connectivity

Various models describe connectivity among populations; two of the most applicable concepts at vents are isolation-by-distance and panmixia. In the isolation by distance model, genetic differentiation increases with geographic distance, so that distant populations are less likely to exchange larvae, and genetic differentiation among geographically distant populations will be more pronounced. Vent sites can exist in a stepping stone manner along ridges, with active sites and their resident populations separated by distances of a few to hundreds of kilometres. Many vent species follow the isolation by distance model, as reviewed by Vrijenhoek (2010), including the amphipod *Ventiella sulfuris* (France et al., 1992), polychaete worm *Alvinella pompejana* (Hurtado et al., 2004) and tube worms *Tevnia jerichonana* (Hurtado et al., 2004) and *Riftia pachyptila* (Black et al., 1994; Coykendall et al., 2011). In the case of isolation by distance, there may be a stepping-stone supply of larvae, and it is possible a site further down the chain may be starved of recruits if an intermediary site is removed through mining activity.

Panmixia occurs when gene flow among populations is so high that there are no significant genetic differences between populations. This model has been suggested for a number of vent species, including some bathymodiolid mussels (*Bathymodiolus platifrons*, *B. japonicas*, *Bathymodiolus thermophilus*: Craddock et al., 1995; Miyazaki et al., 2013); the mussel *Gigantidas gladius* (Boschen et al., 2015b); limpet *Lepetodrilus nux* (Nakamura et al., 2014); and shrimp *Rimicaris exoculata* (Teixeira et al., 2012).

Because patterns in genetic connectivity can operate on scales from organisms on a chimney to vent fields within a region, connectivity surveys should consider a nested design to investigate the multiple scales of connectivity (Thaler et al., 2011). Patterns in connectivity also change over time and vary between regions, so that similar species in different regions and different species within the same region may demonstrate different models of connectivity. When assessing the impacts of mining activities on genetic connectivity, it is necessary to conduct new connectivity investigations for each mining region and for a number of species to capture the range of connectivity patterns present.

4.5. Metapopulation dynamics: expansion and contraction

Metapopulations can change over time, with populations getting larger (expansion) or smaller (contraction) in response to changes in the environment and the supply of recruits (Excoffier et al., 2009). These changes can be detected through shifts in genetic variability. Demographic (population) expansion of metapopulations is often associated with an increase in genetic variability, but reduced genetic structure within the metapopulation network. A metapopulation that has undergone demographic expansion typically has a few common and numerous less common versions of a sequence that only differ from the

common sequence(s) by a few changes. Many vent populations demonstrate this expansion signal (Vrijenhoek, 2010) that can arise as new habitat created through hydrothermal activity is rapidly colonised by the larvae of neighbouring populations.

Metapopulation contraction is often associated with a decrease in genetic variability overall. Such decreases in genetic variation could indicate that one or more populations no longer contributes recruits to the metapopulation. Vent populations can undergo metapopulation contractions as a result of natural reduction in vent habitat availability through changes in hydrothermal and volcanic activity (Lutz et al., 1994; Tunnicliffe et al., 1997). Mining activities could also lead to metapopulation contraction through damaging the habitat of a resident population, which could reduce or completely remove a source of new recruits to the metapopulation. As a result, sites in the surrounding area would have reduced larval exchange and the regional genetic diversity may diminish. When a population undergoes rapid reduction in population size, this is known as a genetic bottleneck (Peery et al., 2012). In such cases, although the population may recover in terms of numbers of individuals, the genetic diversity of the population is considerably lower than it was prior to the disturbance. Bottlenecks have been observed for vent species, such as the shrimp *Chorocaris* sp. 2 (now *C. variabilis* Komai and Tsuchida 2015) in the Manus Basin (Thaler et al., 2014).

4.6. Metapopulation dynamics: sources and sinks

Not all populations contribute equally to the metapopulation in terms of recruits. Populations that contribute many recruits to subsequent generations are known as source populations, whilst populations that contribute few, if any, recruits are sink populations. Source populations help maintain the genetic diversity of the regional metapopulation and could be important for the recolonization of mined sites. As such, source populations should be protected from mining activity to ensure that they continue to provide new recruits to the network of vent site populations (Tunnicliffe et al., 2014).

Self-recruitment also occurs within vent populations (Metaxas, 2004; Mullineaux et al., 2005) and may be particularly important in sustaining isolated populations, such as those on hydrothermally active seamounts along volcanic arcs (Metaxas, 2011). It is important to identify vent sites with moderate to high levels of self-recruitment, as mining activities at these sites will result in the loss of populations if they are predominantly sustained by self-recruitment. There are several population statistical models that can use genetic information to detect the source populations for immigrant recruits along with the level of self-recruitment.

Different species may have different larval characteristics or behaviours, so that although one vent site may be a source population for one species, it is not necessarily a source for other species. Equally, connectivity and habitat conditions can change over time so that a population may change between acting as a source or a sink. Understanding the source-sink dynamics of, and identifying self-recruiting populations within, a network of vent sites is an essential step in determining the risk that mining activities could pose to any one population. Ultimately this information is key to identifying suitable set-aside sites that are protected from mining activity and will help maintain the genetic diversity and population connectivity of the region.

5. Set-asides

Marine protected areas established to preserve seafloor communities from the impacts of mining activities are a strong environment management option, as exemplified in the Environmental

Management Plan of the International Seabed Authority (ISA) (International Seabed Authority, 2012). One such form of protected areas are set-aside sites. Set-asides are selected to have similar physical and biological characteristics to the mine site to maintain regional biodiversity and should form a coherent network, with high levels of genetic connectivity among sites to facilitate recolonization, if habitat regeneration occurs (International Seabed Authority, 2011; Van Dover et al., 2012; Collins et al., 2013b). Set-asides are analogous to temporary marine protected areas and conform to the concept of 'preservation reference zones' defined by the International Seabed Authority as "areas in which no mining shall occur to ensure representative and stable biota of the seabed" (International Seabed Authority, 2010).

Testing the suitability of a potential set-aside should consider the importance of the site in maintaining biodiversity in the region through metapopulation connectivity. Multiple set-asides may be required to maintain connectivity in the event that the mined site is a principal node of regional connectivity. A secondary consideration is the capacity of the set-aside to act as a source of recruits for recolonization of the mine site, thus biodiversity of the mine site ideally should be adequately represented at the set-aside and sources for recruits identified.

The scale of SMS mining operations is expected to be relatively small, for example the Solwara 1 deposit offshore of Papua New Guinea is only 0.112 km² and the majority of sedimentation impacts are expected to occur with 1 km of the mine site (Coffey Natural Systems, 2008). Patterns of genetic connectivity can occur on scales considerably larger than those of mining activity; some vent species demonstrate panmixia over thousands of km (Craddock et al., 1995; Teixeira et al., 2012; Miyazaki et al., 2013). Given that genetic connectivity often operates on a larger scale than mining operations, and that a source site could even be outside of a mining operator's licence block (the area in which a company is allowed to conduct mining activities and can establish set-aside sites), there needs to be wider investigation into connectivity of key species that can address the regional scale. This could be achieved through cooperation between operators in adjacent licence blocks in terms of data sharing (both biological and environmental). For this to be effective, there would also need to be a degree of transparency, data standardisation, data quality control and quality assurance, to be enforced by the mining regulatory bodies. As such, designating a suitable set-aside site or a network of sites may need to involve multiple stakeholders and contractors across licence blocks.

6. Tools for genetic assessment

There is a suite of genetic tools available for measuring biodiversity and connectivity that can inform decisions on set-aside site selection. Studies on genetic connectivity are predominantly frequency-based analyses using DNA sequences or markers. Connectivity can be deduced by temporal and spatial changes in the frequency of different versions of genes (alleles) within populations and the pattern and magnitude of genetic variation within and among populations. Technological advances including robotization and next generation sequencing are constantly reducing costs and processing times so that genetic tools can be used in routine management, such as in Alaskan salmon fisheries (Larson et al., 2014). Within the deep-sea, genetic tools were used to support the proposal for a network of areas of particular environmental interest set aside from polymetallic nodule mining in the Clarion-Clipperton Zone in the north Pacific (Smith et al., 2008).

Genetic diversity can be measured both in mitochondrial DNA (mtDNA) sequences (e.g. *COI* or other suitable mitochondrial genes) and at appropriate nuclear DNA markers (microsatellites, single nucleotide polymorphisms (SNPs) or other suitable nuclear

markers). Mitochondrial markers are well suited for measuring genetic diversity among and within species, while nuclear markers generally have more statistical power to detect genetic variation within a species (i.e. population structure, contemporary connectivity and demographics). All sequence data from genetic studies should be made available in publically accessible repositories to allow for effective regional management. These repositories include BoLD (<http://www.barcodeoflife.org/>) for COI barcodes (see below), GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) for sequence data and DRYAD (<http://datadryad.org>) for frequency-based data such as microsatellites and SNPs.

Because DNA extraction only requires small amounts of tissue, and in order to maximize the value of each sample, sampling for genetics should be conducted in concert with other biological sampling. Samples taken for DNA analysis should be preserved in either >95% non-denatured ethanol or frozen at -80°C or -20°C . If neither of these is available, 20% dimethyl sulfoxide (DMSO) is acceptable for short-term storage and transport. Thereafter, samples should be rapidly transferred to ethanol or frozen. The steps required from identification of key species through sampling, genetic analysis and data storage are discussed in the sections below and summarised in Fig. 1.

6.1. Species identification – DNA barcoding

The first steps of sampling both mine and potential set-aside sites are survey-based collection and then preservation of faunal samples for morphological taxonomy, which can usually identify

most species, as well as those that benefit from further genetic analysis. DNA barcoding can support traditional morphological identification of species, especially when dealing with apparently identical taxa that are genetically distinct (cryptic species). DNA barcoding can also assist in identification when resolution of species identification is poor due to a lack of taxonomic studies and associated identification keys. Accurate identification of specimens is crucial to understand the composition of vent communities and any changes over space and time. Representative specimens of all taxa that have been identified from the survey(s) should be deposited and retained in open access reference collections in national research institutes, museums or universities. Records of species occurrences and any associated metadata should be lodged with Ocean Biogeographic Information System (OBIS: www.iobis.org).

The cytochrome-c-oxidase subunit I (COI) serves as the standard barcoding gene to identify individuals to species for most eukaryotic animals (Hebert et al., 2003; Bucklin et al., 2011). This is currently the most universal approach to barcoding and the obtained sequences can be rapidly identified using online resources, such as Basic Local Alignment Search Tool (BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). COI barcoding works by using variation in the genetic sequence to identify a species (Folmer et al., 1994; Geller et al., 2013). In cases where COI is not sufficiently informative to identify organisms to species level, an alternative gene should be utilised. Whilst barcoding can be exceedingly useful in confirming identification if the sequence is known, it should be recognised that our knowledge of deep-sea diversity is still

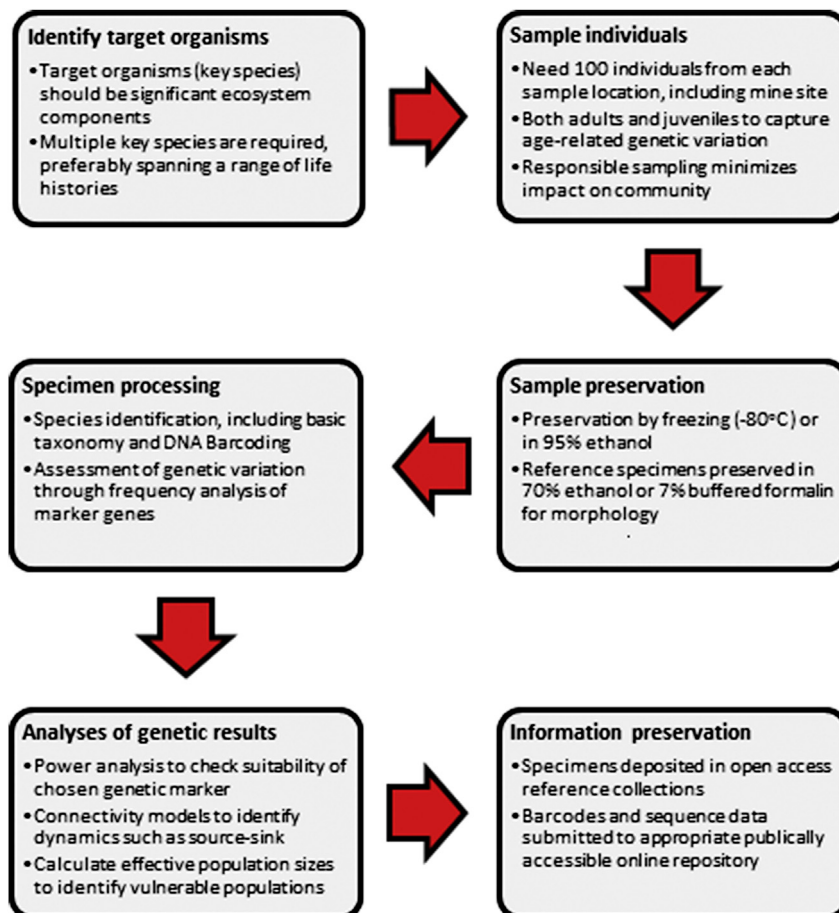


Fig. 1. The basics of sampling, preservation and analysis for genetic connectivity among potential set-aside and mine sites.

developing and that not all DNA sequences will be identifiable; the tool will grow stronger as more sequences are submitted.

6.2. Population structure and connectivity

Due to the rapid development of genetic methods to detect and quantify genetic variation, it is not possible to recommend a best technology or genetic marker for assessing population structure. The pros and cons of different genetic marker types, and the analyses applied to these markers, have been extensively reviewed (Broquet and Petit, 2009; Marko and Hart, 2011). However, it is necessary to ensure that the chosen genetic technology has adequate statistical power to detect population genetic parameters. Power analyses should be performed to ensure that the chosen technology and framework can detect and measure even low levels of genetic differentiation and connectivity that can provide information on set-aside suitability (e.g. Putman and Carbone, 2014).

In addition to sequence-based mitochondrial (e.g. COI) analysis, frequency-based nuclear DNA markers (e.g., microsatellites, SNPs) should be employed on a subset of key species to establish the population structure and connectivity among fauna at different sample sites within the deposit (Smith et al., 2008; Thaler et al., 2011). Both mtDNA and nuclear DNA markers should be assessed because these two genomes may represent different evolutionary pathways. Demographic parameters such as effective population sizes (see Section 4.3), genetic bottlenecks (see Section 4.5), and source-sink dynamics (see Section 4.6) will all provide information on the health of populations and avoid locating set-asides in sub-optimal locations. There is a suite of statistical frameworks, software packages and freeware available to conduct these assessments (Excoffier and Heckel, 2006).

Because species may have different patterns of connectivity, several species (we recommend five for practicality) should be analysed. These key species should ideally represent different life history strategies to capture the range of larval behaviours and dispersal trajectories (see Hilário et al., 2015 for examples), which will influence the connectivity of populations. The targeted key species should also be significant ecosystem components (SECs) that have ecological importance to the vent ecosystem (O et al., 2015). The criteria for SECs are detailed in Table 1.

Once key species have been identified, sampling should commence at the appropriate spatial scale, ideally with a nested design to assess multiple scales of connectivity. The spatial scale of sampling will be determined in part by the relative proximity of vent habitat, the dispersal distances of key species (if known) and

logistical constraints regarding mining lease extent. For example, samples could be taken at multiple patches on a chimney or vent orifice, from multiple vents within a site and from multiple sites within a region (Thaler et al., 2011). Each sample should ideally consist of 100 individuals (e.g., 50 from each of two cohorts). Sampling multiple cohorts enables analysis of temporal stability in connectivity, whilst a total of 100 individuals will reduce the probability of unpredictable or random effects. Sampling should be conducted in accordance with the InterRidge Code of Conduct for scientific sampling at hydrothermal vents (Devey et al., 2007; InterRidge, 2009), so that sampling is minimal and only comprises what is needed for the study. Extra caution should be taken when sampling at all potential set-aside sites to minimize disturbance and impacts from sampling.

6.3. Species detection, inventory and monitoring – environmental DNA (eDNA)

Environmental DNA (eDNA) is the total DNA present in an environmental sample, such as a set volume of water or sediment (Taberlet et al., 2012). The use of eDNA is non-invasive and does not require the sampling of animals. Instead eDNA can be derived from microbes, sloughed cells, faeces, blood or gametes present in an environmental sample (Bohmann et al., 2014). The eDNA approach does not require taxonomic expertise and allows for rapid processing, in parallel, of large numbers of samples. As such, it could be a beneficial accompaniment to traditional biological sampling as part of biodiversity assessments or environmental monitoring of SMS deposits.

Environmental DNA samples should be rapidly frozen (−80 °C) or, if a water sample, filtered and the filter preserved in 100% ethanol prior to DNA extraction. Sampling replication should be conducted to incorporate local heterogeneity, whilst multiple sub-replicates should be used while extracting eDNA to incorporate microscale heterogeneity. Although 18S rRNA currently appears to be the best-suited genetic marker to obtain an overview of the non-microbial organisms present, additional markers specific to targeted groups can be used to obtain a better resolution (Tang et al., 2012). The genetic markers should be sequenced on an appropriate high throughput-sequencing (HTS; e.g. Illumina, Ion Torrent) platform. At the time of writing, no standardised methodology exists for eDNA sampling or analyses. Therefore, an eDNA approach should be combined with traditional visual identification of biological samples and DNA barcoding, which also enables validation of eDNA results.

Table 1

The criteria for Significant Ecosystem Components (SECs), as established during the process of risk assessment for the Canadian Endeavour Hot Vents MPA (following “valued ecosystem components” in O et al., 2015). Application of the considerations is context specific e.g. “endemic” may refer to one/few sites compared to the region.

SEC type	SEC considerations
Species	<ul style="list-style-type: none"> • Nutrient importer/exporter • Specialised or keystone role in the food web • Habitat creating species • Rare, unique, or endemic species • Sensitive species • Depleted (listed) species
Habitat	<ul style="list-style-type: none"> • Biogenic habitat types • Sensitive habitats • Habitats supporting rare, unique or endemic species • Habitats supporting critical life stages • Habitats providing critical ecosystem functions or services
Ecosystem/community	<ul style="list-style-type: none"> • Ecologically significant community properties • Functional groups that play a critical role in ecosystem functioning • Ecological processes critical for ecosystem functioning • Sensitive functional groups

6.4. Assessing microbial diversity – microbial metagenomics

Metagenomics can be used to assess the microbial diversity of marine sediment samples (Spang et al., 2015) and could be a useful addition to traditional biological sampling approaches in the assessment of set-asides. Samples collected during sediment sampling or other sources can be analysed using high throughput-sequencing of 16S rRNA. Because many, if not most, microbes remain undescribed, we cannot provide an in-depth description of this community but it is possible to characterize the overall community at the level of major taxa and metabolisms (Campbell et al., 2013). Changes in the microbial communities over time can also be detected and characterised. Samples used for metagenomics ideally need to be stored in -80°C .

7. Using genetic techniques in the set-aside selection process

As discussed in Section 5, set-aside areas should, as a minimum, have biological characteristics similar to the mine site to maintain regional diversity and, where multiple sites are selected, should contribute to a coherent network to maintain connectivity in the regional metapopulation. Incorporating genetic parameters into set-aside design demonstrates effective and responsible environmental management by providing information essential for designing mitigation strategies that lower ecological risk from mining activities.

Proposing and establishing suitable set-aside(s) occurs during the baseline survey phase of a mining project; Collins et al. (2013b) recommend a three-stage process for baseline surveys. Stage 1 involves the definition of the physical characteristics of the site, including mapping of the seafloor and describing current regimes; Stage 2 involves determining biological and chemical characteristics of the sites, including characterising habitats, assemblages and dominant large fauna using video surveys; Stage 3 involves targeted sampling, including species identification and population connectivity assessments of key species. We incorporate genetic conservation parameters to extend the criteria of Collins et al. (2013b) with regards to Stage 3, and add a fourth stage in which the set-aside is designated and subsequently monitored (Fig. 2). Collins et al. (2013a) present a detailed account of the studies required in Stages 1–3.

Genetic tools can be used in Stage 3 to help characterise the mine site and proposed set-aside sites and to determine the suitability of proposed sites. Set-aside suitability should be tested using morphological identification of species and assemblages (Collins et al., 2012) but should also be augmented by barcoding, supplemented by eDNA analysis and microbial metagenomics. The suitability of set-asides in terms of their connectivity can also be assessed through the use of genetic markers that can provide important information on effective population size, source-sink dynamics and connectivity models for vent-endemic populations (Thaler et al., 2011, 2014).

Genetic tools can also be utilised in Stage 4, during the monitoring phase that follows set-aside designation. After a mine site and set-aside have been selected, both sites need to be monitored for recovery at the mine site, for any mining impacts at the set-aside, and for the continued effectiveness of the set-aside site(s). For the set-aside(s) to remain effective they need to be free from impacts resulting from mining activities, retain similar biological characteristics to the mine site (taxonomic and genetic composition and structure) and to continue to act as a source population for key species. The monitoring phase should utilise the same genetic tools that were used in Stage 3 to characterise the sites and assess their suitability.

Specific recommendations for selection of set-asides, with

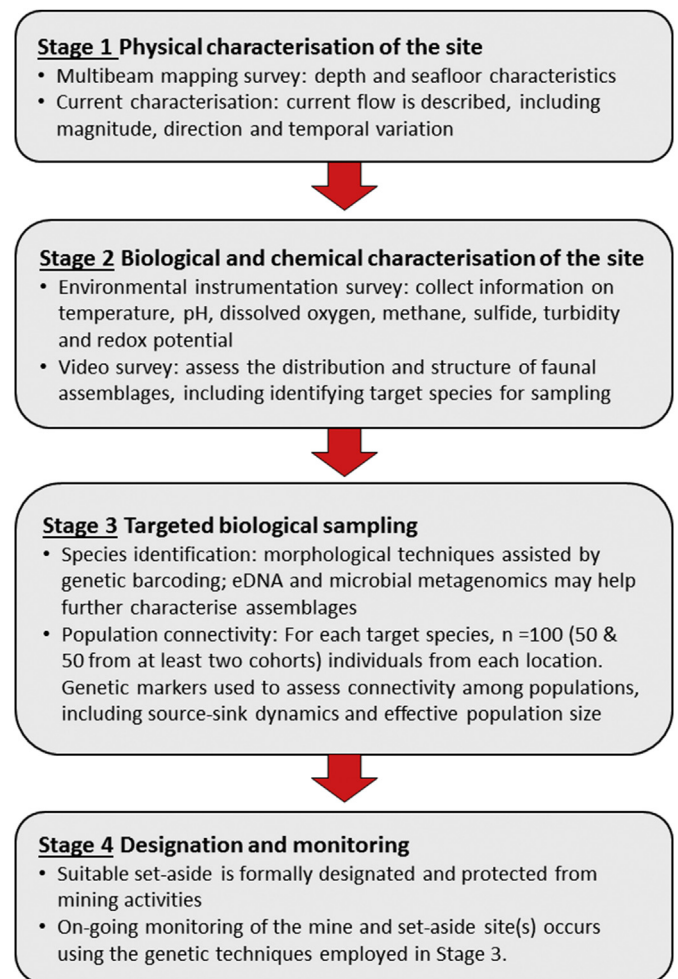


Fig. 2. The four-stage process for baseline survey involved in establishing a set-aside, developed from Collins et al. (2013b) to include genetic techniques.

respect to genetic connectivity within the regional metapopulation are as follows;

- Source populations of key species should be present at the set-aside; if different key species have source populations at different sites then multiple set-asides may be required.
- The set-aside populations of key species should have equal or greater genetic diversity (number of alleles) and statistically similar genetic composition (types of alleles) to populations at the mine site.
- The effective population size at the set-aside should be equal to or larger than the effective population size at the mine site.
- Set-asides should occur at a location where they remain unimpacted by mining activities and where the regional circulation in combination with larval dispersal patterns allow them to act as sources of recruits to the metapopulation.

8. Set-aside suitability checks for environmental managers and regulators

To enable informed decisions to be made on the suitability of set-aside areas for an SMS mining project proposal, critical components should be submitted to relevant government or international agencies during the environmental impact assessment

approval process. Suggested components include a map, relevant data and a report.

The map of the proposed set-aside should include: the set-aside (location, size and boundary), the proposed mine site (location, size and boundary), licence block boundaries of the operator and other operators in the region, existing deep-sea set-asides, other Marine Protected Areas and any other relevant information. All environmental and biological data regarding the proposed set-aside area, and any sites studied but not proposed as set-asides, should be submitted to the relevant agency in an electronic and accessible format. The report should address the selection process (see Section 7) and answer the questions detailed in Table 2.

9. Conclusions

Hydrothermal vent communities rely on hydrothermal activity to survive and are particularly at risk from SMS mining activities that alter fluid flows. Hydrothermal habitat is patchy, and sometimes ephemeral, so that habitat change or loss poses a considerable threat to the persistence of vent species that are often endemic to the biogeographic region. Nearly all vent species (which are mainly sessile or of limited mobility) depend on larval exchange to

maintain populations; in turn the success of larval recruitment depends on access to suitable habitat. The proposal of suitable set-aside vent areas is a critical step in the environmental impact assessment process for mining operations. Suitable set-asides can serve two functions. Firstly, set-asides will enable the preservation of communities similar to those lost through mining; secondly, they could conserve source populations of key species that help maintain regional genetic diversity and may facilitate recolonization of the mine site.

Choosing the most suitable set-aside site can be aided by a suite of genetic tools that can help to assess the diversity of SMS deposit communities and also determine population connectivity of key species in the region. These connections can be measured by examining the genetic variability of populations and used to determine the source areas of recruits and the amount of exchange among populations. Molecular approaches are rapidly becoming more accessible and more informative and any environmental assessment will benefit from these analyses. We outline approaches with mitochondrial and nuclear markers that are currently employed to assess genetic diversity and connectivity but recognize that new innovations may supplant them. These tools can help identify potential set-aside sites, assess the suitability of a

Table 2
A recommended set-aside suitability checklist for regulators and environmental managers.

Question	Requirement
Location and environmental characteristics	
<i>Are there existing set-aside areas near the proposed mine area?</i>	<ul style="list-style-type: none"> Proposed mine site is located so as not to impact existing set-aside areas Mine site is not a key source population for existing set-asides (see Section 4.6) An existing set-aside may be sufficient for proposed mine site Set-aside(s) is located within proponent's exploration licence area or other area designated for set-asides
<i>Is the proposed set-aside located within the proponent's licence block?</i>	
<i>Is the set-aside sufficiently far away from the expected impacts of the mine site?</i>	<ul style="list-style-type: none"> Set-aside(s) is located outside impact zone of mining activities
<i>Does the set-aside have similar venting activity to the proposed mine site?</i>	<ul style="list-style-type: none"> Generally, venting activity is equal or greater at the set-aside(s) than the mine site Inactive mine sites require inactive set-asides
Methods used for survey	
<i>What methods were used to study the site?</i>	<ul style="list-style-type: none"> Appropriate tools are used to identify overall community composition, to species level where possible and determine population structure and connectivity (see Section 6) The same methods are used to study the mine site and set-aside(s) Key species are Significant Ecosystem Components and ideally span a range of life histories (see Section 6.2)
<i>What criteria were used to select key species?</i>	
Biodiversity	
<i>What are the key species and habitats studied?</i>	<ul style="list-style-type: none"> Assessment includes megafauna (>2 cm), macrofauna (<2 and > 0.5 cm) and sediment infauna representative of key habitats at both the mine and set-aside sites. Species are identified morphologically and genetically (see Section 6.1) Set-aside site(s) have similar biodiversity to the mine site Communities at the set-aside site are similar to those at the mine site with the vast majority of species at the mined site being present at the set-aside site(s) A network of connected set-aside areas may be required to capture the biodiversity of the mine site
<i>Does the set-aside have similar biodiversity to the mine site?</i>	
Genetic diversity and connectivity	
<i>Does the set-aside have genetic diversity equal to or greater than the mine site?</i>	<ul style="list-style-type: none"> The set-aside populations of key species have equal or greater genetic diversity and similar genetic composition to populations at the mine site The effective population sizes for key species at the set-aside are equal to or larger than the effective population size for key species at the mine site The mine site is connected to the set-aside site(s) if the purpose of the set-aside is to repopulate the mine area
<i>Is the set-aside genetically connected to the proposed mine site?</i>	
<i>Have source and sink populations been identified?</i>	<ul style="list-style-type: none"> Within the network of metapopulation sites, source populations have been identified for protection from mining activity and/or to be used as set-asides Sink populations are not generally considered appropriate set-asides sites as they are not able to contribute recruits to the metapopulation Sites that are genetically unique and/or are dependent on high levels of self-recruitment for their survival are identified and protected from mining activity
<i>Have unique and/or self-recruiting populations been identified?</i>	
Archiving, monitoring, and national guidelines	
<i>Where have the samples been stored?</i>	<ul style="list-style-type: none"> When collected from within an EEZ, all samples remain property of the State The location of samples is recorded to enable potential future analysis Reference specimens are located in open access reference collections (see Section 6.1). The data collected to assess set-aside suitability are uploaded to a publicly accessible repository (see Section 6) Set-aside(s) are monitored during mining and for a designated period afterwards to ensure they have not been impacted by mining and that they continue to function effectively as set-asides If national guidelines or requirements exist these are addressed
<i>Where have the data been uploaded?</i>	
<i>What are the details of the monitoring plan?</i>	
<i>Does the set-aside reflect national guidelines?</i>	

set-aside in terms of biodiversity and connectivity, and identify vulnerable populations that may require protection from mining activity. As with traditional biological approaches, genetic tools have a place in monitoring programmes to assess recolonization of the mined site and in continued assessment of the suitability of set-asides. We suggest that environmental managers should stay abreast of new advances to ensure the most appropriate genetic tools are deployed.

Because genetic connectivity may operate at large scales, the most suitable set-aside site(s) may occur outside of the licence block. As such, it is strongly recommended that operators within a region pool resources and share biological and environmental data to identify the most biologically appropriate set-aside site or network of sites. Policies of transparency and open access of biological and environmental data should be enacted by regulatory bodies to ensure there is a co-ordinated regional approach to deep-sea environmental management.

Ultimately, this document aims to provide the background necessary to understand how and why appropriate genetic tools can assist in the selection of set-aside areas and support subsequent monitoring. It is critical that all stakeholders continue to develop processes that will support environmental managers and regulators in the new challenges presented by deep-sea mining, particularly at SMS deposits where faunal populations of hydrothermal vents are at risk.

Author contributions

The majority of manuscript editing was undertaken by REB, PCC and VT. Each section was initially co-written by a group of authors and later edited by the group as a whole. Abstract: REB; Introduction: REB, VT; Section 2: AMe, VT, REB; Section 3: JPAG, AMe, JC; Section 4: JPAG, JC, FS, REB; Section 5: PCC, REB, JL; Section 6: JC, JPAG, PCC, FS; Section 7: REB, PCC; Section 8: AS, JL; Section 9: VT, REB. The tables and figures were prepared by the following authors; Table 1: VT; Table 2: AS, REB; Fig. 1: VT, REB; Fig. 2: REB, PCC. AMc provided important edits throughout the manuscript regarding appropriate presentation for the target audience and on marine conservation aspects. JL provided useful edits to the manuscript regarding the industry perspective. All authors helped to draft and approve the manuscript. All authors read and approved the final manuscript.

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