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Experimental assessment of the response of meiobenthos to disturbance associated with atmospheric carbon increase and mineral extraction

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For the world is changing:

I feel it in the water,

I feel it in the earth,

and I smell it in the air.

Treebeard, The Lord of the Rings by J.R.R. Tolkien

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My work as a PhD student is coming to an end and the final thesis is written. It feels like throwing the ring in the fires of Mount Doom at the end of a long journey[1]. But I couldn't have done it without the help of many people that supported me on this journey and who deserve a special mentioning.

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[1] The Lord of the Rings movies and soundtrack were often playing in the background while I was writing my thesis which actually helped me to focus. Also, today, on 03.01.2018 J.R.R. Tolkien would have become 126 years old.

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Samenvatting

De oceaan bedekt 70 % van het aardoppervlak en biedt ons een rijkdom aan goederen en diensten. Tijdens de voorbije twee eeuwen nam de menselijke invloed op de oceanen aanzienlijk toe en vormde geleidelijk aan een bedreiging voor de stabiliteit van dit grote ecosysteem. Naast de overexploitatie van visbestanden, extractie van minerale bronnen en vervuiling van de oceaan door chemicaliën en plastiek verandert de mens het mariene ecosysteem ook indirect door de toename aan broeikasgassen, voornamelijk CO_2, in de atmosfeer (Halpern et al., 2008).

Door de gasuitwisseling tussen de oceanen en de atmosfeer lost CO_2 op in het zeewater. Hierdoor verschuift het zuur-base-evenwicht van het mariene carbonaat systeem naar een zuurdere toestand (Zeebe en Wolf-Gladrow, 2001). Deze door de mens geïnduceerde verschuiving in het carbonaat systeem in de zee heet oceaanverzuring (OA). Verhoogde atmosferische CO_2 concentraties dragen bij tot het broeikaseffect waardoor meer warmte in de atmosfeer van de aarde wordt behouden, wat op zijn beurt de stralingsforcering verhoogt (Lyman et al., 2010). Als gevolg hiervan worden het land en de oceanen geleidelijk warmer (Lyman et al., 2010). Voorspellingen duiden erop dat kustzones en hun biologische gemeenschappen de sterkste verstoringen zullen ondervinden ten gevolge van bovengenoemde stressoren (Halpern et al., 2008). Zonder het matigen van de oorzaken wordt er verwacht dat de atmosferische CO_2 concentraties van de huidige 400 ppm tot meer dan 1000 ppm zullen stijgen, over een tijdspanne van 100 jaar (Collins et al., 2013). Om globale verstoring tegen te gaan of te voorkomen werden er verschillende mitigatiestrategieën voorgesteld zoals de opslag van atmosferisch CO_2 in ondergrondse geologische reservoirs, zoals uitgeputte olie- of gasvelden (Metz et al., 2005). Hoewel veelbelovend, draagt deze technologie ook de risico's van zware verzuring van het zeewater met zich in geval van een technische fout of lekkage van CO_2 door scheurtjes in het moedergesteente en het overliggende sediment (Damen et al., 2006).

Een geheel andere vorm van menselijke verstoring in onze oceanen wordt veroorzaakt door het ontginnen van minerale bronnen. Dit omvat de directe extractie van minerale gesteentes uit de diepzeebodem, maar ook het storten van restmateriaal afkomstig uit landmijnen in het mariene milieu. Potentiële effecten op het milieu als gevolg van dit afvalstorten zijn onder meer het verstikken en begraven van de benthische fauna met potentieel toxische substraten, veranderingen in de korrelgrootte van het sediment en modificaties van de biochemie in de zeebodem (Ramirez-Llodra et al., 2015). De extractie van minerale bronnen uit de zeebodem gaat gepaard met het verwijderen van hard substraat en sediment, compactie van het sediment, fragmentatie en modificatie van het habitat, verspreiding van sedimentwolken en daaropvolgende depositie, maar ook de

mogelijke mobilisatie van zware metalen en verhoogde toxiciteit als gevolg (Gollner et al., 2017).

Het doel van dit doctoraatsonderzoek was het nagaan van de responsen van benthische gemeenschappen, in het bijzonder van meiofauna (meercellige dieren in de grootteklasse van 1 mm - 32 µm), op antropogene stressoren die gepaard gaan met de toename van atmosferische CO_2 (Hoofdstuk 2 en 3) en extractie van minerale bronnen (Hoofdstuk 4 - 7). In sommige experimenten werd ook de interactie met macrofauna en microbiota opgenomen (Hoofdstuk 2 en 4). De biotische responsen werden onderzocht via verschillende experimentele benaderingen, waaronder experimenten met één soort, mesokosmos experimenten en *in situ* experimenten.

Hoofdstuk 1 introduceert de lezer in de verschillende onderwerpen en bijhorende stressoren die in dit proefschrift werden onderzocht en beschrijft de kenmerken van de mariene meiofauna. Verder geeft dit hoofdstuk een overzicht van de verschillende methoden en uitdagingen bij het uitvoeren van experimenteel, marien onderzoek.

De eerste twee hoofdstukken (**Hoofdstukken 2** en **3**) focussen op de gevolgen van oceaanverzuring en -opwarming op benthische gemeenschappen in zachte substraten uit een subtidaal en een intertidaal gebied. In **Hoofdstuk 2** werden responsen van bivalven, meiobenthos en bacteriën op een breed spectrum van pCO_2 waarden over een periode van 12 weken onderzocht met als doelstelling om effecten van zeewaterverzuring te onderzoeken die gerelateerd zijn aan toekomstige oceaanverzuring en potentiële CO_2-lekkage tijdens de opslag van koolstofdioxide in ondergrondse reservoirs. Effecten van hoge pCO_2 op bivalven waren soortspecifiek en grootte-afhankelijk. Tekenen van schelpdesintegratie in de kokkel *Cerastoderma edule* waren reeds meetbaar bij 1.500 µatm (pH 7,7) en kleine kokkels vertoonden een verhoogde mortaliteit bij een lagere pCO_2 dan de grotere, volwassen kokkels. Stervende kokkels migreerden naar het sedimentoppervlak, wat een visuele indicatie van hoge pCO_2 opleverde. De gemeenschapsstructuur van meiofauna en van de bacteriële gemeenschap op het hoogste pCO_2 niveau van 24.400 µatm (pH 6,4) verschilde van de Controle (pH 7.8). Terwijl de densiteiten van nematoden en hun gemeenschapsstructuur vergelijkbaar bleef tussen alle behandelingen, reageerden minder dominante taxa gevoeliger op de pH veranderingen in het zeewater. Densiteiten van meiobenthostaxa met een kalk-exoskelet daalden significant bij >6.000 µatm (pH 7,0), terwijl Gastrotricha een zeer tolerante en opportunistische respons op verhoogde pCO_2 vertoonden met sterk verhoogde densiteiten bij een pCO_2 van 24.400 µatm. Deze resultaten hebben aangetoond dat zelfs kleine veranderingen in pCO_2 verstoringen op meerdere

grootteklassen kan veroorzaken in subtidale zanderige bodems waar bivalven domineren. Het onderzoek benadrukt ook het belang van zeldzame taxa en biotische interacties in onderzoek naar de gevolgen van oceaanverzurings.

Cumulatieve effecten van zeewaterverzuring (ΔpH = -0.4) en -opwarming (ΔTemp = -3 ° C) beïnvloedden de meiofauna gemeenschapssamenstelling in intertidale sedimenten na 8 weken blootstelling aan de veranderde abiotische factoren (**Hoofdstuk 3**). Net als in hoofdstuk 2 waren de responsen van nematoden gematigd, terwijl andere, minder dominante taxa significante densiteitsverschillen tussen behandelingen vertoonden. Een gereduceerde pH en een verhoogde temperatuur resulteerde in een densiteitsverhoging van Platyhelminthes, verminderde densiteiten van Copepoda en Nauplii en een volledige afwezigheid van Gastrotricha in vergelijking met de experimentele controle. Mogelijks vloeien deze verschillen voort uit een combinatie van groepsspecifieke gevoeligheid voor de veranderde abiotische condities en veranderingen in biotische interacties, zoals predatiedruk door Platyhelminthes en predatorische nematoden op andere meiofauna. Beide studies (Hoofdstukken 2 en 3) onderstrepen het belang van biotische interacties tussen meerdere grootteklassen en trofische niveaus voor de beoordeling van de milieueffecten die geassocieerd zijn met scenario's van oceaanverzuring en -opwarmings in de nabije toekomst. Verder pleiten we voor de studie van minder dominante taxa, aangezien de responsen van deze organismen een betere aanwijzing kunnen zijn voor de invloeden van oceaanverzuring dan responsen van de dominante meiofauna (bijvoorbeeld nematoden).

De volgende hoofdstukken hebben betrekking op enkele mogelijke gevolgen van het ontginnen van minerale bronnen op bathyale en diepzeegemeenschappen en in het bijzonder met de gevolgen van de depositie van grof en fijn substraat en toxiciteit van koper.

In een korte termijn (11 - 15 dagen) mesokosmos experiment leidde de depositie van verschillende hoeveelheden mijnafval en natuurlijk sediment op een bathyale gemeenschap uit een Noorse fjord tot een verminderd zuurstofverbruik van de bodemgemeenschap, verminderde opname van ^{13}C-gelabelde algen en een verminderde koolstofverwerking, parameters die het functioneren van het ecosysteem beschrijven (**Hoofdstuk 4**). De eerste tekenen van een verminderd functioneren van het ecosysteem waren reeds zichtbaar bij 0.1 cm depositie met mijnafval en werden sterker bij depositie met verhoogde hoeveelheden substraat. Bovendien vertoonde de meiofauna een verticale migratierespons, mogelijks als gevolg van verminderde zuurstofconcentraties in het natuurlijke sediment. Deze respons was echter ook geassocieerd met een verhoogde mortaliteit van nematoden. Ondanks enkele algemene overeenkomsten vonden we ook verschillen

tussen de depositie met mijnafval en met sediment, voornamelijk met betrekking tot het zuurstofverbruik van de sedimentgemeenschap, de zuurstofpenetratiediepte en de koolstofopname van de biota. Dit suggereert dat het effect van substraatdepositie op sedimentprocessen afhangt van het type substraat en benadrukt het belang van sedimentkarakteristieken voor de beoordeling van de effecten van begraving. We concluderen dat de onmiddellijke depositie van zeer kleine hoeveelheden substraat al op een relatief korte termijn de werking van benthische gemeenschappen in bathyale fjorden kan verminderen.

In **Hoofdstuk 5** onderzochten we de gevolgen van depositie van kleine deeltjes van vermalen mangaanknollen op een *in situ* sedimentgemeenschap uit een abyssaal mangaanknollengebied in de zuidoostelijke Stille Oceaan. Experimentele depositie van het substraat resulteerde in de verdeling van een 2 cm dikke laag grof substraat over het sedimentoppervlak. We bemonsterden het sediment na een incubatietijd van 11 dagen, vergelijkbaar met het vorige experiment, analyseerden de structuur van de meiobenthos- en nematodengemeenschap, en meetten de koperopname in verschillende nematoden om een eerste indruk te krijgen van mogelijke toxische effecten van het vermalen mangaanknollensubstraat. Vergelijkbaar met het vorige hoofdstuk werd een groot deel van de meiofauna in het toegevoegde substraat gevonden wat een indicatie is voor actieve migratie in een potentieel ongeschikte leefomgeving. Proporties van copepoden en nauplii in het toegevoegde substraat waren hoger dan die van nematoden en resulteerden waarschijnlijk uit de hogere mobiliteit en kolonisatiepotentieel van deze dieren. De samenstelling van de nematodengemeenschap onthulde geen duidelijke verschillen tussen de toegevoegde substraat laag en de onderliggende lagen waardoor het migratierespons van nematoden niet soortspecifiek was. De koperopname door nematoden was vergelijkbaar in dieren uit controle sedimenten en uit het mangaanknollen substraat. Het vinden van een verticaal ontsnappingsrespons dat mogelijks gepaard gaat met verhoogde mortaliteit van benthos, zoals beschreven in hoofdstuk 4, kan significante implicaties hebben voor de risicobeoordeling van mijnbouw in de diepzee.

De effecten van abiotische factoren die relevant zijn onder diepzeecondities, zoals hydrostatische druk en temperatuur, op metaaltoxiciteit in de mariene nematode *Halomonhystera disjuncta* werden in **Hoofdstuk 6,** d.m.v. acute toxiciteitstests, onderzocht. Hoge hydrostatische druk (10 MPa) verhoogde de gevoeligheid van de nematoden aan koper terwijl hun gevoeligheid door verlaagde temperatuur werd verminderd (10 °C vergeleken met 20 °C). Wij concludeerden daarom dat toxiciteitseffecten in de diepzee enkel kunnen worden beoordeeld wanneer rekening wordt gehouden met de effecten van beide stressoren en dat bestaande

gegevens uit ondiep water niet zomaar naar en diepzeecontext kunnen worden veralgemeend.

Vervolgens probeerden we om de gevoeligheid van een abyssale sedimentgemeenschap op depositie met koper gecontamineerd sediment te beoordelen (**Hoofdstuk 7**). Dit werd bereikt door een *in situ* gemeenschap uit een abyssaal mangaanknollengebied bloot te stellen aan kunstmatig sediment dat gecontamineerd was met verschillende koperconcentraties (0 - 20 mg L^{-1} Cu^{2+}). Deze concentraties omvatten de dodelijke koperniveaus voor nematoden die in Hoofdstuk 6 werden bepaald. Na 96 uur werd het sediment bemonsterd. De structuur van de meiobenthosgemeenschap werd geanalyseerd en we bepaalden het kopergehalte in verschillende nematoden uit het controlesediment en uit het sediment met de hoogste koper concentratie. Opnieuw vertoonde de meiofauna een verticale migratie en werden de organismen in hoge densiteiten in het toegevoegde sediment gevonden, ongeacht de kopercontaminatie. Vergelijkbaar met hoofdstuk 5 waren copepoden en nauplii succesvoller in het koloniseren van het nieuwe sediment dan nematoden. De analyse van nematoden koperopname liet zien dat dieren uit het gecontamineerd sediment lagere waarden toonden dan nematoden uit het controle sediment. We hebben onze experimentele methode kritisch beoordeeld en geven toe dat de oplosbare koperfractie mogelijks tijdens het aanbrengen van het sediment uit het substraat werd gewassen of dat de koper door sorptieprocessen van het mangaan-oxihydroxide- en ijzeroxide-rijk natuurlijk sediment niet meer biobeschikbaar was. Lage koper biobeschikbaarheid in het sediment kan de verminderde koperwaarden in nematoden verklaren die blootgesteld waren aan het kunstmatige sediment. Terwijl deze studie de moeilijkheden van (diepzee) experimenteel onderzoek illustreert, rezen er ook nieuwe vragen met betrekking tot metaaltoxiciteit die verder onderzoek stimuleren (bijv. waarom is het kopergehalte in blootgestelde nematoden lager en niet gelijk aan dat van controledieren?).

Het onderzoek dat in dit proefschrift wordt gepresenteerd, verduidelijkt het grote potentieel van experimentele studies om in grote mate bij te dragen tot het begrijpen van algemene patronen die verband houden met milieuverstoringen op verschillende ruimtelijk-temporele schalen. De beperkingen en uitdagingen van experimenteel onderzoek, alsmede de implicaties van onze resultaten in termen van risicobeoordeling en monitoring van verstoorde gebieden, worden in **Hoofdstuk 8** geïntegreerd en in het kader van eerder onderzoek besproken.

Uit onze resultaten is gebleken dat structurele veranderingen van de benthische gemeenschap en in het bijzonder de verticale positionering in het sediment een goede, eerste indicator zijn van negatieve milieueffecten, die voordelen bieden voor

de biomonitoring van verstoorde gebieden. Bovendien benadrukt de differentiële gevoeligheid van verschillende meiofaunagroepen het belang van ecosysteem-gebaseerde benaderingen die toelaten om rekening te houden met biotische interacties tussen grootteklassen en trofische niveaus. Echter, deze studies zouden gepaard moeten gaan met soortspecifieke studies die informatie geven over de exacte mechanismen van stressoren op de fysiologie van deze soorten. Tot slot werden in verschillende experimenten die in deze thesis worden weergegeven cumulatieve effecten van stressoren geïdentificeerd, wat het belang van multi-stressor experimenten in toekomstig onderzoek benadrukt.

Summary

The ocean covers 70 % of the Earth's surface and provides us with a wealth of goods and benefits. Since the last two centuries, human impact on the oceans has considerably increased, threatening the stability of this large ecosystem. Apart from the overexploitation of fish stocks, extraction of mineral resources and pollution of the ocean with chemicals and plastics, humanity also indirectly alters marine ecosystems through the exceeding increase of greenhouse gasses and more particularly of CO_2 (Halpern et al., 2008).

Through gas exchange between the ocean and the atmosphere, CO_2 dissolves in the seawater where it shifts the acid-base equilibrium of the marine carbonate system towards a more acidic state (Zeebe and Wolf-Gladrow, 2001). This anthropogenically induced shift of the ocean's carbonate system is called ocean acidification (OA). Increased concentrations of CO_2 in our atmosphere additionally contribute to the greenhouse effect through which more heat is retained on the Earth's surface, thus increasing its radiative forcing (Lyman et al., 2010). As a consequence, the land and the oceans gradually become warmer (Lyman et al., 2010). Coastal zones and their biological communities are predicted to exhibit the strongest perturbations from above mentioned stressors (Halpern et al., 2008). Unmitigated, the atmospheric CO_2 concentrations are predicted to rise from current 400 ppm to over 1000 ppm by the year 2100 with devastating effects on this planet (Collins et al., 2013). Therefore, mitigation strategies are put forward such as the storage of atmospheric CO_2 in underground geological reservoirs like depleted oil or gas fields (Metz et al., 2005). Although promising, this technology also bears risks of severe seawater acidification in case of a pipe failure or CO_2 leakage through faults in the cap rock and overlying sediment (Damen et al., 2006).

Another source of anthropogenic disturbance that affects our oceans is the environmental stress associated with mineral extraction. This includes the direct extraction of extensive mineral resources from the seafloor at great depths but also the deposition of waste material (tailings) from land-based mines into the marine environment. Potential environmental impacts following tailings deposition include smothering and burial of benthic fauna with (potentially toxic) substrates, changes in sediment grain size and modifications of sediment biochemistry (Ramirez-Llodra et al., 2015). The extraction of mineral resources from the seafloor on the other hand will be associated with removal of hard substrate and sediment, sediment compaction, habitat fragmentation and modification, sediment plume dispersal and subsequent re-deposition but also the possible mobilization and toxicity of heavy metals (Gollner et al., 2017).

The objective of this PhD research was to investigate responses of benthic communities with particular focus on meiofauna (1 mm - 32 µm metazoan size

fraction of the benthic fauna) to anthropogenic stressors relating to the increase of atmospheric CO_2 (Chapter 2 and 3) and mineral extraction (Chapter 4 - 7). In some experiments, the interaction with macrofauna and microbiota is included, too (Chapter 2 and 4). Responses were investigated through different experimental approaches including single-species, mesocosm and *in situ* experiments.

Chapter 1 introduces the reader to the different topics and associated stressors that were investigated in this thesis and briefly describes the characteristics of marine meiofauna. Furthermore, this chapter provides an overview of different methods and challenges in experimental, marine research.

The first two results-chapters (**Chapter 2** and **3**) focus on the impacts of seawater acidification and warming on benthic soft-sediment communities from a coastal, subtidal and an intertidal area, respectively. In **Chapter 2** responses of bivalves, meiobenthos and bacteria to a broad range of pCO_2 [2] were examined over a period of 12 weeks to investigate effects of seawater acidification in scenarios of future ocean acidification and potential CO_2 leakage from carbon capture and storage. Effects of high pCO_2 on bivalves were species-specific and size-dependent, signs of shell dissolution in the cockle *Cerastoderma edule* already occurred at 1,500 µatm (pH 7.7) and mortality of small bivalves occurred at lower pCO_2 than that of larger, adult cockles. Moribund cockles migrated to the sediment surface which presented a visual indication of high pCO_2. Meiofauna and bacterial community structure was significantly changed at the highest pCO_2 level of 24,400 µatm (pH 6.4). While nematode density and community structure remained similar over all treatments, less dominant taxa responded more sensitive to the changes in seawater pH. Densities of calcifying meiobenthos declined significantly at >6,000 µatm while Gastrotricha showed a very tolerant and opportunistic response to high pCO_2 exhibiting significantly higher densities at 24,400 µatm. These results revealed that even low pCO_2 may induce disturbances in bivalve dominated sandy communities on multiple trophic levels and emphasized the importance of rare taxa and biotic interactions in ocean acidification research.

Cumulative effects of seawater acidification (ΔpH=-0.4) and warming (ΔTemp=+3 °C) significantly altered meiofauna community composition in intertidal sediments after 8 weeks of exposure (**Chapter 3**). Similar to previous chapter, nematode responses were moderate while other, less dominant taxa showed significant density differences between treatments. As such, reduced pH and elevated temperature resulted in elevated densities of predatory Platyhelminthes, reduced densities of Copepoda and Nauplii and complete absence

[2] pCO_2 describes the partial pressure of CO_2

of Gastrotricha compared to the experimental control. We hypothesized that these differences result from a combination of group-specific sensitivity to the changed abiotic conditions and changes in biotic interactions such as predation pressure of Platyhelminthes and predatory nematodes on other meiofauna. Both studies (Chapter 2 and 3) underline the importance of biotic interactions between multiple size classes and trophic groups for the assessment of environmental change resulting from near future ocean acidification and warming scenarios. Furthermore, we advocate the study of less dominant taxa since responses of these organisms may present a more sensitive indication of environmental stress related to ocean acidification compared to the dominant meiofauna (e.g. nematodes).

The next Chapters deal with some of the potential impacts of mineral extraction on bathyal and deep-sea soft-sediment communities and more particularly with the impacts of deposition with coarse and fine substrate and copper toxicity.

In a short-term (11 - 15 days) mesocosm experiment, burial of a bathyal community from a Norwegian fjord with different amounts of mine tailings and natural sediment resulted in reduced community oxygen consumption, ^{13}C-labelled algal uptake and carbon processing which are relevant measures of ecosystem functioning (**Chapter 4**). First signs of reduced ecosystem functioning were already visible at 0.1 cm mine tailings and were aggravated at burial with increased amounts of substrate. Furthermore, meiofauna showed a vertical migration response possibly as a result of reduced oxygen concentrations in the natural sediment, however, this migratory response was associated with an elevated mortality of nematodes. Despite some general similarities, distinct differences between the substrates used in this study were found with regard to sediment oxygen consumption, oxygen penetration depth and carbon uptake. This suggests that the effect of burial on sediment processes depends on the type of substrate and emphasizes the importance of sediment characteristics for the assessment of burial impacts. We conclude that instantaneous burial with very small amounts of substrate may already impair the functioning of benthic communities in bathyal fjords on the short term.

In **Chapter 5** we have tested the effects of burial with small particles of crushed polymetallic nodules[3] on an *in situ* sediment community from an abyssal nodule field located in the South East Pacific. Experimental deposition resulted in a 2 cm layer of the coarse substrate over the surface area of the deployed enclosure corrals. We sampled the sediment after an incubation time of 11 days, comparable

[3] Polymetallic nodules are small, cauliflower-shaped mineral deposits covering the abyssal seafloor at depths between 4000-6000 m

to the previous experiment, and analysed the structure of the meiobenthos and nematode community and measured copper uptake in several nematodes to gain a first impression of potential toxic effects of the crushed nodule substrate. Similar to the previous chapter, a high proportion of meiofauna was found in the added substrate indicating active migration into a potentially unsuitable habitat. Proportions of copepods and nauplii were higher in the added substrate than those of nematodes, pointing towards a higher mobility and colonization potential of these animals. Nematode genus composition revealed no apparent differences between the community in the added substrate layer and underlying layers meaning that the migration response of nematodes was not species specific. Nematode copper uptake was similar in animals from Control sediments and from the crushed nodule layer. The finding of a vertical escape response potentially accompanied by elevated faunal mortality, similar as was found in Chapter 4, may have significant implications for the risk-assessment of deep-sea mineral extraction.

The effects of abiotic factors relevant under deep-sea conditions such as hydrostatic pressure and temperature on metal toxicity, were assessed by means of acute toxicity tests on the marine nematode *Halomonhystera disjuncta* in **Chapter 6**. High hydrostatic pressure (10 MPa) significantly increased sensitivity of the nematodes to copper while sensitivity was reduced at low temperature (10 °C compared to 20 °C). We therefore concluded that toxicity assessments in deep-sea organisms should be done under consideration of the effects of both stressors and that existing data from shallow water species cannot easily be generalized to a deep-sea context.

Subsequently, we tried to assess the sensitivity of an abyssal sediment community to burial with copper contaminated sediment (**Chapter 7**). This was done by exposing an *in situ* community from an abyssal nodule field to sediment that was contaminated with a range of copper concentrations (0 – 20 mg L^{-1} Cu^{2+}), widely covering lethal levels for nematodes determined in Chapter 6. After 96 h we sampled the sediment and analysed the structure of the meiobenthos community and the copper content in several nematodes from the Control sediment and from sediment with the highest copper concentration. Again, all meiofauna showed an active migration behaviour and were found in high abundances in the added sediment regardless of the copper contamination. Similar to Chapter 5, copepods and nauplii were more successful in colonizing the new sediment compared to nematodes. Interestingly, analysis of nematode copper uptake revealed lower values in animals from the contaminated sediment than in nematodes from the Control sediment. We critically reviewed our experimental method and concede

that the added substrate potentially became depleted of copper through out-washing of the soluble fraction from the artificial sediment during its application or through sorption processes by the manganese-oxihydroxide and iron-oxide rich natural sediment. Low copper bioavailability in the sediment may explain the reduced copper values in nematodes exposed to the artificial sediment. While this study exemplifies the difficulties in (deep-sea) experimental research it also reveals new questions regarding metal detoxification (e.g. why is nematode copper content in exposed nematodes lower and not equal to control animals?), stimulating further investigation.

The research presented in this thesis indicated the high potential of experimental studies to substantially contribute to the understanding of general patterns associated with environmental disturbances on different spatio-temporal scales. The limitations and challenges of experimental research as well as the implications of our results in terms of risk assessment and monitoring of disturbed areas are integrated in **Chapter 8** and discussed in the light of previous research.

Altogether, our results revealed that structural changes of the benthic community and particularly their vertical positioning in the sediments, may be a powerful indicator of first signs of environmental stress, offering advantages for the biomonitoring of impacted sites. Furthermore, the differential sensitivity of different meiofauna groups emphasises the importance of applying an ecosystem-based approach which also allows to account for biotic interactions among size classes and trophic levels. However, these approaches should be accompanied by single-species studies that inform about the exact mechanisms of stressors on the physiology of different species. Finally, cumulative effects of stressors have been identified in different experiments presented here which strongly support the prioritization of multi-stressor experiment in future research.

Chapter 1 General Introduction

The Earth as we know it has been altered by humans for roughly 8,000 years with profound effects on the environment. In the more recent history, anthropological influences extended to such a degree that it has been suggested to mark this time period as an entirely new epoch, the Anthropocene (Crutzen, 2006). In order to define a new epoch, certain criteria must be met. These include that changes have to occur globally and that they can be traced in the geological record as stratigraphic material (rock, glacier, sediment)(Lewis and Maslin, 2015). Evidences of such permanent marks have been collected worldwide (Waters et al., 2016). Naming the Anthropocene and, especially, defining its starting point will have wider, sociopolitical implications and will alter the way people perceive themselves as part of the Earth as a system (Lewis and Maslin, 2015).

Major global anthropogenic modifications to the Earth and its climate have been caused by the invention and use of chlorofluorocarbons, testing and use of thermonuclear weapons, exploitation of mineral resources and the vast increase in the release of greenhouse gases predominantly caused by the burning of fossil fuels, cement production, and land use change (Crutzen, 2006; Le Quéré et al., 2014; Waters et al., 2016). Since the beginning of the industrial revolution halfway the 18th century, CO_2 concentrations have risen to levels that exceed those of the past 800,000 years and are continuing to increase at an unprecedented rate (Hartmann et al., 2013; Lüthi et al., 2008). As a result, the carbonate system in the oceans is modified, leading to a widespread phenomenon called ocean acidification (OA, see next section) (Rhein et al., 2013). Furthermore, the overall increase of greenhouse gases and other factors such as deforestation have led to a warming of the land and ocean surface by 0.85 °C over the period from 1880 – 2012 (Hartmann et al., 2013). This warming is predicted to continue to average temperature rises between 0.3 °C and 4.8 °C by the end of this century, depending on the Representative Concentration Pathway (RCP) defined in the report of the Intergovernmental Panel on Climate Change (IPCC)(Collins et al., 2013; IPCC, 2013). To alleviate the most devastating consequences, the international community has agreed to reduce global carbon emissions and try to limit global temperature rise to 2 °C in the Paris Climate Agreement of the United Nations Framework Convention on Climate Change in December 2015.

The transition to a low-CO_2 society to meet the UN's targets will require an increased production of known renewable energy resources and the development of new technologies to advance global energy production, but also to commence mitigation strategies. However, production of this new infrastructure to move away from non-renewable energy sources requires large amounts of another finite resource: rare earth elements (REE) and other metals (Vidal et al., 2013).

Provided that recovery efficiencies of mineral resources will improve with the development of new technologies, some metal reserves like copper, may suffice to support global demand for over 5000 years (Kesler and Wilkinson, 2008). In contrast, other metals like hafnium, antimony, platinum or metals that look promising for new solar power technologies like gallium, indium or tellurium may run out in a very short time of 5-15 years if they are extensively used in renewable energy technology production (Cohen, 2007; Teske et al., 2016). Next to that, the geopolitical situation and uneven distribution of minerals on earth is driving countries and industries to look for alternative sources to satisfy their mineral demand (Herrington, 2013).

The oceans, covering more than 70 % of the world's surface area, contain a wealth of living and non-living resources and provide valuable ecosystem services[4] to mankind (Petersen et al., 2016; UNEP, 2006). In the largest marine biome, the deep sea, polymetallic nodules are found in vast areas of the abyssal plains, cobalt-rich ferromanganese crusts cover the flanks of seamounts, and active and non-active hydrothermal vents are aligned along mid-ocean ridges and other volcanically active areas (Petersen et al., 2016). These untapped mineral deposits harbour large amounts of economically valuable minerals such as zinc, copper, nickel, silver, gold or REE (Hein et al., 2013; Petersen et al., 2016). The extraction of these marine resources will almost certainly impact the structure and functioning of the benthic ecosystem in the deep sea with unknown consequences in the long term (Gollner et al., 2017; Wedding et al., 2015).

Impacts resulting from increased atmospheric carbon concentrations (e.g. OA and warming) and mineral resource exploitation on marine, benthic communities are the main subjects of this thesis. What follows is a more detailed description of stressors, potential environmental harm and knowledge gaps in both topics.

1.1. Atmospheric carbon increase

1.1.1. Ocean acidification

Our ocean, atmosphere, biosphere and lithosphere are tightly linked via biogeochemical cycles. The carbonate cycle refers to the flux of carbon through different compartments (Berner and Berner, 2012). In this cycle, the ocean acts as a moderator of atmospheric CO_2 increases and as a net carbon sink via sedimentation of particulate organic and inorganic carbon (Emerson and Hedges,

[4] Ecosystem services: A broad term describing benefits people obtain from ecosystems and including supporting (e.g. nutrient cycling), provisioning (e.g. food, freshwater), regulating (e.g. Climate, water purification) and cultural (e.g. spiritual, educational) benefits. (Millennium Ecosystem Assessment, 2005)

2008). In water, inorganic carbon dioxide exists in four different forms: aqueous dioxide (CO_2), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-})(Zeebe and Wolf-Gladrow, 2001). These four forms of carbon are related as described by the following equilibrium (1).

$$CO_2\,(atmos) \leftrightarrow CO_2\,(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad (1)$$

Through the exchange of gaseous CO_2 at the water-air interface, carbon dioxide enters the ocean in the aqueous phase where it reacts with water to form carbonic acid, which in turn can dissociate to bicarbonate and carbonate with the formation of H^+. The pH of freshwater and seawater is described by the simple function $pH=-\log_{10}[H^+]$, thus the carbon chemistry in the ocean is tightly linked to its pH and vice versa. When CO_2 concentration in the atmosphere increases, so will the dissolution of this gas into the surface ocean, leading to a shift of the carbon equilibrium to the right of the equation causing an increase in $[H^+]$ concentrations. As a consequence, pH decreases meaning that the seawater will become more acidic. The term "ocean acidification" (OA) describes the decrease in surface seawater pH as a result from anthropogenically elevated carbon dioxide concentrations in the atmosphere (see Fig. 1.1).

Increasing H^+ concentrations, furthermore, result in a decrease of carbonate concentration in the seawater and a projected pH drop of 0.3 – 0.4 units by the year 2100 would result in a 150% increase in H+ and a decrease in CO_3^{2-} by 50% (Doney et al., 2009; Orr et al., 2005). In the ocean, the formation of calcium carbonate mainly depends on the availability of carbonate ions and the saturation state is defined as (2),

$$\Omega = \frac{[CO_3^{2-}][Ca^{2+}]}{K'_{sp}} \qquad (2)$$

where K'_{sp} is the stoichiometric solubility product which is dependent on temperature, salinity, pressure and the particular mineral phase (Doney et al., 2009). Evidenced by time series measurements, the saturation state of calcite and aragonite -different crystal forms of calcium carbonate- in the ocean has declined continuously since pre-industrial times (Doney et al., 2009).

In the ocean, temperature, pressure, salinity and alkalinity of the seawater play an important role in determining the seawater pH in addition to the partial pressure of CO_2 (pCO_2) and, therefore, the intensity and speed of ocean acidification varies regionally (Rhein et al., 2013; Zeebe and Wolf-Gladrow, 2001). In general, polar regions are less well buffered against pCO_2 and $[H^+]$ changes, because of the higher solubility of CO_2 in cold waters (Zeebe and Wolf-Gladrow, 2001), whereas subtropical gyres show a stronger buffering capacity (Egleston et al., 2010). This observation coincides well with the observed surface pH reductions of 0.10 in the

Northern Atlantic since the mid-1800s compared to 0.05 in the subtropical South Pacific (Rhein et al., 2013).

Due to the generally strong buffering capacity of the ocean, pH in the marine environment has remained very stable between a pH of 8.0 and 8.3 over the past 25 million years (Widdicombe and Spicer, 2008). However, in coastal environments, the amplitude of daily, seasonal and annual pH shifts can be high, approaching pH changes that are predicted to occur globally by 2100 (Hofmann et al., 2011) and coastal organisms have adapted to these pH fluctuations. Nevertheless, ocean acidification is occurring at a fast rate and in combination with other stressors, such as hypoxic events and pollution, coastal environments may experience larger pH shifts than what is predicted on a global scale (Duarte et al., 2013). In the past, events such as the Permo-Triassic mass extinction have been correlated to rapid seawater acidification (Clarkson et al., 2015).

Calcite and aragonite form the main constituent of shells and skeletons of marine organisms and a reduction of the calcite and aragonite saturation in the water column, especially hampers calcification of marine shelled-organisms, making this group especially vulnerable to ocean acidification (Gazeau et al., 2013; Hendriks et al., 2010; Orr et al., 2005). But also non-calcifying marine organisms may experience physiological challenges resulting from shifts in external seawater pH. In water breathers, the regulation of the internal acid-base balance is dependent on ion exchange mechanisms, especially of H^+ and bicarbonate carriers (Pörtner et al., 2004). Under hypercapnic conditions, i.e. conditions of elevated pCO_2, the organisms will strive to re-establish their old or a new acid-base balance, a process that consumes energy through increased ion transport over the cell membranes (Pörtner et al., 2004). Thereby, hypercapnia can alter metabolic rates and energy allocation, causing severe long-term effects on individual and population level (Fabry et al., 2008; Pörtner et al., 2004; Sokolova et al., 2012). Yet, the exact mechanisms of how marine animals cope with increased surrounding pCO_2 (hypercapnia) and resulting physiological and ecological consequences are still under debate and seem to depend most on the life style, activity and life stage of organisms rather than on their phylogeny (Byrne, 2011, Pörtner et al., 2004; Widdicombe and Spicer, 2008).

1.1.2. Warming of the ocean

Next to the uptake and regulation of carbon dioxide from the atmosphere, the ocean also absorbs a large portion of the solar radiation on the planet, making it a major contributor to the regulation of the earth's climate. Ocean temperature increases if solar irradiation exceeds the thermal radiation back to space (radiative forcing(Lashof and Ahuja, 1990)). From 1971 to 2010, the global upper ocean

(>75 m water depth) has warmed by 0.11 °C per decade, equivalent to heating of 0.42 W m^{-2} (over the entire Earth surface) or 63 % of the increase in the global energy inventory (Rhein et al., 2013). The majority of scientists acknowledges that this increased heat budget of the Earth is a result of anthropogenic activities and an increase of greenhouse gases in particular (Bindoff et al., 2013). An increase in greenhouse gases in the atmosphere increases radiative forcing, meaning that more energy from the sun is retained in the atmosphere which ultimately leads to a warming of the Earth's surface (Lashof and Ahuja, 1990).

Although to a lesser extent, the deep ocean also contributes 30 % to the change in energy inventory and has warmed by 0.015 °C during the past 40 years with the highest increases in heat content observed in the deep Southern Ocean (Rhein et al., 2013). It has also been shown that in times of low heat uptake by the surface ocean (<300 m water depth), the deep ocean takes up significantly more heat (Meehl et al., 2011). Based on four climate change scenarios, an increase in ocean surface temperature of 0.5 – 1.5 °C is expected in the upper 1000 m by 2100 (Collins et al., 2013). Strongest increases are predicted to occur in the tropical and subtropical regions and in the deep Southern Ocean (Collins et al., 2013).

The mechanisms and some of the effects of ocean acidification and warming on the marine environment are summarized in Fig. 1.1. As this figure points out, both stressors change at the same time and do not occur in isolation. In this thesis we,

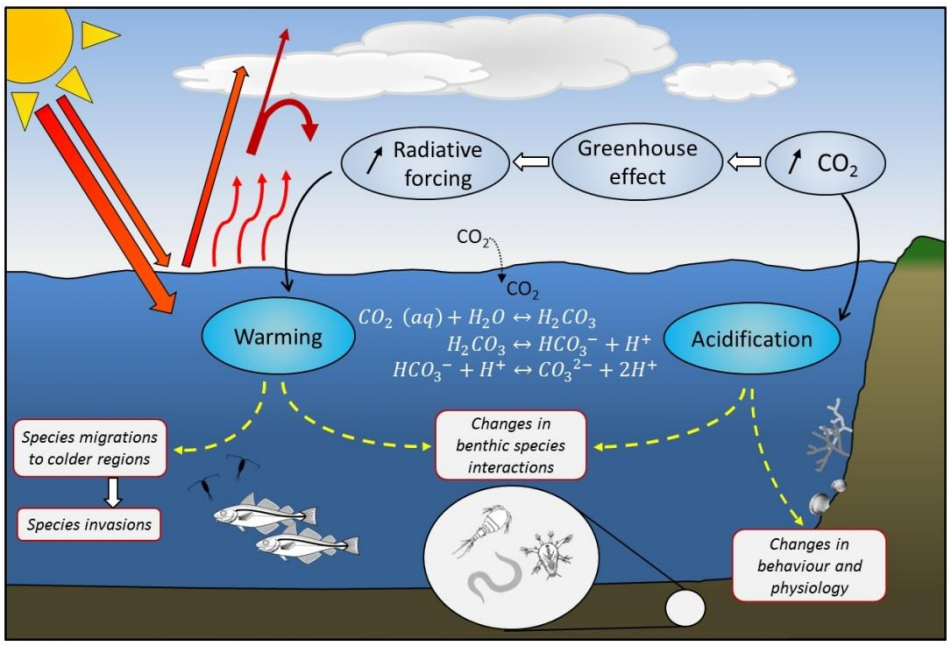

Figure 1.1 Schematic overview of the mechanisms of ocean acidification and warming with some of the most important impacts on the marine environment.

therefore, address the cumulative effects of both, ocean acidification and warming on the marine benthos (Chapter 3). Ocean acidification research is rapidly expanding and many studies have been performed on single species and species assemblages, but studies on the combined effects with other stressors, and temperature change in particular, are largely underrepresented in this branch of research (Wernberg et al., 2012).

1.1.3. Sub-seabed carbon capture and storage

To reduce the adverse ecological and economic effects of global change, including ocean acidification and warming as a result of rising CO_2 emissions, different mitigation options have been proposed (Marchetti, 1979). A reduction in fossil fuel use, increased use of renewable energy sources and improved efficiency of energy conversion and devices are the most obvious measures to reduce carbon emissions. However, this may not be sufficient to prevent the predicted atmospheric CO_2 peak with CO_2 concentrations rising from 350 ppm to 1000 ppm or more in the next 100 years (Collins et al., 2013). Therefore, additional carbon capture and storage (CCS) is suggested as a mitigation method acting on time scales of hundreds to thousands of years (Metz et al., 2005). Several options for CCS exist, including direct injection into the deep sea or sub-seabed storage in reservoir rocks and in depleted oil or gas fields (Metz et al., 2005).

Direct injection into the deep sea is based on the concept that carbon dioxide undergoes a phase change from gas to liquid at high pressures accompanied by a sharp decrease in volume, which allows storage of large amounts of carbon in a limited space (Holloway, 2005). Injection of CO_2 at the bottom of well-selected deep-ocean basins would create CO_2 lakes and the deep-ocean circulation would prevent outgassing of the CO_2 to the atmosphere for the next centuries. The whole water body of the ocean possesses an enormous buffering capacity with regard to CO_2, however, since ocean acidification is a process happening at the ocean-atmosphere interface, much of this buffering capacity, particularly that of the deep ocean remains untapped. The injection of CO_2 into the deep-ocean would accelerate the equilibration of increased pCO_2 of the surface ocean with the deep sea, a process that would otherwise take centuries (Barry et al., 2004). Another method, sub-seabed storage of carbon dioxide, will be accomplished by the injection of CO_2 in porous reservoir rocks (see Fig. 1.2). The CO_2 replaces parts of the pore-liquid and migrates to higher parts of the reservoir rock where it is trapped by an impermeable cap rock to ensure stable and long-term storage (Holloway, 2005). In case of a leak into the marine environment, however, the CO_2 will immediately react with the surrounding water causing localized acidification of sediment pore water and seawater, varying in severity depending on duration and

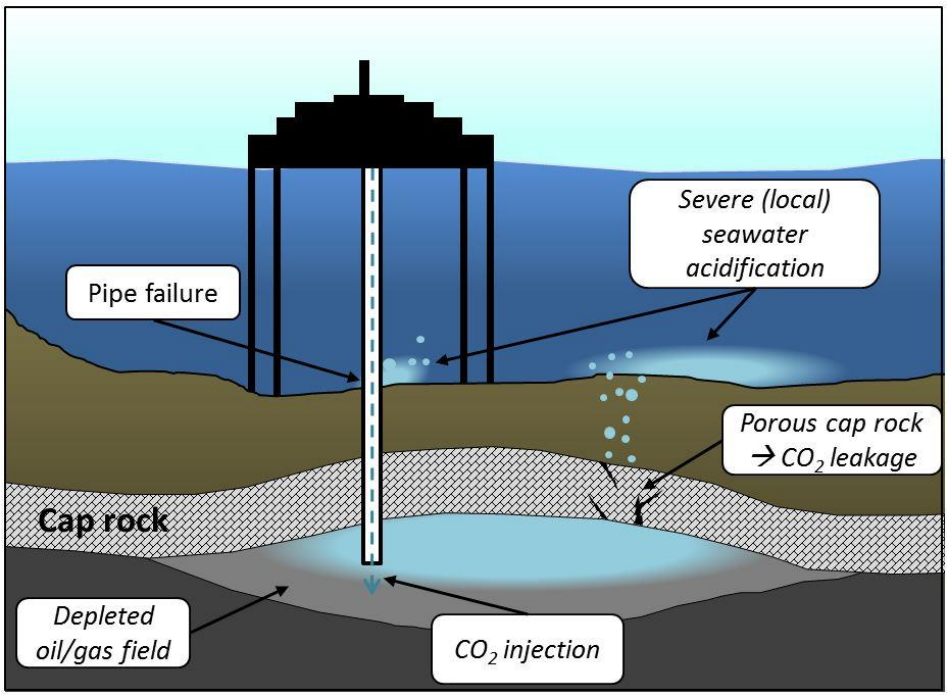

Figure 1.2 Simplified and schematic presentation of the implementation of CCS and potential associated risks for the marine environment.

type of the leakage (Blackford et al., 2009, 2008). At this point, the possible impacts on the marine environment arising from potential leaks due to e.g. sediment faults, permeable cap rocks or pipeline failure require to be studied and evaluated carefully before considering CCS implementation on a large scale.

Despite being a relatively secure method of carbon storage for very long time scales, environmental impacts in case of an accident need to be anticipated and methods for efficient monitoring and detection of leaks need to be developed. The research conducted in Chapter 2 reports the responses of a bivalve dominated infaunal community to a very broad range of pCO_2 to investigate impacts of ocean acidification but also of pCO_2 changes that may occur during leakage events from CCS. This will increase our insight in the physiological and behavioural impacts of strongly elevated pCO_2 on different taxonomic groups.

1.2. Exploitation of deep-sea mineral deposits

Already during the "HMS *Challenger*" expeditions (1872 - 1876), mineral deposits, called polymetallic nodules, were discovered in the deep sea (Murray and Renard, 1891). These are round or cauliflower shaped rocks that are primarily formed by the accumulation of metals around manganese- and iron-oxides and contain

increased concentrations of minerals like manganese, copper, cobalt, nickel and REE (Hein et al., 2013; Hein and Koschinsky, 2014; Koschinsky and Halbach, 1995). However, it was only until 1977 that their high economic value has been assessed by Mero (1977).

In that same year, another geological feature of the deep seafloor has been discovered: hydrothermal vents[5] (Corliss et al., 1979). These geological features are formed in areas of high volcanic activity such as mid-ocean ridges, back-arc basins or submarine volcanic arcs (Hannington et al., 2005). There, the seawater in the cracks of the rock reacts with the magma in the sub-seafloor forming a hot, acidic hydrothermal fluid that is rich in dissolved metals which precipitate when the hot fluid meets colder surrounding seawater (Petersen et al., 2016). This continuous precipitation as a result of the volcanic activity creates massive domes of metal enriched rock that are often referred to as seafloor massive sulphides (SMS).

A third, promising marine mineral resource are cobalt-rich ferromanganese crusts, or polymetallic crusts, which are formed by the precipitation of hydrated manganese and iron oxides to rocky surfaces at depths between 400 to 7000 m (Hein et al., 2013). Those precipitates acquire trace metals via surface sorption, resulting in metal enriched deposits containing high amounts of economically interesting cobalt and nickel, though also tellurium and REE can be found in elevated concentrations (Hein et al., 2013). Due to the differences in location and properties of these three mineral deposits (polymetallic nodules, seafloor massive sulphides and polymetallic crusts), mining will require the use of different kinds of equipment. While polymetallic nodules may be collected off the surface of the seafloor, mining of SMS and polymetallic crusts will rather be a three dimensional operation, comparable to open pit mining on land.

As polymetallic nodules are found at very great water depth (>4000 m), most of the nodule fields lie in the area beyond national jurisdiction (ABNJ). In the United Nations Convention on the Law of the Sea (UNCLOS), this part of the seafloor is defined as "the Area" and is considered "common heritage of mankind" (UNCLOS, Art. 137). In this treaty, the International Seabed Authority (ISA) has been assigned as an independent legislative body to regulate all activities in the Area. However, in case of SMS and polymetallic crusts, deposits may lie within the Exclusive Economic Zone (EEZ) of a country which, therefore, owns the sovereign rights over this resource and may issue licences for mining but, at the same time, also carries the responsibility of proper environmental management. In 2011, Papua New Guinea issued a mining licence for SMS mining in the Bismarck

[5] Hydrothermal vents are also known as black or white smokers due to the frequent presence of chimney-like structures and the "smoke-like" emission of hydrothermal fluids.

Sea to Nautilus Minerals Inc. (Solwara-1 project) and mining is projected to start in early 2018 (Nautilus Minerals, 2015). This first deep-sea (1600 m) mining operation of SMS is closely watched by the international community.

The most commercially attractive area for polymetallic nodule mining is located in the central North Eastern Pacific, between the Clarion and the Clipperton Fracture Zone (CCZ) at 4000 -5000 m water depth (International Seabed Authority, 2017a; Wedding et al., 2015). Currently, the ISA has issued 17 exploration licences of which 3 are pending signature (International Seabed Authority, 2017b, Fig. 1.3). A licence for polymetallic nodule exploration is set for a 15 year span and allows the contractor exclusive rights over one particular area in the CCZ (max. 150 000 km² in the first half, after which half of the area is taken as a reserve area for developing countries) (International Seabed Authority, 2017a). In this period the contractor is obliged to gather baseline scientific data on the environment present in his area and on the best available technology and practices for deep-sea mining, which all have to be reported to the ISA. In the Belgian claim area, equipment tests with a prototype, that may be up-scaled to an industrial sized mining device, are planned to happen within the next two years (Deme-Group, 2017).

Mining of seabed resources will inevitably affect the marine environment and its

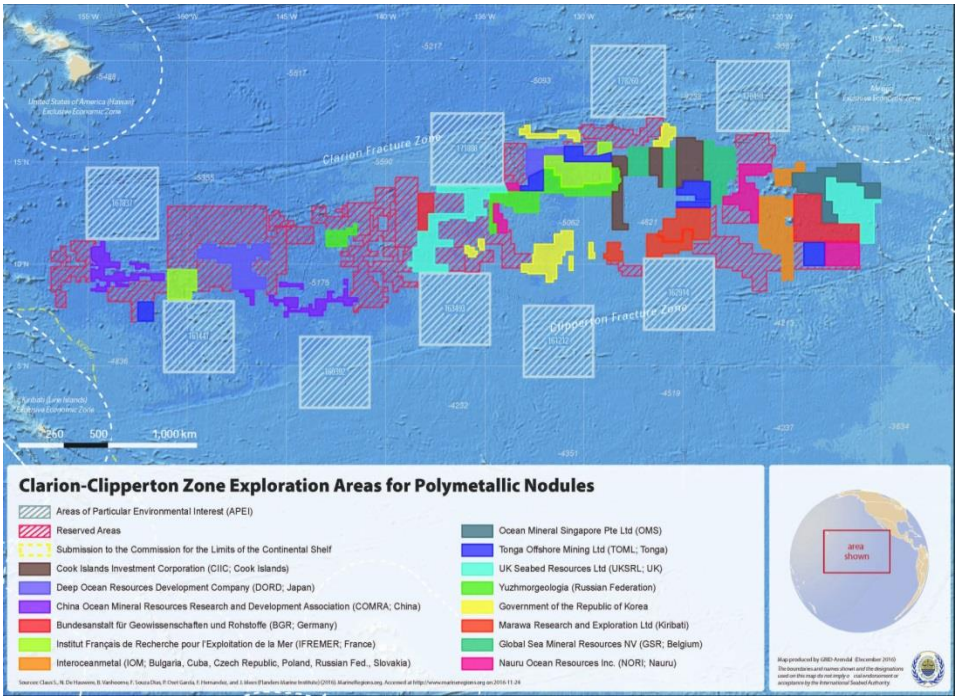

Figure 1.3 Map of concession areas for polymetallic nodules in the CCZ. White boxes show Areas of Particular Environmental Interest (APEI). Source: International Seabed Authority, https://www.isa.org.jm/contractors/exploration-areas, accessed 15.08.2017.

inhabitants. In the case of polymetallic nodules, collection of the nodules by the mining device will result in direct pressures such as the removal of hard and soft substrate, sediment compaction and noise and light pollution (Oebius et al., 2001). Next to that, the mining operation will also create sediment resuspension into a sediment plume with subsequent blanketing of adjacent areas and mobilization and release of elevated concentrations of potentially toxic elements may occur during extraction or after processing of the minerals (e.g. through the release of extraction water or tailings to the water column) (Boschen et al., 2013; Koschinsky et al., 2001a, 2001b; Thiel, 2001, Fig. 1.4) (Oebius et al., 2001). These pressures may in turn result in the fragmentation and modification (i.e. change of chemical properties or sediment composition) of the remote and specialized habitats in the deep sea, and impact the biological communities (Vanreusel et al., 2016). Despite the high international attention, ecosystems of the targeted areas remain poorly studied and mechanisms of resilience and recovery of the benthic fauna are largely unknown (Gollner et al., 2017; Wedding et al., 2015).

In the assessment of environmental impact of an activity on an ecosystem two main questions arise: 1) How much disturbance can the ecosystem absorb before undergoing a change (relating to resilience) and 2) How fast, if ever, can the ecosystem return to its initial state (relating to recovery rate). Resilience is the capacity of a system to absorb disturbance and reorganize while undergoing change so as to still retain essentially the same function, structure, identity, and feedbacks (Walker et al., 2004). If a disturbance impacted community structure and function, the communities or populations may, over time, return to their initial state (full recovery), when the disturbance abates (Lotze et al., 2011). According to Boyd et al. (2003), two aspects need to be considered in the assessment of recovery rates of benthic communities: 1) The settlement of new recruits (re-colonization) and 2)

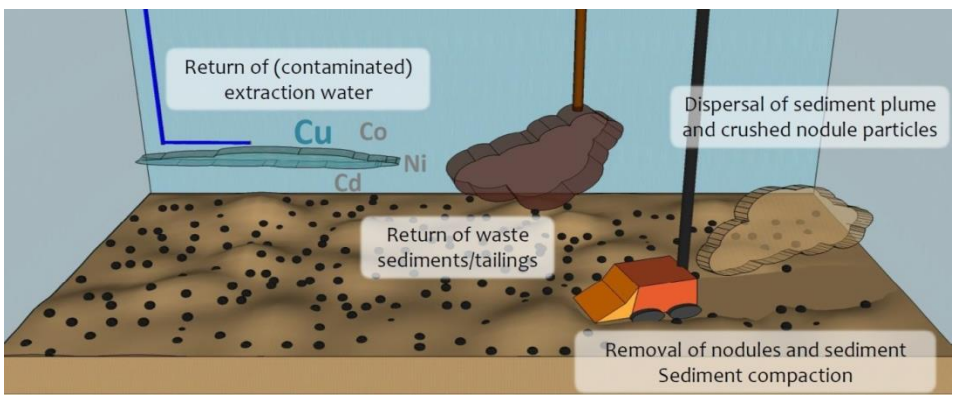

Figure 1.4 Schematic overview of possible impacts resulting from deep-sea mining by the example of polymetallic nodule extraction.

the return of community structure (restoration). Commonly reported attributes of community structure include number of species, abundance, biomass or age structure of populations (Boyd et al., 2003).

In the past, several small scale experiments have been conducted simulating effects of polymetallic nodule extraction, by e.g. disturbing the seafloor with an epibenthic sledge, that were monitored over time (Jones et al., 2017). Gollner et al. (2017) analysed available biological data from these disturbances to assess the recovery of population density and diversity of mega-, macro- and meiobenthos in a meta-analysis. Results suggest that recovery rates vary among taxa, and can be correlated with size class and mobility of the organisms (Gollner et al., 2017). While densities of small, mobile organisms may recover in relatively short time spans (2-7 years), densities of sessile fauna recovered very slow or have not recovered even after a few decades (Gollner et al., 2017). Despite the fact that densities of mobile taxa returned to their initial state, in many cases substantial shifts in community composition occurred and lasted for decades or have not recovered to their original state (Gollner et al., 2017). Furthermore, mobile taxa usually show a higher potential for recovery or are less affected by mining activities through avoidance (Jones et al., 2017). In contrast, sessile taxa rely on hard substrates and the recovery of those organisms after mining is very unlikely (Gollner et al., 2017; Vanreusel et al., 2016). The loss of those species may entail adverse effects on other taxa that live in close association with e.g. sponges and corals (Gollner et al., 2017; Purser et al., 2016). As evidenced by Vanreusel et al. (2016), polymetallic nodule coverage is highly correlated with epifaunal densities and diversity, emphasizing the importance of hard substrate provided by the nodules for the abyssal ecosystem.

Keeping in mind that polymetallic nodules are very slow-growing (<5 mm per million years; Morgan and Cronan, 2000) and that faunal recovery after disturbance is most likely very slow and perhaps incomplete, combined with the fact that mining would affect very large areas, policy makers need to make very well-considered decisions regarding the regulation of polymetallic nodule mining. Adequate environmental protection within a mining context can, however, only be achieved if we possess a good understanding of the state, functioning and recovery potential of the various ecosystems, and on the mining impacts as such. To date, we still lack sufficient data and knowledge to make well informed decisions, especially with regard to large-scale disturbances, sediment plume effects and metal toxicity in deep-sea benthic organisms as well as cumulative effects of multiple stressors and consequences for ecosystem services (Beaulieu et al., 2017; Gollner et al., 2017; Le et al., 2017; Mestre et al., 2014).

In order to increase our understanding of metal toxicity and burial with different substrates such as crushed nodules and sediment, we performed controlled

experiments on abyssal soft-sediment communities or using proxy species from shallow water environments. This baseline experimental research provides first insights in short-term responses and mechanisms of these stressors on marine benthic organisms, and it also identifies knowledge gaps and the potential for further research.

Another consequence of any mining operation, whether it is on land or offshore, is the production of large amounts of tailings - remnants of the ore after extraction of the resource of interest. The proportion of produced tailings to extracted resource varies with ore grade and resource type but can amount up to 99 % for copper mining or a staggering 99.99 % in case of gold mining (Vogt, 2013). To date, huge amounts of tailings have been disposed of in landfills, lakes and riverine systems, but also the disposal in the ocean is practiced in a number of countries (Vogt, 2013). Dam failures from tailings disposal in terrestrial systems have led to devastating environmental consequences in the past (Rico et al., 2008) but economic and esthetical reasons are also put forward when the implementation of submarine tailings disposal (STD) is discussed (Kvassnes and Iversen, 2013; Vogt, 2013). STD can entail a range of environmental impacts on the benthos such as hyper-sedimentation, changes in grain size, smothering of fauna and toxicity of heavy metals or chemicals (Ramirez-Llodra et al., 2015). Although STD operations are accompanied by monitoring studies, mostly, impacts on only one or few aspects of the ecosystem are reported and thorough baseline studies are lacking. Furthermore, to date only few studies have experimentally quantified effects of tailings disposal on benthic ecosystems that might aid the evaluation of threshold values.

This knowledge gap has partly been addressed in a mesocosm experiment reported in Chapter 4 where we investigated burial with different amounts of mine tailings and sediment on a bathyal soft-sediment community from a Norwegian fjord. Next to structural changes of different components of the community, carbon remineralization potential and oxygen consumption were determined as measures of ecosystem functioning.

1.3. Marine meiobenthos

The meiobenthos (or meiofauna[6]) comprises mobile small invertebrates that permanently or temporarily reside inside marine sediments and are retained on a 38 μm (or 32 μm) sieve but pass through a 1000 μm sieve. The definition by size includes both metazoans[7] and protists[8], however, in the context of this thesis I will

[6] In this thesis, the denominations "meiobenthos" and "meiofauna" are used synonymously.
[7] The term Metazoa describes all multicellular animals

focus solely on the metazoan meiofauna, thus excluding e.g. Foraminifera and Radiolaria. Meiobenthos can be further subdivided in temporary and permanent meiobenthos: the temporary meiobenthos only resides inside the sediment for part of its lifetime (usually in larval or juvenile stages), while the permanent meiobenthos inhabits the sediment during their entire life and generally lack a planktonic larval stage (Higgins and Thiel, 1988). From the 33 metazoan phyla, 22 have representatives in the meiofauna of which 5 phyla are exclusively meiobenthic and belong to the permanent meiofauna (i.e. Gastrotricha, Gnathostomulida, Kinorhyncha, Loricifera and Tardigrada) (Higgins and Thiel, 1988). In most marine sediments, meiobenthic assemblages are dominated by free-living nematodes that can comprise >90% of the meiobenthic assemblage, usually followed by harpacticoid copepods (Moens et al., 2013; Schratzberger and Ingels, 2017). Therefore, most meiofauna research has focused on those particular taxa resulting in a vast body of literature on marine nematodes and copepods (Schratzberger and Ingels, 2017). Nevertheless, the role and importance of rare meiofaunal taxa in different ecosystems and with regard to biotic interactions receives increasingly more attention (Bianchelli et al., 2010; Gambi et al., 2010; Pusceddu et al., 2011; Schade et al., 2016).

Due to their small size, the distribution of meiobenthic organisms is largely determined by the biogeochemical conditions in the sediment such as oxygen availability, organic matter content or grain size composition (Ingels and Vanreusel, 2013; Neira et al., 2001; Steyaert et al., 2003; Vanreusel et al., 1995), but also biotic interactions may play an important role (De Meester et al., 2015; Ingels et al., 2014). In most marine habitats, the biomass of meiobenthic organisms is usually lower than that of macro- or megafauna, though in abyssal habitats meiofaunal biomass may exceed that of macrofauna (Rex et al., 2006). Nevertheless, the importance of meiobenthos for benthic ecosystems is rather displayed in their high activity and life cycle turnover rates that significantly contribute to secondary production and food consumption (Coull, 1999; Gerlach, 1971). Apart from that, the presence of meiofauna in marine sediments has been shown to affect other benthic size classes. For example, meiofaunal bioturbation, mucus production and predation pressure stimulate bacterial growth and bacterial nitrification and denitrification (Bonaglia et al., 2014; Coull, 1999). Meiofauna may also interfere with macrofaunal settlement and indirectly structure macrobenthic communities which may lead to modified ecosystem properties and functioning (Piot et al., 2014; Schratzberger and Ingels, 2017; Watzin, 1983).

[8] The term Protista describes all unicellular, eukaryotic, organisms

Next to their relatively high importance for the functioning of benthic ecosystems, meiofauna may serve as a bioindicator of environmental stress with regard to climate change and anthropogenic activity such as pollution by metals or chemicals, physical disturbances and mineral resource extraction (Zeppilli et al., 2015). In addition to that, the ease to sample and culture shallow-water nematodes and copepods, their short life-spans and high fecundity render them very suitable test organisms for laboratory studies (Austen and McEvoy, 1997; Beyrem et al., 2011; Kennedy and Jacoby, 1999; Raisuddin et al., 2007). In the abyssal deep sea, low densities of macrofauna and the often limited number of replicated samples result in low statistical power (De Smet et al., 2017) where the high meiofaunal densities offers a clear advantage. All of the above mentioned characteristics of meiofauna advocate their use in experimental studies. Therefore, this particular group of organisms presented the focus of the research conducted in this thesis.

1.3.1. How does meiofauna cope with environmental stress?

Different biological mechanisms exist to cope with environmental stress: behavioural change to evade or avoid stress, resistance to stress via increased tolerance or physiological plasticity and activation of recovery mechanisms (Huey et al., 2002; Walther et al., 2002). However, all of those responses require energy and therefore induce shifts in the organisms' energy balance whereby less energy may be available for other functions such as reproduction or growth (Sokolova et al., 2012).

Behavioural avoidance can be easily observed in highly migratory organisms such as fish that adjust their migratory routes as a result of rising seawater temperatures with most species showing a poleward migration (Perry et al., 2005; Poloczanska et al., 2013). However, for seafloor associated organisms such as demersal fish or benthic invertebrates, the seafloor bathymetry poses a strong barrier limiting the migratory response (Sundby et al., 2017). These species will have to face the changing abiotic conditions and additional stressors (e.g. invasion of other species, chemical pollution) and need to induce mechanisms of resistance and recovery (Sundby et al., 2017). Similarly, meiobenthic organisms are limited in their avoidance and migratory response to large scale disturbances such as OA and warming (OAW) or deep-sea mining disturbances. However, on a small scale, meiofaunal organisms are able to influence their position inside the sediment by crawling and directing their settlement through swimming motions in the absence of strong currents (Lins et al., 2013; Titelman, 2001; Ullberg and Ólafsson, 2003). In fact, behavioural avoidance of meiofauna on a small scale is shown daily by intertidal nematodes that migrate up and down in the sediment as a response to strong currents, heat, rainfall or to avoid competition and predation (Boaden and

Platt, 1971; Steyaert et al., 2001). Vertical migration patterns of nematodes and copepods have also been reported as a result of shifting oxygen conditions in the pore water and overlying seawater (De Troch et al., 2013; Grego et al., 2014; Hendelberg and Jensen, 1993; Moodley et al., 2000a). Nevertheless, due to their small size, meiofauna remains limited in their dispersion and cannot easily avoid disturbances of large spatial scales.

Through natural selection, living organisms are adapted to the characteristics of their habitat. As such, it has been shown that populations from polluted environments exhibit a higher tolerance to high pollutant concentrations than populations from unpolluted sites (Klerks and Weis, 1987; Wang and Rainbow, 2005). As a result, polluted communities vary from those of unpolluted sites, an observation that has also been made for meiobenthic communities which offers great opportunities for biomonitoring (Gyedu-Ababio et al., 1999). Due to the differential sensitivity of nematode species to environmental stressors, nematode community compositions have been widely used as indicators of environmental health (Bongers and Ferris, 1999; Ekschmitt and Korthals, 2006; Wilson and Kakouli-Duarte, 2009). But also on a higher taxonomic level, compositional changes in the overall meiofauna community may indicate environmental stress. While nematodes have been shown to be very tolerant to hypoxic and anoxic conditions, copepods respond more sensitively (Wetzel et al., 2001). However, also the less dominant meiofauna may give clues to environmental stress. Calcifying organisms such as ostracods or small bivalves may experience enhanced stress from pore water acidification compared to other meiofauna (Orr et al., 2005; Schade et al., 2016). On the other hand, some tardigrade species are known for their high tolerance to environmental stress (Jönsson et al., 2008; Jørgensen and Møbjerg, 2015, Møbjerg et al., 2011; Persson et al., 2011) and may respond less sensitively.

Recovery of meiofaunal communities after disturbance is always difficult to assess and strongly depends on the prevailing abiotic condition, the recovery potential of persisting populations and the colonizing potential of other organisms. As previously mentioned, nematodes show a high tolerance to environmental stress, but very mobile taxa, such as copepods have shown to exhibit a higher colonization potential and may take advantage of disturbance events (Fonsêca-Genevois et al., 2006; Gwyther et al., 2009). Depending on the kind of disturbance and the considered environment, recovery of meiofauna in terms of density and diversity may take days to months in shallow-water habitats (Sarmento et al., 2013; Sherman and Coull, 1980), but may as well take up to several years or decades in severely disturbed deep-sea habitats (Gollner et al., 2017). The differences in responses between shallow-water and deep-sea communities need to be taken into

consideration when studies from both environments are compared and even more when shallow-water studies are used to make inferences for deep-sea communities.

1.4. Experimental work

In this thesis, I report on six experimental studies that were conducted with organisms from very contrasting environments including subtidal and intertidal environments, bathyal fjords and abyssal nodule fields (see 1.4.1). Not only the environments, but also the applied experimental techniques were considerably different between studies. Experimental studies always have to trade-off their ecological relevance against their interpretability which is linked to the degree of control over different factors and variables (Fig. 1.5).

Nevertheless, the different types of experimental studies depicted in Fig. 1.5 are complementary and *in situ* studies would be difficult to interpret if information on lower hierarchical levels (e.g. individual, cell), explaining underlying mechanisms or processes, are missing. Likewise, information obtained on a low hierarchical level is difficult to put in the ecological context without knowledge of e.g. species interactions or responses to environmental factors. Only a combination of different methods may enable us to fully understand ecological processes and perhaps make predictions aided by ecological modelling techniques of these complex processes.

In the choice of the experimental design one also needs to consider different modes of complexity. Here, we may distinguish single stressor experiments (low complexity) from multi-stressor experiments (high complexity). While a single factor may impact communities in a certain way, cumulative effects of multiple stressors can induce additive, synergistic or antagonistic responses that may differ considerably from the effects of each stressor in isolation and, therefore, require increased attention (Kroeker et al., 2017). Finally, depending on the parameter or endpoint of interest, experimental studies require different durations. If for

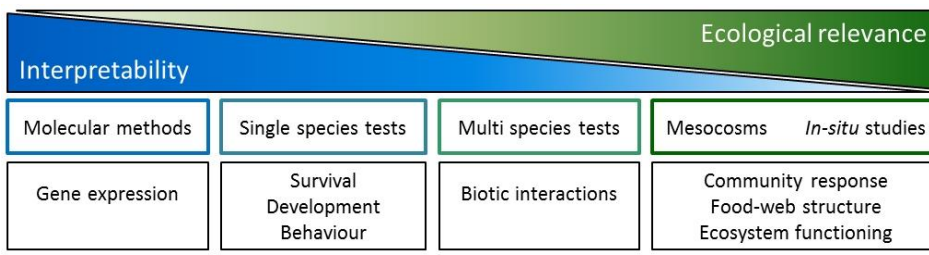

Figure 1.5 Simplified presentation of different experimental methods (top boxes) and examples of measurable variables (bottom boxes) arranged on a scale of interpretability and ecological relevance. Modified from (Höss and Williams, 2009)

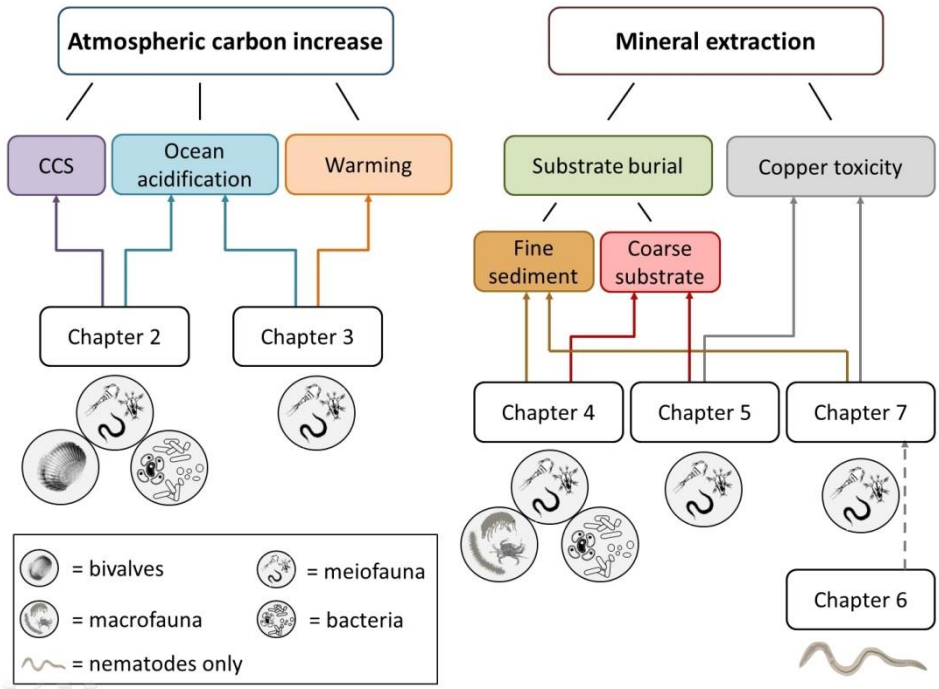

Figure 1.6 Schematic overview of the experiments conducted in this thesis and their relationship to the different impact scenarios. Circles indicate the different benthic size classes considered in each experiment. The dashed line indicates that the work presented in Chapter 6 was preliminary to the experiment in Chapter 7.

example stressor induced mortality is the endpoint of interest, the duration of the experimental study should never exceed the average life expectancy of the organism. The opposite is true if reproductive measures are the endpoint of interest where multiple generations have to be considered.

One of the studies described in this work was conducted as a single species, short-term and multi-stressor experiment under controlled laboratory conditions (Chapter 6, see Fig. 1.6). Due to the high control over the abiotic conditions, this kind of experimental work allowed us to study mechanisms affecting the organism when one or several factors are manipulated. However, the conditions under which the experiment is conducted are very artificial and do not represent natural conditions that could potentially lead to divergent responses of the organism due to e.g. food limitation, competition or predation.

Mesocosm experiments may offer a solution where the aim is to enclose (part of) an ecosystem in the laboratory or in the field while still being able to control and manipulate certain factors of interest. In this thesis, I will follow the definition of Stewart et al. (2013) where mesocosms are defined as "…experimental enclosures from one to several thousands of litres,…" and, thus, also include studies that

define their setup as microcosms. Mesocosm experiments were performed in Chapters 2, 3 and 4 where multiple trophic levels in a naturally occurring assemblage were studied including meio- and macrofauna and microbiota. This approach considerably increases ecological relevance compared to single species experiments and as miniature ecosystems, the results obtained from these experiments may even be up-scaled to explain or predict large-scale patterns (Benton et al., 2007; Spivak et al., 2011).

Finally, open incubations under *in situ* conditions allow evaluation of stress responses of communities under naturally occurring variability and disturbance. However, their high ecological relevance due to low interference of the scientists in natural patterns comes at the cost of low interpretability because of the large number of uncontrolled and potentially interfering factors. In the deep sea, organisms living deeper than 2000 m generally do not survive recovery from the seafloor without the use of expensive hyperbaric sampling equipment (Pradillon and Gaill, 2007). In those cases, *in situ* experiments are a preferred option to study responses of abyssal assemblages, as described in Chapters 5 and 7. The mortality associated with sample retrieval from the deep seafloor also poses challenges with regard to mortality assessments as staining would need to be performed *in situ*, which is logistically extremely challenging for meiofauna. As a consequence, short-term experiments are difficult to interpret with respect to organism survival. However, they are often the only option since research cruises are tightly scheduled and often do not revisit the same areas. Furthermore, long incubation times come at the risk of interference of unknown disturbances with the experimental setup (abiotic, e.g. passing eddies, temporary oxygen depletion or biotic, e.g. disturbances by megafauna) that may afterwards be difficult to account for in the data analysis.

1.4.1. Introduction to the environments studied in this thesis

The experimental work in this thesis focusses on four different environments which are chosen in relation to the importance of the studied stressors for those particular environments.

In Chapter 2 we examine the effects of a broad range of pCO_2 on a bivalve dominated coastal community from a sandy, subtidal (1-2 m) area in the South-Western Baltic Sea, mores specifically, the Kiel Fjord. In this experiment, a small increase in pCO_2 is representative of an ocean acidification scenario while larger pCO_2 increases induced seawater acidification scenarios that may only occur during leakage events from CCS. In the Baltic Sea, several geological areas were prospected and found suitable for storage of CO_2 in sub-seabed reservoirs (Mortensen, 2016; Shogenova et al., 2009). Furthermore, the Baltic Sea experiences

large periods of hypoxia which, through heterotrophic degradation of organic matter and resulting dissolved inorganic carbon production (DIC), can amplify the effect of ocean acidification (Melzner et al., 2013). Through these processes, seawater pCO_2 may reach maximum values of 3400 – 4500 µatm in coastal, hypoxic habitats (Melzner et al., 2013).

This interaction of hypoxia and ocean acidification also relates to estuarine ecosystems such as the Paulina intertidal mudflat in the Westerscheldt estuary, which is the study area under investigation in Chapter 3. Estuaries and coastal shallow habitats seem to be more vulnerable to acidification and warming than the open ocean because of the shallow water body and their tight connection with the land. As such, nutrient input by surface runoff and riverine discharges may result in additional acidification of the system through enhanced organic matter production and subsequent degradation or the input of acid substances (Hofmann et al., 2011). The pH in the Westerscheldt estuary is very variable but shows a decreasing trend since the mid 1990s (Provoost et al., 2010) underlining the importance to study ocean acidification effects in this particular area.

Norway is one of the few countries worldwide to conduct submarine tailings disposal in fjord systems (Ramirez-Llodra et al., 2015). As such, fjord benthic communities, from depths well below the euphotic zone, are most likely to be affected by this anthropogenic disturbance. For this reason, a bathyal (200 m), soft-sediment community from a Norwegian fjord was chosen for the experiment reported in Chapter 4.

Finally, responses of deep-sea communities to disturbances resulting from deep-sea mining activities can best be assessed through studies on abyssal (4200 m) communities from areas where the targeted resources occur. Therefore, experiments described in Chapter 5 and 7 have been carried out *in-situ* at an abyssal nodule field site in the Peru Basin, South-East Pacific.

1.5. Interdisciplinary and international framework of this research

This research was conducted in an international framework with support from different European projects. The ECO_2-project (Sub-seabed CO_2 Storage: Impact on Marine Ecosystems), funded by the European Union 7th Framework Programme, brought scientists from multiple disciplines together to assess the impacts of sub-seabed carbon capture and storage. Apart from the monitoring of two pilot projects of CCS in the North Sea and the Barents Sea, this project also stimulated experimental research on the impacts of severe seawater acidification on

marine environments and the investigation of CO_2 vents as natural analogues of strong CO_2 leakage scenarios.

Additional support to study ocean acidification effects and species responses to environmental change was provided by the UGent BOF GOA project "Assessing the biological capacity of ecosystem resilience", where emphasis is put on different experimental approaches to investigate resilience of marine communities.

During the MIDAS project (Managing Impacts of Deep-Sea Resource Exploitation), an international consortium of scientists, policy makers, NGO's and industry representatives carried out research in a multidisciplinary setting to investigate impacts of deep-sea mineral extraction. The research included e.g. diversity assessments in targeted areas, experimental research and stakeholder surveys, and the results were integrated to assist policy makers in the development of environmental regulations for deep-sea mineral extraction.

Three research cruises on the German research vessel RV *Sonne* supported, expanded and continued some of the research started in MIDAS. These research cruises were made possible through the European project Joint Program Initiative Oceans (JPIO) − Ecological Aspects of Deep-Sea Mining that ran partly in parallel with the MIDAS project and ends in 2017. This project focussed I) on the assessment of mining impacts on the biodiversity, community structure and resilience of benthic fauna, II) on the employment of state-of-the-art sampling and measurement equipment, and III) on the use and enhancement of modern mapping techniques.

These different, European projects provided a very stimulating, international environment and vital financial and technical support to carry out the research presented in this thesis.

1.6. Rationale and outline of this thesis

Through different experimental approaches, this thesis aims to evaluate the impacts of two different scenarios of anthropogenic disturbance on the meiobenthic communities. The connections between all chapters and the studied environmental stressors are visualized in Fig. 1.6. The effects of atmospheric carbon increase can be subdivided into three different stressors. Firstly, acidification of the seawater due to CO_2 exchange at the atmosphere-water-interface and subsequent shift in the acid-base equilibrium in the ocean (see section 1.1.1); Secondly, the indirect effect of ocean warming due to increased radiative forcing resulting from higher greenhouse gas concentrations (see section 1.1.2); and thirdly, the active removal of carbon from the atmosphere and

storage in geological sub-seabed reservoirs and potential seawater acidification in case of a CO_2 leak into the ocean (see section 1.1.3).

In a **Chapter 2** we addressed the effect of ocean acidification and severe seawater acidification from a CCS leakage scenario by applying a broad range of pCO_2 to a subtidal soft-sediment community including three naturally occurring bivalve species and a natural meiofauna and microbial community. This experiment was performed under natural temperature fluctuations, however, at the end of this century not only ocean acidification will increase but also the temperature of the surface oceans. Therefore, ocean acidification research should aim to measure effects of both stressors. In a mesocosm experiment, we exposed an intertidal soft-sediment community to reduced pH and elevated temperature to investigate the mentioned cumulative effects on the structure of the meiobenthic community (**Chapter 3**). Furthermore, we applied a Trypan blue staining technique to also account for nematode mortality.

Inside marine sediments, abiotic conditions differ from those in the water column and temporal and spatial fluctuations in pore water pH are often occurring phenomena (Pörtner et al., 2004; Zhu et al., 2006). CO_2 content of sediment pore water is usually higher than that of the overlying seawater, mainly due to high microbial activity (Silburn et al., 2017), and abiotic conditions (pH, oxygen saturation) vary with sediment depth resulting in distinct vertical distributions of infaunal communities. Therefore, we paid special attention to also measure the effects of reduced seawater pH and elevated temperature on pore water pH and vertical distribution of the meiobenthos (Chapter 3). This experiment substantially contributes to the understanding of the cumulative effects of both stressors on shallow-water soft-sediment communities and sediment geochemistry.

The effects of mineral extraction, and more particularly the impacts of burial with toxic and non-toxic sediment and tailings on bathyal and deep-sea species, are addressed in Chapters 4 to 7. We used different approaches to increase our understanding of the impacts on the level of the entire benthic ecosystem, the meiobenthic community and the organism level. On an ecosystem level we investigated the impacts of burial with tailings and native sediment on the ecosystem functioning of a bathyal soft-sediment community from a Norwegian fjord in terms of oxygen consumption and remineralization of fresh organic matter (**Chapter 4**). While effects on the recovery of macrobenthic communities after tailings disposal is often reported, information on other benthic compartments and on the functioning of the ecosystem following tailings deposition are largely unknown (Ramirez-Llodra et al., 2015). Chapter 4 partly fills this knowledge gap and provides indications of threshold values for tailing deposition with regard to ecosystem functioning of Fjord assemblages. Similar to Chaper 3, the assessment

of nematode mortality was an added value to the analysis of the meiobenthos structure in the different treatments.

The threshold values identified for Norwegian fjord communities may also give indications of the responses of other deep-sea communities. In view of deep-sea mining, impacts of large sediment plumes and re-deposition of suspended matter are expected over large areas (Murphy et al., 2016). This urges scientists to identify impacts and thresholds of substrate burial on abyssal nodule field communities. In **Chapter 5** we report the results of an *in situ* experiment at abyssal depths in the Peru Basin, South-East Pacific, testing the burial with crushed nodule particles. Abrasion and distribution of small particles from polymetallic nodules during mining or transport is likely, due to the very brittle character of the minerals. Similar to the tailings burial reported in Chapter 4, the crushed nodule particles are much coarser than the natural sediment on the abyssal plain which may potentially affect meiobenthic community structure. Durations of both experimental incubations were similar allowing better comparison between both studies (Chapters 4 and 5).

Because of the high metal content in nodules and surrounding sediment as well as in other targeted deep-sea minerals, toxicity effects on deep-sea biota are a major concern of deep-sea mining, and knowledge on the impacts of abiotic factors prevailing in the deep-sea on metal toxicity is limited (Mestre et al., 2014). We addressed this issue in a laboratory experiment where the effects of high hydrostatic pressure (10 MPa representing a water depth of ~1000 m) and low temperature (10 °C vs. 20 °C) on the sensitivity of the cultured, intertidal nematode *Halomonhystera disjuncta* to copper were measured using acute toxicity tests (endpoint: survival, **Chapter 6**). While it was not feasible to use a deep-sea species in this three-stressor lab experiment, the use of an intertidal species that shows close phylogenetic and ecological links with a bathyal nematode (Van Campenhout et al., 2013, 2014) can still provide valuable information on the potential mechanisms of copper toxicity under varying abiotic conditions. In a mining scenario, however, metal toxicity comes along with sediment burial and again, cumulative effects of both stressors on benthic biota can be expected. To account for these cumulative effects, we conducted an *in situ* experiment at the Peru Basin (site cfr. Chapter 5) where copper contaminated sediment was distributed onto the natural abyssal sediment (**Chapter 7**).

The combination of different experimental approaches, but also the overlap between investigated stressors and methods revealed some important insights in the response of meiofauna from contrasting environments to the different stressors associated with increased atmospheric carbon and mineral extraction.

Our research and the findings are elaborated in detail in the subsequent chapters. The final chapter (**Chapter 8**) aims to integrate and discuss the findings of our own research in the light of previous research and based on that I will provide some recommendations for future research and for the monitoring of impacted sited in the different scenarios.

Chapter 2 Simulated leakage of high pCO_2 water negatively impacts bivalve dominated infaunal communities from the Western Baltic Sea

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Abstract

Carbon capture and storage is promoted as a mitigation method counteracting the increase of atmospheric CO_2. However, at this stage, environmental consequences of CO_2 leakage from sub-seabed storage sites are still largely unknown. In a 3-month-long mesocosm experiment, this study assessed the impact of elevated pCO_2 levels (1,500 to 24,400 µatm) on *Cerastoderma edule* dominated benthic communities from the Baltic Sea. Mortality of *C. edule* was significantly increased in the highest treatment (24,400 µatm) and exceeded 50%. Furthermore, mortality of small size classes (0-1 cm) was significantly increased in treatment levels ≥6,600 µatm. First signs of external shell dissolution became visible at ≥1,500 µatm, holes were observed at >6,600 µatm. *C. edule* body condition decreased significantly at all treatment levels (1,500-24,400 µatm). Dominant meiofauna taxa remained unaffected in abundance. Densities of calcifying meiofauna taxa (i.e. Gastropoda and Ostracoda) decreased in high CO_2 treatments (>6,600 µatm), while the non - calcifying Gastrotricha significantly increased in abundance at 24,400 µatm. In addition, microbial community composition was altered at the highest pCO_2 level.

We conclude that strong CO_2 leakage can alter community composition in multiple size classes of benthic infauna, likely due to high mortality of the dominant macrofauna species *C. edule*.

2.1. Introduction

Carbon dioxide (CO_2) is one of the most important natural greenhouse gases on Earth, playing a vital role in regulating the global heat budget and, thus, Earth's climate system. Currently, the concentration of atmospheric CO_2 is increasing at an unprecedented rate (Field et al., 2014), a process mainly caused by human actions (Le Quéré et al., 2014). Anthropogenic CO_2, which enters the ocean, causes ocean acidification through an increase in dissolved inorganic carbon resulting in a decrease in pH. To reduce anthropogenic CO_2 emissions to the atmosphere and mitigate climate change, the World Energy Outlook suggested long-term sequestration of CO_2 (CCS) into sub-seabed geological structures as one suitable strategy (IEA, 2010). Utilization of this technique, however, depends on the successful management of storage risks. These risks are still poorly constrained and current research is directed to obtain reliable information to establish a legal framework for environmentally safe application of CCS and efficient monitoring techniques (Hawkins, 2004; Keating et al., 2010).

One of the main potential risks of CCS is CO_2 leakage through e.g. fracture zones or pipe failure leading to acidification of the pore and bottom water or water column, respectively (Blackford and Gilbert, 2007). The extent of impacts of acidified seawater on the surrounding marine ecosystem will depend on the scale and intensity of leaks, the local hydrodynamic regime and the duration of exposure (Blackford et al., 2009). Models that evaluated different potential leakage scenarios in the southern part of the North Sea predict temporary, local seawater pH changes ranging from <-0.12 in case of a continuous diffuse seepage up to >-1.0 pH units in a continuous point source leakage scenario (Blackford et al., 2009, 2008). The latter represents the extreme case of a major geological fault break or an unrecognized pipe failure with outgassing of high amounts of CO_2 into the marine environment (Blackford et al., 2014, 2012; Taylor et al., 2014; Widdicombe et al., 2015). Experimental release of ca. 4 tons of CO_2 via sediment injection during the 37 day long experiment in a Scottish Sea Loch resulted in maximum changes in sediment pH by 0.8 pH units, varying between 8.0 and 7.2 (Blackford et al., 2014).

Studies that have investigated the influence of elevated seawater pCO_2 on benthic organisms primarily used short- to medium-term exposures and single species experiments and pCO_2 levels relevant for ocean acidification (OA) forecasts. These forecasts predict global average seawater pH shifts of -0.3 units by the year 2100 (Gattuso et al., 2015), however, coastal environments may experience even larger pH shifts (Hofmann et al., 2011; Melzner et al., 2013). The influence of medium-term exposure to high pCO_2, that may result from CO_2 leakage at CCS sites, on

communities is not well investigated and threshold levels need to be identified. The sensitivity of marine organisms to varying pCO_2 levels and accompanying seawater pH variations differs between taxa, resulting in very diverse responses to elevated internal CO_2 concentrations (hypercapnia) (Pörtner et al., 2004). The general response of animals to hypercapnia is related to disturbance and regulation of the intra- and extracellular acid-base balance (Pörtner et al., 2004; Seibel and Walsh, 2003, 2001). Those processes are energy consuming and can result in growth reduction, decreased metabolic activity, low reproduction rates, behavioural changes or ultimately death (Melzner et al., 2013; Pörtner et al., 2004; Stumpp et al., 2011). Calcifying organisms are particularly susceptible to acidified seawater due to reduced aragonite and calcite saturation of the water which can lead to enhanced dissolution of unprotected skeleton components and reduced availability of inorganic carbon for calcification (Andersson et al., 2011; Dupont et al., 2010; Orr et al., 2005). Non-calcifying, infaunal invertebrates (e.g. meiofauna, and more particular the dominant nematodes) with a low mobility and bacterial communities are naturally exposed to large fluctuations of pore water pH and pCO_2 (Widdicombe et al., 2011; Zhu et al., 2006), and are therefore likely to be more tolerant to high pCO_2 (OA) (Pörtner et al., 2004).

Only a handful of studies have exposed sediment communities in their natural composition to elevated seawater pCO_2, primarily due to the great logistic effort necessary to collect and maintain such communities in the laboratory and to control the carbonate system with sufficient accuracy during experiments. Negative effects of medium-term (20 weeks) exposure to high seawater pCO_2 (pH 7.3-5.6) have been reported on macro- and meiofauna diversity and community structure (Widdicombe et al., 2009). In addition, Widdicombe and Needham (2007) could show that even though seawater acidification did not alter nereid worm burrow size or structure, sediment nutrient fluxes were changed after five weeks of acidification exposure, supposedly due to changes in bacterial communities. Moreover, since the burrowing activity of e.g. echinoderms has a strong influence on the biogeochemistry of sediments and the composition of meiofauna communities (Dashfield et al., 2008), it seems likely that changes in macrofauna abundance in response to elevated seawater pCO_2 can have strong repercussions on ecosystem processes in the sediment (Widdicombe et al., 2009; Widdicombe and Needham, 2007).

Up to date, no comprehensive study has addressed the impact of elevated seawater pCO_2 and acidification on benthic bivalve dominated communities thriving in shallow, sandy coastal sediments in the North Atlantic region. Bivalves are of ecological importance as they filter large amounts of water, deposit particulate organic matter onto and into the sediment and provide settlement habitat for other

organisms, making them irreplaceable key players in the benthic-pelagic coupling (Graf, 1992; Riisgård et al., 2011). Furthermore, they play a key nutritional role for many fish and migratory bird species (Beukema et al., 2010; van Gils et al., 2006).

A range of recent studies on several (mainly epibenthic) bivalve species established that calcification, growth, filtration and metabolism are negatively impacted by elevated seawater pCO_2, often already at moderately decreased pH >7.5 (ca. <5,000 μatm). In addition, increased occurrence of oxidative and acid-base regulatory stress has been observed in bivalves (Gazeau et al., 2013; Melzner et al., 2013; Thomsen et al., 2013; Tomanek et al., 2011). Detrimental external and internal shell dissolution occurs in several species when exposed to acidified seawater for prolonged time intervals (weeks), with less severe effects observed in species that possess a thick and intact periostracum (Melzner et al., 2011; Ries et al., 2009; Thomsen et al., 2010; Tunnicliffe et al., 2009). However, biological impacts such as bioturbation (Dashfield et al., 2008; Widdicombe and Needham, 2007; Zhu et al., 2006) or interactions between and among different taxa and trophic groups (Dashfield et al., 2008; Hale et al., 2011; Ishida et al., 2013; Widdicombe et al., 2009) can result in very diverse responses of the biological community when compared to single species/ taxon studies. Mesocosm experiments which address the effect of seawater acidification on shallow water benthic species report an overall higher sensitivity of macrofauna while meiofauna, with nematodes as dominant and commonly reported taxon, remain unaffected or even increase in abundance (Dashfield et al., 2008; Hale et al., 2011; Kurihara et al., 2007; Widdicombe et al., 2009). Laboratory experiments confirm a strong resistance of dominant meiofauna taxa to pH changes as expected to occur as a result of OA by the year 2300 (pH 7.4; Kurihara et al., 2007). However, when the pH is further reduced to values that could potentially occur in response to a severe CCS leakage scenario (<pH 7), a reduced survival of nematodes has been observed (Takeuchi et al., 1997). Microbial communities are important for the coastal ecosystem as they are primarily responsible for organic matter remineralisation, nutrient cycling and function as food source for grazing marine organisms (Liu et al., 2010; Marinelli and Waldbusser, 2005; Ramette, 2009; Thompson et al., 2004). Investigations about the direct effects of lowered pH are still inconsistent (Joint et al., 2011; Liu et al., 2010). While some studies observed differences in abundance of a few genera or rare taxa, others found a change in bacterial biomass production with increasing pCO_2 (Coffin et al., 2004; Kerfahi et al., 2014; Roy et al., 1993; Tait et al., 2014).

In this study, we conducted a mesocosm experiment using natural *C. edule* dominated sandy communities from the Western Baltic Sea utilizing a flow-through seawater design with optimized food supply. Next to the cockle

Cerastoderma edule, the co-occurring soft-shell clam *Mya arenaria,* and the Baltic tellin *Limecola balthica* (Taylor et al., 1973) were studied in this experiment. *C. edule* and *L. balthica* live within the top two to five centimetres of the sediment, while *M. arenaria* occurs to sediment depths of up to 50 cm (Möller et al., 1985). Generally, all three species are widespread in tidal flats and shallow coastal areas and serve as an important link between primary producers and consumers. Owing to their high densities in the sediment (often >1,000 individuals m^{-2}) and considerable mobility and activity (Flach, 1996) they exert a strong influence on infaunal communities and biogeochemical processes, e.g. by increasing primary production and fertilization of microphytobenthos through NH_4^+ excretion (Flach, 1996; Swanberg, 1991). To predict marine benthic ecosystem vulnerability to potential chronic leakages from CCS storage sites, experiments need to utilize a high pCO_2 range, realize a multispecies approach and intermediate exposure durations. Therefore, six pCO_2 levels ranging between 900 µatm (control) and 24,400 µatm, corresponding with pH values between 7.8 and 6.4, were applied over a time of 12 weeks. Here, the lowest pCO_2 treatments fell in the range of predicted future ocean acidification scenarios (ΔpH=-0.1 and -0.4) while higher pCO_2 levels (>6000 µatm) are only likely to occur on a local scale as a result of CO_2 leakage.

In search for an indicator species useable in future CCS site assessments, experiments focused on the most abundant bivalve *C. edule*. We hypothesized that *C. edule* would be very sensitive to elevated pCO_2, as it lives close to the sediment surface. *C. edule* mainly occurs in relatively unpolluted areas while e.g. *L. balthica* can also be found in polluted and hypoxic areas (McGreer, 1982), suggesting a higher resistance of *L. balthica* to unfavourable environmental conditions. To extend the common single-species approach to an ecosystem investigation and to test for the role of *C. edule* as an important ecosystem engineer, the impact of high pCO_2 on benthic meiofauna and bacterial communities was also assessed. We hypothesized that elevated pCO_2 and changes in bivalve abundance and fitness would impact meiofaunal and microbial communities.

2.2. Materials and methods

2.2.1. Experimental setup

Sandy communities were exposed to six different seawater pCO_2 regimes for a total of three months (17.12.2011 – 06.03.2012) in a climate - controlled room. Six header tanks were continuously supplied with filtered seawater from Kiel Fjord, each one connected to six experimental units (EU) ensuring continuous seawater supply (Fig. S2.1). This design is a randomized design (B4) according to (Cornwall and Hurd, 2015). Each EU consisted of a round plastic container with a volume of

12.5 L containing ca. 9.5 L of sediment and an overlying water column of ca. 3 L. The lower 10 cm of the sediment consisted of sieved sand taken from a local beach (Kiel, Falckenstein: 54°23,66 N; 10°11.56 E) while the upper 10 cm consisted of surface sediment from the station at which the experimental animals were sampled to resemble natural conditions as well as to provide naturally occurring microbial and meiofauna communities. Bivalves and sediment were sampled in Kiel Fjord at Falckenstein with a Van Veen grab in 1-2 m depth using the vessel FK Polarfuchs on November 21st 2011 and kept in holding basins at 9°C before being placed in EUs. Density of infauna bivalves was determined during the sampling process. 1 m² of sediment at Falckenstein was found to contain 146 *M. arenaria*, 9 *L. balthica,* and 1,040 *C. edule*. In order to simulate a natural size distribution in our laboratory experiment, we sieved the collected sediment (1 mm mesh width). All bivalves were taken out and replaced by a defined number per EU: 5 *M. arenaria* (size classes: 0.5-1 cm: 2 animals; 1-1.5 cm: 2 animals; 2-2.5 cm: 1 animal), 1 *L. balthica,* and 40 *C. edule* (size classes: 0-0.5 cm: 3 animals; 0.5-1 cm: 18 animals; 1-1.5 cm: 11 animals; 1.5-2 cm: 7 animals; 2-2.5 cm: 1 animal). Small gastropods (exclusively *Hydrobia* spp.) were abundant with ~10 individuals per EU. Due to their small size (<0.5 mm) they were randomly distributed within all EUs with the sieved sediment. Due to the natural low diversity of the Baltic, the density of other macrofauna individuals was <1 individuals per m². These low abundant species (e.g. nereid polychaetes, pharid bivalve species) were excluded from the experiment. The experimental units were kept in a seawater flow-through system for two weeks under control conditions prior to the experiment to allow proper acclimatization of biogeochemistry and the faunal community. Seawater pH was maintained in the header tanks using a pH feedback system (IKS Aquastar, iksComputersysteme GmbH, Karlsbad, Germany). Treatment levels were achieved through continuous addition of acidified water from the header tanks into the overlaying seawater of each EU and included levels of 900 µatm (control, pH 7.8 calculated on NBS scale), 1,500 µatm (pH 7.7), 2,900 µatm (pH 7.4), 6,600 µatm (pH 7.0), 12,800 µatm (pH 6.7), and 24,400 µatm (pH 6.4) (Tab. S2.1). 900 µatm was used as a control due to the high background pCO_2 in Kiel Fjord (Thomsen et al., 2013, 2010). To support the bivalve nutritional needs unicellular algae (*Rhodomonas* sp.) were cultured as described in Thomsen et al. (2010) and added continuously into the header tanks via a peristaltic pump, thus maintaining a stable concentration of 3,500 – 4,000 (Tab. S2.1) cells ml^{-1} within header tanks (Tab. S2.1).

A flow rate of 100 ml min^{-1} was provided to each EU from the respective header tank via gravity feed. Throughout the experiment, pH, salinity, temperature, and flow rate were measured daily in each replicate. Salinity and temperature fluctuated

in accordance with naturally occurring changes in Kiel Fjord seawater (14.6-20.5 psu and 4.3-8.9°C, respectively). Light conditions were similar for all EUs with light intensities of 5.53 up to 7 µmol $s^{-1}m^{-2}$ for 9 hours per day. Dead animals were removed daily and behaviour of bivalves (presence/ absence on the sediment surface) was noted every other day starting in the third experimental week. Carbonate chemistry and algae concentration in the EUs were measured weekly. Dissolved inorganic carbon (C_T) was measured using an Automated Infrared Inorganic Carbon Analyzer (AIRICA, Marianda, Kiel, Germany). Seawater chemistry (pCO_2 and calcium carbonate saturation state) was then calculated according to the guide to best practices for ocean CO_2 measurements (Dickson et al., 2007), using CO2SYS (Lewis and Wallace, 1998) with pH (NBS scale) and C_T, temperature, salinity, and first and second dissociation constants of carbonic acid in seawater (Roy et al., 1993).

2.2.2. Bivalve sampling and processing

At the end of the experiment, all but four *C. edule* specimen per EU were frozen at -20°C. Shell-free dry mass was measured according to Thomsen et al. (2013). To survey shell dissolution, five randomly selected *C. edule* from each treatment were analyzed using a stereomicroscope (40-fold magnification). In addition, scanning electron microscopy (SEM) was used to examine external shell dissolution at a higher resolution. For that purpose, three shells of the 900-6,600 µatm CO_2 treatments (as signs of dissolution were obvious at higher levels) were mounted separately on SEM pedestal stubs, coated with gold-palladium and examined using scanning electron microscopy (Nanolab 7, Zeiss, Oberkochen, Germany and HitachiS4800, Hitachi High - Technologies Europe, Krefeld, Germany).

The extent of oxidative stress in the lipid fraction of the whole body tissue was estimated by analysing the malondialdehyde (MDA, a marker of lipid peroxidation) content in four *C. edule* specimens per EU, which were stored frozen at -80°C prior to measurement. The entire tissue mass of each bivalve was separately processed via grinding in liquid nitrogen. Fifty milligrams of ground tissue powder of each of the four specimens from the same EU were pooled together to obtain sufficient tissue amounts for the analysis (one pool per EU). MDA concentration of each replicate was determined following the protocol of (Uchiyama and Mihara, 1978). Approximately 100 mg tissue-powder was homogenized with 0.2% phosphoric acid in a 1:5 ratio (sample/ phosphoric acid). In the next step an equivalent volume of 2% phosphoric acid was added. One blank (homogenate + 0.2 ml 3 mM hydrogen chloride) and two samples (homogenate + 0.2 ml TBA solution) were adjusted to a pH of 1.6 (with HCl or NaOH) and were incubated at 100°C for one hour. After cooling, 0.5 ml of butanol was added, samples were vortexed for 40

seconds and centrifuged at 1,000 g for 5 minutes. The supernatant was collected into fresh vials and centrifuged for 5 minutes at 14,000 g. Samples and blanks were transferred to a 96 well plate and the extinction was measured in a plate reader (Plate Chameleon, Hidex, Turku, Finland) at 532 and 600 nm. Tissue concentration of MDA was calculated following equation (1).

$$(1)\ C_{tiss} = \frac{C_{MDA} \times V_{But} \times V_{Extr}}{V_{aliq} \times W}$$

C_{MDA}: MDA concentration [nmol ml^{-1}]

V_{But}: volume of butanol [ml]

V_{Extr}: extraction volume [ml]

V_{aliq}: volume of homogenate [ml]

W: weight of tissue [g]

2.2.3. Meiofauna sampling and processing

The uppermost centimetre of each EU was sampled after six and twelve weeks using a small corer (Ø 2.5 cm) and stored in 4% buffered paraformaldehyde until further extraction of the meiofauna. For this purpose, samples were washed on two stacked sieves in order to separate the macrofauna fraction (on a 1mm sieve) from the meiofauna fraction (on a 38 µm sieve). Subsequently, meiofauna was extracted by triple density gradient centrifugation (3000 rpm) with colloidal silica gel LUDOX HS40 Dupont (specific gravity 1.18) as a flotation medium (Heip et al., 1985), fixed in 4% buffered formaldehyde solution and stained with Rose Bengal. Meiofauna was counted under a stereo microscope (50x magnification) and identified to higher taxonomic level consulting e.g. (Higgins and Thiel, 1988). Only nematodes that were in a good shape and showed no clear signs of degradation (loose cuticle, lack or damage of internal structures, biofilm presence, flattened or dehydrated habitus) were counted. For nematode identification, 100 nematodes were picked out per sample, transferred to glycerine following the protocol of (De Grisse, 1969) and subsequently mounted on paraffin-ring glass slides. Nematodes were identified to genus level following a pictorial key (Warwick et al., 1998) and the NeMys database (Vanaverbeke et al., 2014) under a microscope (Leica DMR, 10x - 100x magnification).

2.2.4. Microbial community sampling and processing

After six and twelve weeks, one corer sample (Ø 2.5 cm, 1 cm depth) per EU was collected, transferred into 50 ml plastic tubes and stored frozen at -20°C for bacterial community analysis. The samples were subjected to total community

DNA extraction using the FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA) including an additional heating step to increase yield and final elution of the DNA in TE-buffer. Benthic bacterial community structures were determined by means of the high-throughput fingerprinting technique ARISA (Automated Ribosomal Intergenic Spacer Analysis), following a previously published procedure by (Ramette, 2009) with slight modifications: Final concentrations of PCR ingredients within 50 µl-reactions were 0.4 µM of each primer, 0.1 mg ml^{-1} BSA, 250 µM of each dNTP (peqGOLD Kit; Peqlab, Erlangen, Germany), 1x Buffer S with 1.5 mM $MgCl_2$(Peqlab), 1.0 mM extra $MgCl_2$ (Peqlab) and 2.5 U peqGOLD*Taq*-DNA-Polymerase (Peqlab). The forward primer was labelled with FAM at its 5'-end. For each sample (EU), three PCR replicates were prepared. For two samples (EUs) a successful amplification could not be validated, they were therefore excluded from further analyses.

2.2.5. Statistical analyses

Univariate, parametric and non-parametric analyses were performed using the program R version 3.0.2. (R Core Team, 2013). Multivariate, non-parametric analyses were performed with the program Primer version 6.1.11 with the add-on software Permanova+ version 1.0.1 (Anderson et al., 2008; Clarke and Gorley, 2006). Regression analysis (ANCOVA) was performed using Prism 6 software (GraphPad Software, Inc.). A significance level of α=5% was chosen for all statistical tests. Significant test results are reported in the results, statistical information can be found in the supplementary tables.

To test for differences in *C. edule* behaviour, a permutational ANOVA (PERMANOVA), with the factor EU nested in treatment, was used as a non-parametric solution to a repeated measures analysis. *C. edule* mortality at the end of the experiment between treatments and the fraction of dissolved shells between treatments was tested using a Kruskal-Wallis test combined with a Kruskal multiple comparisons test since assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were not met. Mortality between different size classes within each treatment was tested with a Kruskal-Wallis test. For this, animals were pooled in two groups, large (1-2,5 cm shell width) and small (0-1 cm shell width). The influence of pCO_2 on malondialdehyde (MDA) content was tested using a one-way analysis of variance (ANOVA) and a Tukey HSD post hoc test. The difference in shell-free dry mass between treatments was tested with an analysis of covariance (ANCOVA), using log shell - free dry mass and log shell width. As no significant interaction was found for replicate and treatment (nested ANOVA, EU nested in CO_2 treatment, p-value = 0.8411), all measured animals (n=479) of each treatment were used in the regression analysis.

Meiofauna community analysis was based on calculated densities (individuals 10 cm^{-2}) while nematode community analysis was based on relative abundances and calculated densities. Due to an unbalanced (varying number of replicates among treatments, n=3-6) and non-normal distributed dataset, Permanova was chosen as an ANOVA approach for repeated measures to test for differences in total meiofauna densities and meiofauna and nematode community structure between treatments, time and interaction of both, with EU nested in treatment. When the number of unique permutations was lower than ten a Monte Carlo (MC) test was applied to calculate the p-value. Meiofauna densities were square root transformed and Bray-Curtis resemblance matrices were calculated. A pairwise-comparison test was executed for factors that differed significantly. The PermDisp tests always assured homogeneity of data dispersion unless mentioned otherwise. A principal coordinate analysis (PCO) on distances among centroids was performed on the square-root transformed meiofauna densities to visualize the results. Permanova was repeated for the meiofauna dataset after excluding Nematoda and a SIMPER analysis was performed to reveal the taxa that contributed most to the differences between treatments. The taxa with the most impact on dissimilarities were tested each by a univariate Permanova analysis as described above. For taxa with empty samples a dummy variable was added to avoid undefined values when calculating the Bray-Curtis resemblance matrix as suggested by (Clarke et al., 2006) . Meiofauna diversity indices (Shannon-Wiener index, Simpson index and Pielou's evenness index) were calculated and tested for significant differences between treatments with Permanova.

Quality assessment of raw bacterial community profiles and binning (2-3 replicates per sample) were done as previously reported (Ramette, 2009). Merged community profiles were generated in R (v.2.13.2; (R Core Team, 2013)) by using a custom script and considering Operational Taxonomic Units (OTUs) that occurred at least twice (Ramette, 2009). Non-metric Multidimensional Scaling (nMDS) was used to represent dissimilarity matrices based on Bray-Curtis or Jaccard coefficients into a reduced space (Legendre and Legendre, 1998). Analysis of similarity (ANOSIM) was conducted with the PAST software (Version 1.76; (Hammer et al., 2001)). To test for the effect of pCO_2 treatment and time on bacterial community composition, multivariate ANOVA and variation partitioning were conducted in R.

2.3. Results

2.3.1. Bivalve community response

Out of the three investigated bivalve species, the cockle *C. edule* was the most abundant and most sensitive to acidified seawater. As the abundance of the other two bivalve species was very low and no mortality was recorded, we focus on *C. edule* in the following sections. Throughout the experiment, cockles were either buried with open siphos or lying on the sediment surface. Under control conditions and at 1,500 µatm, cockles were mostly buried (Fig. 2.1). In the highest CO_2 treatment (24,400 µatm), cockles migrated towards the surface (Fig. 2.1). At 12,800 µatm, some cockles were observed on the surface, however always at a lower abundance than in the highest treatment. The distribution of cockles on the surface was significantly influenced by CO_2 treatment (p(perm) = 0.0001; Tab. S2.2). Although time and the interaction between time and treatment were significant (p(perm) = 0.0001), 63% of the variance were explained by the factor treatment. In the 24,400 µatm treatment, 50% of cockles were located on the sediment surface on day 50 (Fig. 2.1).

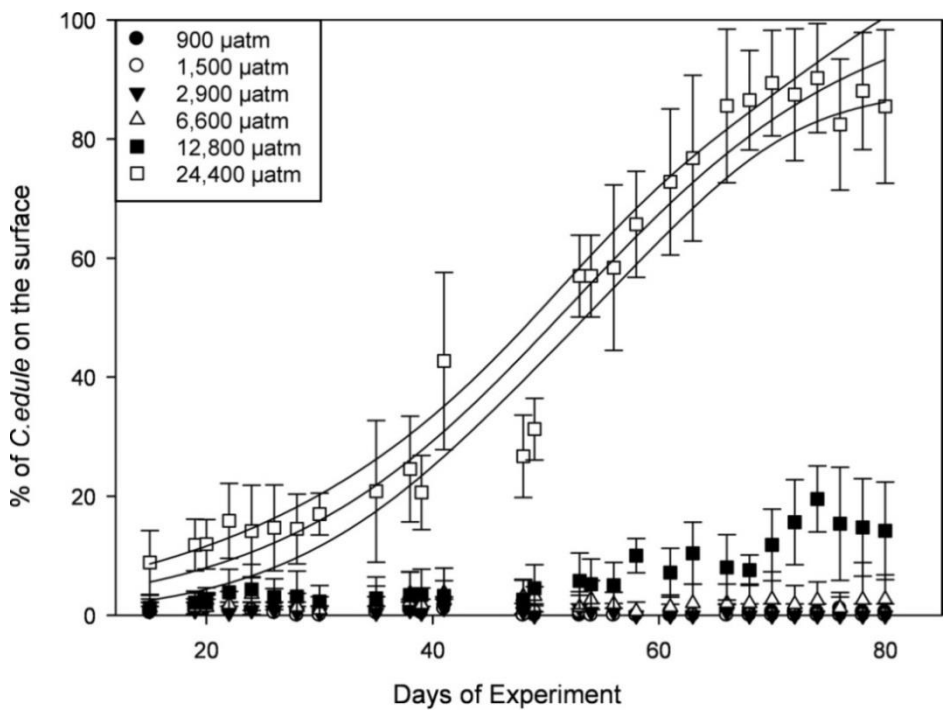

Figure 2.1 *C. edule* behaviour. Average abundance of non-buried *C. edule* over the complete experimental phase in % of total, curve fitted for 24,400 µatm including 95% confidence interval.

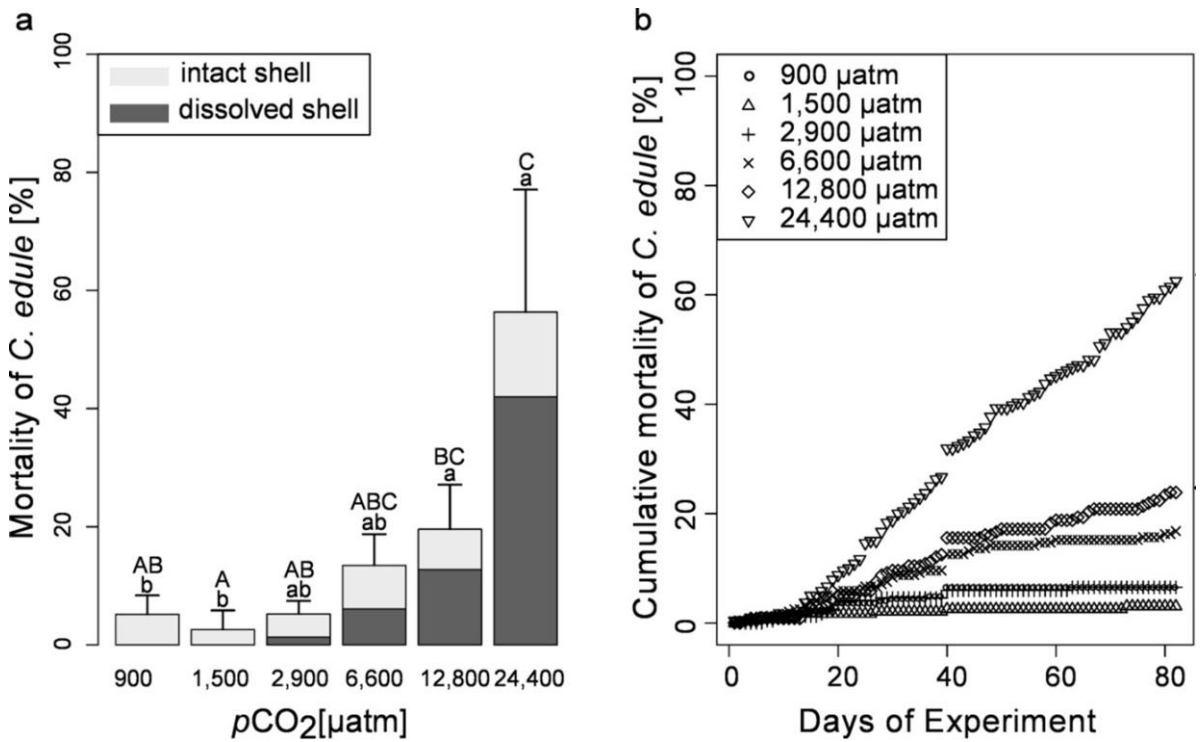

Figure 2.2 a) Bars showing *C. edule* mortality over the entire experimental duration (mean ± SD); colour coding showing fraction of corroded vs. intact shell (white = intact, grey = corroded); letters indicate significant differences between treatments. (b) Cumulative mortality plotted over the duration of the experiment. 50% mortality at 24,400 µatm was reached at day 68.

Mortality of *C. edule* increased with seawater pCO_2 with 50% mortality in the highest treatment after 68 days (Fig. 2.2). A significantly elevated mortality could be shown for the 24,400 µatm group compared to the control, 1,500 µatm, and 2,900 µatm groups (Kruskal-Wallis multiple comparison test, $p<0.05$, Tab. S2.3, S2.3a). Mortality, when averaged over all size classes, tended to increase in 12,800 µatm as well (total mortality ca. 15%). However, this increase was not significant by the end of the experiment. Smaller individuals reacted more sensitively towards high pCO_2 (Fig. 2.3). There were no differences in mortality between size classes in the control, 1,500 µatm, and 2,900 µatm treatments. At 6,600, 12,800 and 24,400 µatm, mortality in the smaller size class (0-1 cm) was significantly higher than mortality of cockles in the larger size class (1-2.5 cm) (Kruskal-Wallis multiple comparison test, $p<0.05$, Tab. S2.4).

The comparison of intact shells against shells with signs of dissolution showed an increase in shell corrosion of *C. edule* with increasing pCO_2 (Fig. 2.2, Fig. 2.4). Shells from the control treatment had an intact periostracum and were not characterized by shell dissolution (N=3 of 3 observations). Shells from ≥1,500 µatm were characterized by signs of external dissolution (SEM analysis, N=3 of 3 observations, Fig. 2.4).

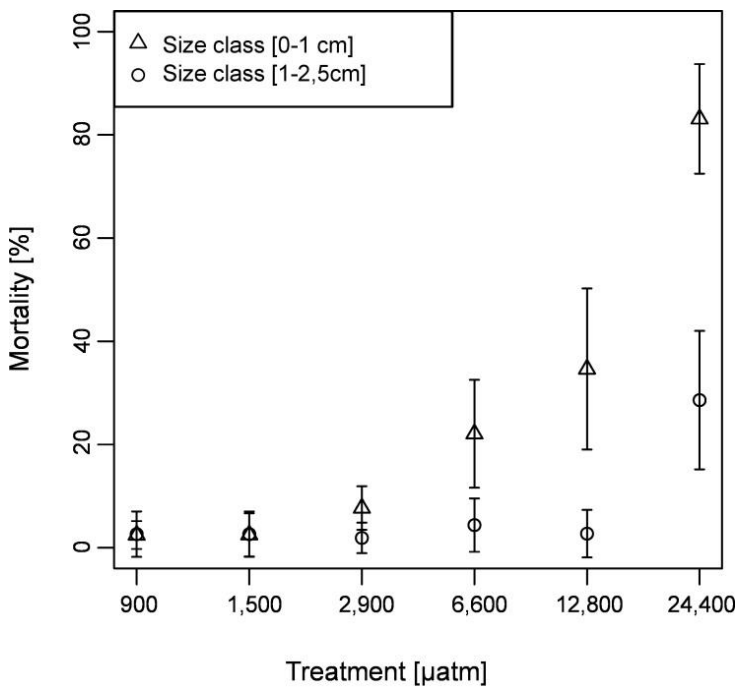

Figure 2.3 Cumulative mortality of different *C. edule* size classes during the experiment plotted for each treatment

Stereo microscopic images demonstrated visible signs of shell dissolution for all treatments above 2,900 µatm, with increasing severity in higher pCO_2 treatments. For some cockles (<5%), exclusively small animals (<0.7 cm) holes were already visible at 2,900 µatm.

Cockles maintained under high pCO_2 (6,600, 12,800 and 24,400 µatm) were characterized by severe shell damage. In the 6,600 µatm treatment, 72% of dead cockles, mostly small individuals, contained holes in their shells. In the two highest treatments (12,800 µatm and 24,400 µatm) >85% of deceased cockles were characterized by holes in their shells. This rate of shell dissolution was significantly higher when compared to the control and 1,500 µatm treatment (Kruskal-Wallis, $p<0.05$, Tab. S2.3, S2.3b). Whole body malondialdehyde (MDA) concentrations,

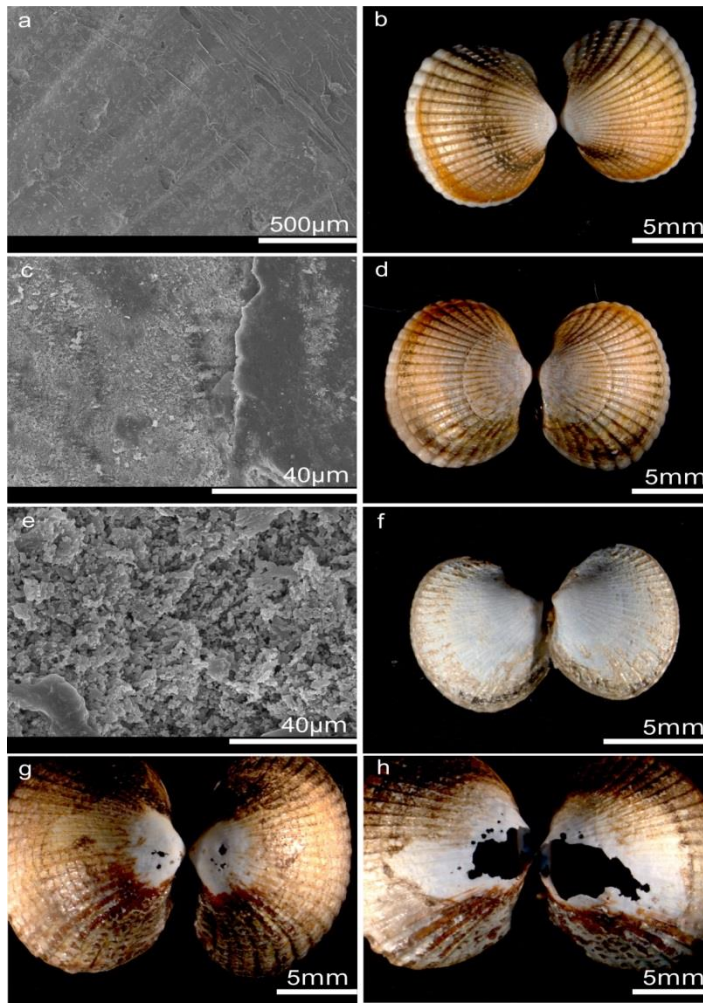

Figure 2.4 Shell corrosion in *C. edule*. (a, b) control (900 µatm), no shell corrosion visible on the outside of the shell. (c, d) 1,500 µatm, shell corrosion on the outside of the shell. (e, f) 6,600 µatm, shell dissolution on the outside of the shell. (g; h) 24,400 µatm, strong dissolution signs, holes.

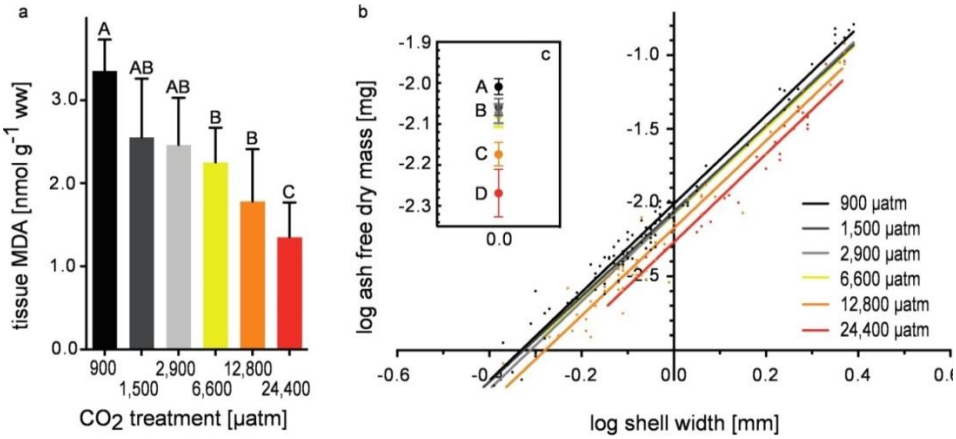

Figure 2.5 *C. edule* condition and MDA accumulation. (a) average of tissue MDA content [nmol g^{-1}ww] for the different treatments. Means and standard deviation, letters indicate significant differences between treatments. (b) regressions of log shell-free dry mass plotted against log shell width, single data points plotted for the 900, 12,800 and 24,400 µatm treatment (equations in Supplementary Tab. S5) (c) y intercepts of regressions of shell free dry mass and 95% confidence interval

an indicator for oxidative stress, were significantly lower in the 6,600 µatm, 12,800 µatm and 24,400 µatm groups when compared to the control (Fig. 2.5a, Tukey HSD, $p<0.05$). Additionally, MDA values of the 24,400 µatm treatment were significantly lower than values measured in 1,500 µatm and 2,900 µatm animals.

Slopes of log shell free dry mass vs. log shell width linear regressions (900-24,400 µatm) were not significantly different between treatments ($F_{(5,467)}=0.49$, $p=0.7851$, Fig. 2.55b), while y-intercepts were significantly different ($F_{(5,472)}=32.5$, $p<0.0001$). It appears that y-intercepts of all treatment levels (1,500-24,400 µatm) are significantly lower (no overlap in 95% confidence intervals) than that of the control group (900 µatm), indicating reduced body condition at elevated seawater pCO_2 (Fig. 2.55c). Slopes of control (900 µatm) and field animals collected just prior to the experiment were similar ($F_{(1,110)}=0.15$, $p=0.7021$). Y-intercepts were significantly different between these two groups ($F_{(1,111)}=17.94$, $p<0.0001$), with slightly reduced condition in the control animals vs. field collected animals (Tab. S2.5). *M. arenaria* and *L. balthica* remained burrowed during the entire experimental duration in all treatments and no mortality was observed for these two species.

2.3.2. Meiofauna community response

A total of twelve meiofauna groups were found. The four most abundant taxa were Nematoda (72.5 ± 7.5 %), Gastrotricha (11.0 ± 7.1 %), Copepoda (5.9 ± 4.5 %), and crustacean nauplii (2.6 ± 3.2 %). Total meiofauna densities ranged from 218 to 988 ind. 10 cm^{-2}. At the end of the experiment, significantly higher total meiofauna densities were found in the 24,400 µatm treatment compared with all

other treatments, except for the 1,500 and 2,900 µatm treatment (Pairwise tests (Treatment*Time), p(MC) ≤ 0.0495, Tab. S2.6, Tab. S2.7).

Meiofauna community composition significantly differed based on the factors treatment (Main test, p(perm) = 0.0004, PermDisp<0.05) and time (Main test, p(perm) = 0.0022). The meiofauna community of the 24,400 µatm treatment had a significantly different composition compared to the assemblages in all other treatments (p(perm) ≤ 0.0201, Fig. 2.6, Tab. S2.7). Evenness and diversity of the total meiofauna community decreased with time (p(perm) ≤ 0.0275, PermDisp <0.05) but did not differ between treatments.

The densities of nematodes (352.43 – 1733.64 ind. 10cm-²), did not consistently differ over time or between treatments. It was only occasionally significantly different between the 6,600 µam and 1,500 µatm treatment after six weeks, and between the 24,400 µatm and 12,800 µatm treatment after twelve weeks (see Tab. S2.7). A total of 36 nematode genera were found. The overall most abundant genera (>5%) were *Ascolaimus* (34.7 % ± 2.2 %), *Metachromadora* (13.2 % ± 1.0 %), *Hypodontolaimus* (9.4 % ± 1.0 %), *Microlaimus* (5.9 % ± 0.5 %) and *Enoplolaimus* (5.6 % ± 0.6 %). Multivariate analyses of relative and total densities of nematode genera did not reveal any significant difference between samples according to the pCO_2 level, time or a combination of both. Samples exhibited an overall high evenness (Pielou's evenness index) ranging from 0.60 to 0.89 but univariate

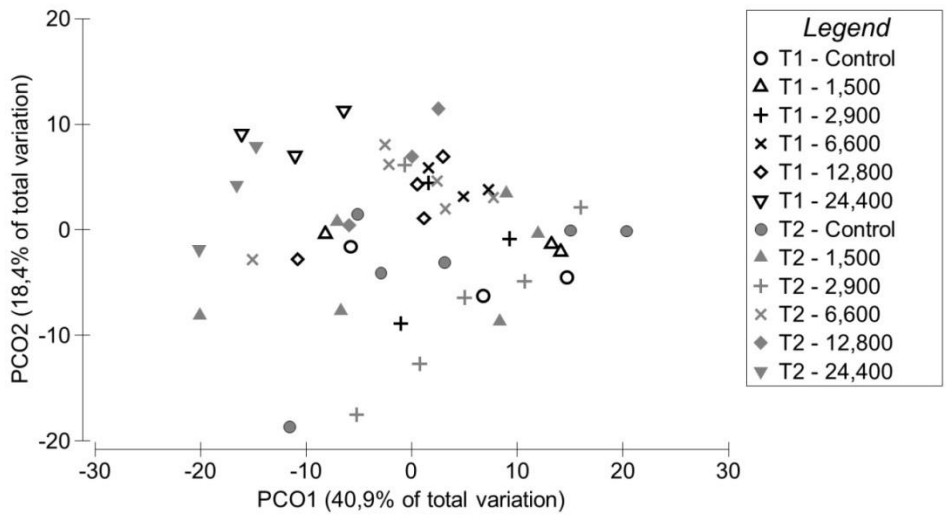

Figure 2.6 Principal coordinate analysis (PCO) plot of distances among centroids on the basis of the Bray-Curtis measure on square root transformed meiofauna densities. Symbols indicate the factors "time" (T1 = after six weeks and T2 = after twelve weeks) and "treatment". Axes refer to a non-arbitrary quantitative scale.

measures based on densities and relative abundances (genus richness, Shannon-Wiener index, Simpson index, Pielou's evenness index) did not differ between samples based on the factors treatment, time or a combination of both.

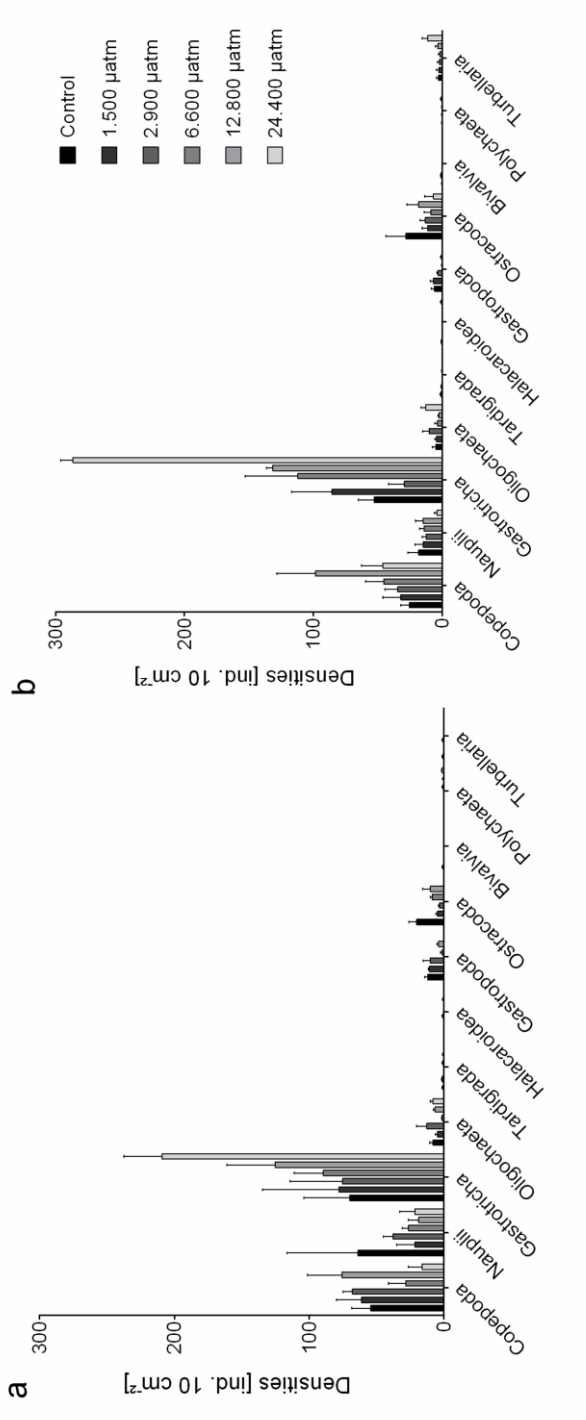

Figure 2.7 Average meiofauna densities excluding nematodes after (a) six weeks and (b) twelve weeks plotted per treatment in number of individuals per 10 cm^2.

When excluding Nematoda from the community composition analysis, Permanova revealed significant differences between treatments (p(perm) = 0.0002, PermDisp < 0.05) and over time (p(perm) = 0.0023). Differences occurred between the 24,400 µatm treatment and all other treatments (p(perm) ≤ 0.0298). According to the SIMPER analysis, Gastrotricha contributed most to these differences (25.53 – 41.08 %), followed by Copepoda (24.19 – 11.25 %), Ostracoda (14.98 – 9.88 %) and Gastropoda (11.73 – 7.40 %). Gastrotricha, Copepoda, Nauplii and Ostracoda contributed most to the difference over time (22.40 %, 18.70 %, 15.47 % and 11.77 %, respectively).

Univariate analyses of the densities of the taxa contributing most to the differences revealed diverse responses (Fig. 2.7). Copepoda densities were not impacted by treatment and time, whereas nauplii densities decreased significantly from the first to the second sampling time point (p(perm) = 0.0246). Gastrotricha densities were increased in the 24,400 µatm treatment compared to the control, 2,900 µatm and 6,600 µatm treatment (Pairwise test, p(perm) ≤ 0.0305) and densities of the 12,800 µatm treatment were increased compared to the control and 2,900 µatm group (p(perm) = 0,0171 and p(perm) = 0,034, respectively) but did not differ from the 1,500 µatm and 6,600 µatm treatment. Ostracoda densities increased over time (Main test, p(perm) = 0.0385) but were significantly lower in the 24,400 µatm treatment compared to the control, 1,500 µatm and 6,600 µatm treatment (p(perm) ≤ 0.0163). Gastropoda densities decreased over time (p(perm) = 0.0001, permDisp < 0.05) and differed between treatments (p(perm) = 0.0097) with decreased densities in all treatments ≥ 6,600 µatm (p(perm) ≤ 0.0269) when compared to treatments ≤ 2,900 µatm. At six weeks, Turbellaria were absent in >80% of the samples, while after twelve weeks densities ranged between 1 and 9 individuals per sample with a presence in >50% of all samples.

2.3.3. Bacterial community

Community fingerprinting of benthic bacterial communities by ARISA indicated similar average operational taxonomic unit (OTU) numbers for all treatments and time points, i.e. for 6 and 12 weeks respectively 196±9 and 179±14 (900 µatm), 191±13 and 196±8 (1,500 µatm), 190±15 and 174±17 (2,900 µatm), 188±12 and 179±19 (6,600 µatm), 184±17 and 183±13 (12,800 µatm), and 184±11 and 189±12 (24,400 µatm). The percentage of shared OTUs among the different EUs for a given treatment and time could be as low as 53-70% (max. 60-78%), and varied between 70-83% for joint OTU profiles (one per treatment and time point). NMDS did not show a separation of bacterial communities according to CO_2 treatment and/or time (data not shown). However, similarity analyses indicated a significant difference between bacterial communities thriving at 900 µatm (control)

and 24,400 µatm (abundance data only, Bonferroni-corrected $p<0.05$ after 6 and 12 weeks, group separation R<0.5, Tab. S2.8), as well as between 1,500 µatm and 24,400 µatm (abundance and presence-absence data, Bonferroni-corrected $p<0.05$ after 6 and 12 weeks, group separation R<0.5). Variation partitioning demonstrated that pCO_2 treatment and time had both significantly influenced bacterial community composition, together explaining 11.7% of the observed variation ($p<0.001$). Time alone explained 5% ($p<0.001$) of the community changes and pCO_2 treatment 6.9% ($p<0.011$). The interaction between the two factors was not significant ($p>0.05$).

2.4. Discussion

Our study indicates that persistent (3 month) strong leakage from a sub-seabed CCS site and the subsequent distribution of a high pCO_2 bottom water plume could lead to massive accumulations of *C. edule* on the seafloor and strong reductions in survival, with a high sensitivity especially for smaller bivalve size classes. Changes in microbial and meiofaunal community composition at the highest pCO_2 level indicate a direct or cascading effect of elevated CO_2 concentration on the entire benthic assemblage in a coastal sandy sediment ecosystem. However, we also demonstrate pronounced sub-lethal effects at lower treatment levels that warrant further research attention.

2.4.1. High pCO_2 changes coastal bivalve communities and bivalve behaviour

The observed behavioural changes of *C. edule* as a result of exposure to high seawater CO_2 correspond well with responses observed for the same species during exposure to hypoxia (Diaz and Rosenberg, 1995; Rosenberg et al., 1991). With increasing pCO_2, moribund or weakened *C. edule* accumulated on the sediment surface. The fraction of *C. edule* on the surface of the sediment increased with duration of the experiment. Quite similarly, Widdicombe et al. (2009) observed emersion of infauna echinoderms from the sediment during a high – CO_2 mesocosm incubation when pH values dropped below 6.5.

We observed substantial mortality of smaller size classes of *C. edule* in all treatments >6,000 µatm. This corroborates with previous work on other bivalve species where smaller juveniles appeared to be more sensitive to elevated seawater pCO_2 (Green et al., 2004; Waldbusser et al., 2010). This effect might be related to less favourable area to volume ratios, as smaller animals have to protect a relatively larger surface area from acid-base disturbance and relatively larger shell area from (internal) dissolution. Shell production costs and inorganic carbon demand for calcification are also much higher in smaller bivalves (Thomsen et al., 2013;

Waldbusser et al., 2013). Size-dependant mortality implies that leakage could lead to an alteration of the demographic structure of bivalve communities.

In our study, shell corrosion was evident from 1,500 µatm to 24,400 µatm and signs of severe dissolution and presence of holes were found in most animals exposed to 12,800 µatm and 24,400 µatm. These findings indicate that even moderate degrees of acidification can already lead to non-reversible shell damage. Outer shell corrosion has been observed in a number of gastropod and bivalve molluscs (Hall-Spencer et al., 2008; Ries et al., 2009) and has often been linked to absence of a thick and intact periostracum (Ries et al., 2009). *C. edule* is exposed to the seawater through watercirculation via the sifons and the relatively high position in the sediment (upper 2-5 cm) in comparison to other bivalves, ensuring constant exposure to the acidic conditions of the seawater (Flach, 1996). Furthermore, *C. edule* is characterized by a very thin periostracum (ca. 2 µm) (Harper, 1997) and a shell that is exclusively composed of aragonite, the polymorph of calcium carbonate that is most prone to dissolution (Glover and Kidwell, 1993; Taylor et al., 1973). In contrast, mytilids are protected by a periostracum of >20 µm (Harper, 1997) and can live in seawater which is strongly undersaturated for aragonite (Ω_{arag}<0.2) as long as their periostracum is intact. Corrosion starts to occur when the periostracum is mechanically damaged (Thomsen et al., 2010), leading to complete dissolution of the shell in extreme cases, e.g. in deep-sea hydrothermal vent mussels that live in strongly acidic waters with pH <6 (Tunnicliffe et al., 2009). While bivalves are able to repair holes and fractures in their shells (Mount et al., 2004), it is apparent from our results that *C. edule* in the highly acidified treatments were unable to allocate sufficient resources to shell repair, or that the rate of dissolution simply overwhelmed their repair capacity. Holes in their shells probably lead to massive and energy intensive stimulation of the immune system due to invasion of foreign microorganisms and loss of valuable proteins from the extrapallial fluid, the fluid which is in contact with the inner side of the shell (Kádár, 2008). In summary, progressive shell corrosion, even in the lower treatment levels (i.e. 1,500 µatm and higher), constitutes a large problem as shell integrity is essential for fitness of bivalve species.

One of the toxic effects of elevated oxyradical formation in cells is an increase in lipid peroxidation levels (Maritim et al., 2003). A commonly employed mode of detection is the reaction of lipid peroxidation intermediates with thiobarbituric acid in the so called TBARs assay. One of the major products of lipid peroxidation, but by far not the only one, is malondialdehyde (MDA), which serves as a marker for oxidative stress (Valavanidis et al., 2006). Oxidative stress is frequently also related to metabolism (Del Rio et al., 2005; Finkel and Holbrook, 2000). Previous studies on bivalves suggest a good relationship between high MDA accumulation and

elevated metabolic rate (Abele et al., 2001; McArthur and Sohal, 1982). MDA concentration was low in bivalves exposed to pCO_2>6,600 µatm suggesting that animals exposed to such high CO_2 concentrations have a reduced metabolism. While intermediate levels of acidification (i.e. <4,000 µatm) have repeatedly been shown to cause elevated metabolic rates (Beniash et al., 2010; Thomsen and Melzner, 2010) and increased oxidative stress (Tomanek et al., 2011) in a number of bivalve species, studies using treatment levels of >4,000 µatm have generally found metabolic reduction (Michaelidis et al., 2005; Thomsen and Melzner, 2010).

When employed as a strategy in the long run, metabolic suppression will lead to consumption of endogenous energy stores and reduced fitness. It has been demonstrated in a range of marine invertebrate species that acidification primarily impacts energy allocation processes and that ultimately, sensitivity is defined by depletion of available energy (scope for growth) by basal metabolism or reduced energy uptake (Dorey et al., 2013; Stumpp et al., 2011). In support of this view, all high-CO_2 exposed *C. edule* in our experiment (1,500-24,400 µatm) were characterized by a reduction in body condition, as indicated by reductions in shell-free dry mass in relation to shell width. This effect becomes more pronounced at the higher treatment levels (Fig. 5b, c) and correlates with reduced MDA accumulation. These findings indicate a negative scope for growth, which is unsustainable in the long - run. In a next step, it will be important to investigate aerobic metabolism and energy uptake in this species to better understand the underlying processes leading to energy budget disturbance.

M. arenaria and *L. balthica* survived the complete experimental duration. Even though abundance of these two species was low, the observations confirm our original hypothesis that *C. edule*, the species most sensitive to hypoxic stress, also is the most vulnerable to ocean acidification (Dries and Theede, 1974). *L. balthica* is a species which is generally quite tolerant towards stressful conditions. It occurs deep in the sediment where oxygen availability is low (Darr et al., 2014) and tolerates polluted sediments (McGreer, 1982). Resistance of *M. arenaria* could be due to a thick, protective periostracum (20 µm) (Harper, 1997). Additionally, a greater burrowing depth (Baker and Mann, 1991) might render this species less sensitive to acidified seawater. All bivalves used in this study have a shell consisting of aragonite, the more soluble calcium carbonate polymorph (Harper, 1997; Taylor et al., 1973). While the mineralogy is an important factor in defining susceptibility of bivalves (Ries et al., 2009), there is a high variation in vulnerability between species with the same shell mineralogy (Gazeau et al., 2013). Crystal size and the proportion of organic matrix within the shell play an additional factor in resistance against environmental stress such as acidified seawater (Harper, 1997).

2.4.2. Very high pCO_2 induces shifts in meiofaunal community structure

The findings of this experiment indicate that when very high seawater carbon dioxide levels occur, meiofauna community structure at higher taxon level can change as a result of differential sensitivity. The changes in densities were most severe in some of the less abundant meiofauna taxa that are not often reported. Most acidification studies focused on the dominant taxa such as Copepoda and Nematoda (Dashfield et al., 2008; Hale et al., 2011; Kurihara et al., 2007; Widdicombe et al., 2009), only few other studies emphasized the importance of changes in taxon composition, particularly including less abundant taxa as a proxy for the status of benthic environments (Bianchelli et al., 2010; Gambi et al., 2010; Pusceddu et al., 2011).

In this study, densities of three meiofauna taxa were affected by changing pCO_2 (i.e. Gastrotricha, Gastropoda, and Ostracoda). In accordance with the results of the bivalve analysis, the calcifying Gastropoda and Ostracoda suffered from decreased densities in treatments with high pCO_2 (at >6,600 µatm and >24,400 µatm, respectively). Densities of Gastrotricha on the other hand increased significantly at the highest treatment level. It is, however, impossible to differentiate whether this is the direct result of the seawater acidification having a positive effect on their physiology (growth and reproduction) or whether this group was favoured by indirect factors, such as changes in food availability or space occupation by the disappearance of macrofauna or other taxa. Gastrotricha, as well as many other meiofauna taxa, feed on bacteria and protozoa (Giere, 2009). The divergent microbial community in the highest acidification treatment could be the driver of the change in meiofaunal community structure with a strong increase in Gastrotrich densities. Despite all uncertainties, our study shows that Gastrotricha act as a physiologically tolerant, opportunistic taxon, apparently benefitting from severe seawater acidification.

In a laboratory experiment without macrofauna, (Kurihara et al., 2007) found no differences in the abundance and biomass of nematodes, harpacticoid copepods or harpacticoid nauplii between treatments with 380 ppm CO_2 (control) and 2,400 ppm CO_2 (acidified) over a time frame of 56 days. Several single-species studies reported decreased reproduction and slowed larval development of copepods when seawater pCO_2 is elevated (Fitzer et al., 2012; Kurihara et al., 2004; Kurihara and Ishimatsu, 2008). Interestingly, the decline in densities of nauplius larvae over time in our mesocosm experiment occurred both in the control and acidified treatment indicating that the reproductive success of the copepods was not influenced by increased pCO_2.

Nematodes, the most abundant taxon in our experiment, seemed to be unaffected by the different treatments in terms of community composition and abundance with no significant changes over time. Several experimental studies on nematodes indicate that a certain threshold exists above which nematodes do not suffer from pH reduction in short- to medium-term (days to weeks) experiments. Once pH drops below this threshold (~pH 6), a decline of nematode densities has been observed that can be assigned to direct physiological responses (Takeuchi et al., 1997). (Widdicombe et al., 2009) found significant changes in nematode community structure only after a 20 weeks exposure and only at pH values lower than 6.0, while macrofauna decreased in abundance at much less severe pH changes. Similarly, in a different mesocosm experiment, nematodes remained unaffected following a seven week exposure to seawater pH of 7.5 (Dashfield et al., 2008). In the study of (Takeuchi et al., 1997), nematode survival was monitored over a time frame of one week and only in the most severe treatments with a pH of 5.4 and 5.1 survival rates were significantly decreased with a species specific response related to the activity of the nematodes. Sub-lethal measures such as reproductive success and scope for growth might be more appropriate variables to assess sensitivity during medium-term exposure (Kurihara et al., 2007). In our case nematode densities remained stable in all treatments and between time steps, suggesting that reproduction had most likely taken place. This was supported by the occurrence of juveniles and gravid nematodes.

2.4.3. High pCO_2 impact on bacterial communities

Significant differences between bacterial communities became evident when comparing the highest treatment (24,400 µatm, severe leakage scenario) to the control (900 µatm) and slightly increased CO_2 level (1,500 µatm). This suggests the presence of bacteria adapted to a wide range of natural fluctuations in pCO_2, but also the risk of substantially influencing ecosystem functionality at very high pCO_2. Part of the potentially occurring changes in bacterial community structure might have been masked by the high spatial variability of the bacterial communities, as suggested by low R-values (PAST analyses) and a low percentage of shared OTUs among the EUs of a given treatment and time. The overall average number of OTUs in this study (186±14 OTUs, 0-1 cm sediment depth, all treatments and time points) was slightly higher than the average value obtained from samples taken during a previous study on shallow subtidal sands in the North Frisian Wadden Sea (Böer et al., 2009) (145±45.7 OTUs, 0-5 cm sediment depth, different seasons). Both pCO_2 level and time played a significant role in the observed community shifts, but as the two factors did not significantly interact, at this point, the prolonged pCO_2 treatment can neither be confirmed nor disregarded as a

causal factor. Overall, influential factors might, besides a direct CO_2 influence, include a change in sediment nutrient composition or a change in sediment bioturbation rates mediated by dying cockles. While *C. edule* has previously been shown to significantly influence the microphytobenthic primary production due to release of NH_4^+(Swanberg, 1991), bacterial abundance was not significantly influenced by bio-diffusing activities of *C. edule*. The comparatively minor effects of substantial decrease in *C. edule* density at the highest CO_2 treatment level on the microbial community structure could be explained by a less pronounced impact of *C. edule* on sediment oxygen and nutrient fluxes compared to *M. arenaria* and *L. balthica* which strongly modify sediment chemistry through their burrowing activity and bio-irrigation of the sediments (Michaud et al., 2006, 2009).

However, the loss of an important ecosystem engineer poses unknown risks. A progressive loss of *C. edule* could affect settlement success of other macrobenthic species (e.g. polychaetes) that are otherwise competitively impacted by *C. edule* (Van Colen et al., 2013; Flach, 1996). These species could more strongly impact microbial and meiofauna communities or alter ecosystem functionality during long-term CO_2 leakage. Similar to findings in our experiment, Kerfahi et al. (2014) demonstrated shifts in sediment microbial communities (top 2 cm of the sediment) along CO_2 clines (600 – 1,600 µatm) in the Mediterranean. The authors found abundances of most dominant genera to be unaffected by CO_2, while 5% of genera differed in abundance along the CO_2 cline. Whether these changes can alter the biogeochemical functions of marine sediments still needs to be investigated in future experiments.

We can conclude that experimental medium-term exposure to high seawater pCO_2, which mimics strong leakage from sub-seabed CCS sites, can cause high mortality of *C. edule* and shifts in the composition of meiofauna and microbiota in a shallow sandy sediment environment. The response of bivalves was strongly species-specific and size-dependent. *C. edule* was found to be sensitive to most treatment levels with signs of shell corrosion at pCO_2 ≥1,500 µatm and reduced body condition at CO_2 ≥1,500 µatm, which points at a negative, unsustainable energy budget. This is supported by reduced tissue MDA accumulation at ≥6,600 µatm. Thus, even lower levels of leakage resulting in seawater pCO_2 values of >1,500 µatm could strongly impact *C. edule* dominated sediments in the long - run as they might lead to reduced growth, enhanced mortality and reduced reproductive output. Longer – term ecological studies on *C. edule* are needed to investigate these processes in more detail. The dominant meiofauna taxa tolerated the highest acidification levels. Rare taxa on the other hand were affected by very high pCO_2 illustrating a decrease in calcifying organism abundance and an increase in other, more opportunistic taxa. While calcifying organisms were likely directly affected by

seawater acidification, it is unclear whether the response of other taxa is directly related to acidification or rather a result of cascading effects due to high *C. edule* mortality. Severe leakage from storage sites that result in very high seawater pCO_2 (>20,000 μatm) on a longer time scale could thus lead to significant changes in the overall benthic community structure, with unforeseen consequences for ecosystem health and functions. Lower levels of leakage (<5,000 μatm) have sub – lethal impacts on the dominant macrofauna species and may impact benthic ecosystem function in the long – term. Clearly, more research effort needs to be devoted into studying longer term consequences of exposure to lower seawater and sediment pCO_2 levels to better constrain the risks that are associated with sub – lethal species responses, particularly with respect to potential impacts on sediment meiofauna and bacterial communities.

Acknowledgements

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2.5. Supplementary data

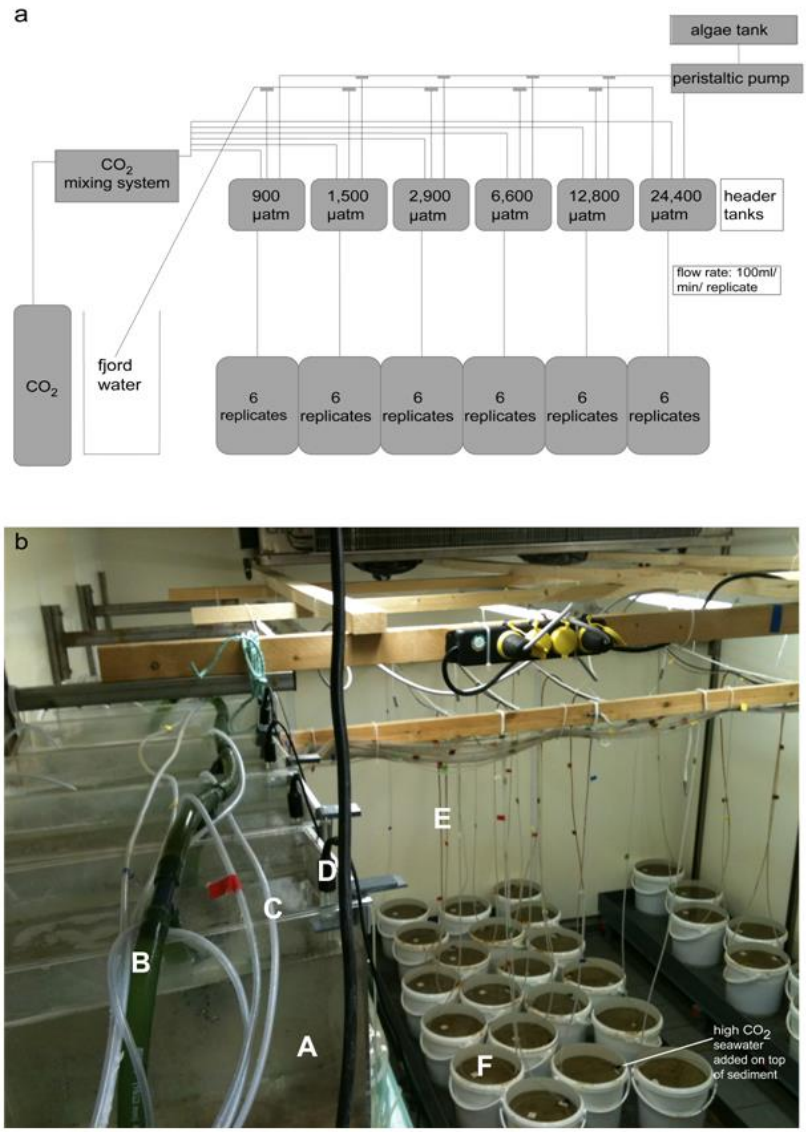

Figure S2.1 (a) Schematic illustration of the experimental setup. (b) Photographic view of the experimental setup; In a temperature controlled climate chamber at GEOMAR, fjord water (B) and algae (*Rhodomonas sp.*) (C) were pumped into header tanks (A) supplying (E) the experimental units (F, six per CO_2 treatment). Six different CO_2 levels were generated through pH controlled (D = pH electrode) CO_2 addition (IKS Aquastar).

Table S2.1 Environmental parameters

	measured (N=20)	Calculated					measured (N=15)	
Treatment	C_T [µmol/kg]	pH (NBS scale)	pCO_2 (µatm)	TA in (mmol/ kgSW)	□Ca	□Ar	Rhodomonas header tank (cells/ml)	Rhodomonas ø per treatment (cells/ml)
900 (±SD)	2038.3 (±130)	7.842	923.69 (± 56,92)	2,032.43	0.86	0.510	4277.30 (± 635.4)	1938.51 (± 946.34)
1,500 (±SD)	2100.3 (±157.7)	7.654	1,461.88 (± 190,91)	2,049.95	0.57	0.340	4401.17 (± 413.5)	2111.42 (± 921)
2,900 (±SD)	2170.2 (±126.7)	7.360	2,882.51 (± 352,93)	2,028.44	0.30	0.170	3652.86 (± 269.4)	1564.41 (± 713.1)
6,600 (±SD)	2377.9 (±165)	6.999	6,630.13 (± 759,50)	2,020.41	0.13	0.080	3504.39 (± 354.2)	1860.39 (± 817.9)
12,800 (±SD)	2638.1 (±258.4)	6.696	12,783.49 (± 1,549,93)	1,936.86	0.06	0.036	3626.80 (± 526.7)	2915.47 (± 2397.4)
24,400 (±SD)	3188.2 (±195.8)	6.396	24,381.04 (± 2,060.83)	1,848.87	0.03	0.017	4260.89 (± 467.9)	3554.18 (± 2170.7)

Table S2.2 Test for the behaviour of C.edule

	df	SS	% explained variance	p(perm)
Treatment	5	306749	**62.9**	**0.0001**
Time	27	33187	**0.68**	**0.0001**
EU (Treatment)	30	1852,8	**0.04**	**0.0001**
Treatment*Time	135	130160	**26.7**	**0.0001**
Total	1007	487420		

Table S2.3 Main test for the cumulative mortality and shell dissolution of *C.edule,* Kruskal-Wallis test

	Main test			
	Factor	df	X^2	p
Cumulative mortality of *C. edule*	Treatment	5	28,81	**0,00**
% dissolved shells of *C. edule*	Treatment	5	26,75	**0,00**

Table S2.3a Kruskal multiple comparison test of mortality of *C.edule* (Kruskal Wallis Analysis, p-value= 0.0000, critical difference 17.85412)

Treatment	900 µatm	1,500 µatm	2,900 µatm	6,600 µatm	12,800 µatm	24,400 µatm
900 µatm						
1,500 µatm	4.3333					
2,900 µatm	0.1667	4.5000				
6,600 µatm	11.5833	15.9167	11.4167			
12,800 µatm	14.2500	**18.5833**	14.0833	2.6667		
24,400 µatm	**22.3333**	**26.6667**	**22.1667**	10.7500	8.0833	

Table S2.3b Kruskal multiple comparison test of shell dissolution of *C.edule* (Kruskal Wallis Analysis, p-value=0.0000, critical difference: 17.85412)

Treatment	900 µatm	1,500 µatm	2,900 µatm	6,600 µatm	12,800 µatm	24,400 µatm
900 µatm						
1,500 µatm	0.000					
2,900 µatm	5.8333	5.8333				
6,600 µatm	13.5000	13.5000	7.6667			
12,800 µatm	**18.3333**	**18.3333**	12.5000	4.8333		
24,400 µatm	**22.3333**	**22.3333**	16.5000	8.8333	4.0000	

Table S2.4 Mortality between different size classes of *C.edule* (Kruskal Wallis Analysis), 2 size classes: small (0-1 cm), large (1-2.5 cm)

Treatment	df	chi-squared	p-value
900 µatm	1	0,01	0,93
1,500 µatm	1	0,04	0,85
2,900 µatm	1	1,13	0,29
6,600 µatm	1	6,68	**0,01**
12,800 µatm	1	8,77	**0,00**
24,400 µatm	1	8,37	**0,00**

Table S2.5 Regression of shell free dry weight (log data). The y-intercept of the Fjord control is slightly lower compared to the experimental control. As the experiment was carried out in winter (T=4.3-8.9°C) and it is known that the condition of *C. edule* decreases in winter (e.g. Newell, R. I. E., Bayne, B. L. Seasonal changes in the physiology, reproductive condition and carbohydrate content of the cockle *Cardium (=Cerastoderma) edule* (Bivalvia: Cardiidae). Marine Biology. 56, (1), 11-19 (1980)) the slightly different condition could be due to winter conditions during the experiment.

Treatment	Equation	r^2	N
900 µatm	Y = 2,994*X - 2,009	0,973	94
1,500 µatm	Y = 2,891*X - 2,058	0,9553	136
2,900 µatm	Y = 2,978*X - 2,074	0,962	91
6,600 µatm	Y = 2,917*X - 2,079	0,9492	64
12,800 µatm	Y = 2,965*X - 2,174	0,9538	66
24,400 µatm	Y = 2,995*X - 2,269	0,9543	28
Fjord Control	Y = 2,879*X - 1,959	0,981	37

Table S2.6 Main test of meiofaunal densities and community composition (square root transformed) as well as nematode community composition (relative abundance and calculated densities)

		Main test				
		df	SS	MS	pseudo-F	p(perm)
Total meiofauna	Treatment	5	683.07	136.61	1.8839	0.1316
	Time	1	54.98	54.98	3.9096	0.0674
	EU(Treatment	25	2081.80	83.27	5.9214	**0.0008**
	Treatment*Time	5	475.15	95.03	6.7575	**0.0033**
	Residuals	12	168.76	14.06		
	Total	48	2870.70			
Meiofauna community composition	Treatment	5	3164.10	632.82	3.0099	**0.0004**
	Time	1	864.39	864.39	6.5242	**0.0022**
	EU(Treatment	25	5613.90	224.56	1.6949	**0.0254**
	Treatment*Time	5	1134.10	226.82	1.7119	0.0633
	Residuals	12	1589.90	132.49		
	Total	48	11586.00			
Meiofauan excl. Nematoda	Treatment	5	7577.60	1515.50	3.4291	**0.0002**
	Time	1	2189.80	2189.80	6.5072	**0.0023**
	EU(Treatment	25	11534.00	461.36	1.3710	0.0979
	Treatment*Time	5	1872.70	374.53	1.1129	0.3744
	Residuals	12	4038.30	336.52		
	Total	48	27137.00			
Nematoda	Treatment	5	644.37	128.87	1.7314	0.1700
	Time	1	125.05	125.05	6.3176	**0.0255**
	EU(Treatment	25	2112.20	84.49	4.2682	**0.0054**
	Treatment*Time	5	521.50	104.30	5.2692	**0.0054**
	Residuals	12	237.53	19.79		
	Total	48	2882.60			
Nauplii	Treatment	5	3376.20	675.25	1.1862	0.3039
	Time	1	3485.40	3485.40	4.0269	**0.0246**
	EU(Treatment	25	12868.00	514.73	0.5947	0.9204
	Treatment*Time	5	2662.20	532.44	0.6152	0.8307
	Residuals	12	10386.00	865.54		
	Total	48	30551.00			

Gastrotricha	Treatment	5	7785.80	1557.20	4.0947	**0.0054**
	Time	1	46.18	46.18	0.2334	0.7109
	EU(Treatment	25	10346.00	413.85	2.0914	0.0664
	Treatment*Time	5	1414.90	282.99	1.4301	0.2666
	Residuals	12	2374.60	197.89		
	Total	48	23926.00			
Ostracoda	Treatment	5	9296.40	1859.30	3.5072	**0.0098**
	Time	1	1884.40	1884.40	4.4909	**0.0385**
	EU(Treatment	25	13762.00	550.47	1.3119	0.2732
	Treatment*Time	5	1292.60	258.52	0.6161	0.7429
	Residuals	12	5035.20	419.60		
	Total	48	32944.00			
Gastropoda	Treatment	5	17720.00	3544.00	14.0640	**0.0001**
	Time	1	2388.10	2388.10	8.7587	**0.0097**
	EU(Treatment	25	6204.90	248.20	0.9103	0.6059
	Treatment*Time	5	1489.20	297.83	1.0923	0.4049
	Residuals	12	3271.80	272.65		
	Total	48	34616.00			
Nematode composition (relative abundance.)	Treatment	1	679.42	679.42	1.4587	0.1859
	Time	5	2327.20	465.44	0.6278	0.9112
	EU(Treatment	12	8896.50	741.37	1.5917	**0.0309**
	Treatment*Time	5	2889.90	577.97	1.2409	0.2076
	Residuals	12	5589.10	465.76		
	Total	35	20382.00			
Nematode composition (densities)	Treatment	1	601.05	601.05	1.3790	0.2096
	Time	5	2619.40	523.88	0.9790	0.5395
	EU(Treatment	12	6421.40	535.12	1.2278	0.1003
	Treatment*Time	5	2503.30	500.67	1.1487	0.2652
	Residuals	12	5230.20	435.85		
	Total	35	17375.00			

Table S2.7 Pairwise tests of the factor treatment of selected meiofauna groups. P(perm)- or, if indicated, p(MC) values are given.

Total meiofauna densities

Treatment*Time after 12 weeks p(MC)	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.4005					
2 900 µatm	0.8379	0.5111				
6 600 µatm	0.4085	0.7809	0.5631			
12 800 µatm	0.5552	0.817	0.6937	0.9653		
24 400 µatm	**0.0466**	0.2711	**0.0495**	**0.0442**	**0.0492**	

Nematode densities

Treatment*Time after 6 weeks p(MC)	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.7595					
2 900 µatm	0.361	0.6511				
6 600 µatm	0.9253	0.6896	0.2893			
12 800 µatm	0.1116	0.4528	0.9128	**0.0396**		
24 400 µatm	0.148	0.4959	0.8873	0.0501	0.8961	

Nematode densities

Treatment*Time after 12 weeks p(MC)	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.3796					
2 900 µatm	0.6314	0.6486				
6 600 µatm	0.4157	0.6337	0.8101			
12 800 µatm	0.8957	0.4267	0.5744	0.2572		
24 400 µatm	0.1219	0.5905	0.2385	0.053	**0.0369**	

Meiofauna Composition

Treatment	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.7912					
2 900 µatm	0.2463	0.3550				
6 600 µatm	0.1545	0.2540	0.0610			
12 800 µatm	0.1344	0.1921	0.0759	0.2253		
24 400 µatm	**0.0038**	**0.0201**	**0.0012**	**0.0006**	**0.00265**	

Gastritricha p(MC)

Treatment	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.7626					
2 900 µatm	0.9857	0.8066				
6 600 µatm	0.2323	0.5180	0.2244			
12 800 µatm	**0.0171**	0.1363	**0.0340**	0.2933		
24 400 µatm	**0.0055**	0.055	**0.0102**	**0.0305**	0.0568	

Ostracoda

Treatment	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.1943					
2 900 µatm	0.3824	0.6143				
6 600 µatm	0.3386	0.6997	0.7181			
12 800 µatm	0.8770	0.3456	0.7747	0.4917		
24 400 µatm	**0.0123**	**0.0163**	0.0615	**0.0041**	0.0644	

Gastropoda

Treatment	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm						
1 500 µatm	0.8381					
2 900 µatm	0.5286	0.6678				
6 600 µatm	**0.0116**	**0.0067**	**0.0079**			
12 800 µatm	**0.0269**	**0.0066**	**0.0049**	0.1533		
24 400 µatm	**0.0108**	**0.0001**	**0.0081**	0.1837	**0.0001**	

Table S2.8 Main Test and pairwise tests of bacterial community composition (PAST analyses)

	Factor	df	%variance explained	F	p
response: Hellinger-transformed ARISA data. (R analyses)	Time + Treatment	2	11.7	5,5858	**0.001**
	Time	1	5.0	4,8737	**0.001**
	Treatment	1	6.9	6,3053	0,011
	Time x Treatment	1	1.3	1,0342	0.38

Bacterial diversity after 6 weeks; Bray-Curtis/ abundance ; R=0.3133 (low group separation)

	900	1,500	2,900	6,600	12,800	24,400
900 µatm						
1,500 µatm	0.264					
2,900 µatm	1	0.177				
6,600 µatm	0.9345	0.0645	0.675			
12,800 µatm	0.249	**0.033**	0.447	1		
24,400 µatm	**0.03**	**0.0285**	0.057	0.822	0.0645	

Bacterial diversity after 12 weeks; Bray-Curtis/ abundance; R=0.2486 (low group separation)

	900	1,500	2,900	6,600	12,800	24,400
900 µatm						
1,500 µatm	1					
2,900 µatm	1	1				
6,600 µatm	0.093	1	1			
12,800 µatm	0.0615	0.1425	1	1		
24,400 µatm	**0.033**	**0.018**	0.2055	0.1845	0.681	

Bacterial diversity after 6 weeks; Jaccard/presence-absence ; R=0.2403 (low group separation)

	900	1,500	2,900	6,600	12,800	24,400
900 µatm						
1,500 µatm	0,414					
2,900 µatm	1	1				
6,600 µatm	1	0,372	1			
12,800 µatm	0,7125	0,186	1	1		
24,400 µatm	0,1425	**0,03**	**0,036**	0,8865	0,108	

Bacterial diversity after 12 weeks; Jaccard/presence-absence; R=0.2893 (low group separation)

	900	1,500	2,900	6,600	12,800	24,400
900 µatm						
1,500 µatm	1					
2,900 µatm	0,8955	1				
6,600 µatm	0,234	1	1			
12,800 µatm	0,0975	0,5475	0,15	1		
24,400 µatm	0,0765	**0,039**	0,066	0,3075	0,0705	

Chapter 3 Combined, short-term exposure to reduced seawater pH and elevated temperature induces community shifts in an intertidal meiobenthic assemblage

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Abstract

In future global change scenarios the surface ocean will experience continuous acidification and rising temperatures. While effects of both stressors on marine, benthic communities are fairly well studied, consequences of the interaction of both factors remain largely unknown. We performed a short-term microcosm experiment exposing a soft-bottom community from an intertidal flat in the Westerscheldt estuary to two levels of seawater pH (ambient pH_T=7.9, reduced pH_T=7.5) and temperature (10 °C ambient and 13 °C elevated temperature) in a crossed design. After 8 weeks, meiobenthic community structure and nematode staining ratios, as a proxy for mortality, were compared between treatments and structural changes were related to the prevailing abiotic conditions in the respective treatments (pore water pH_T, sediment grain size, total organic matter content, total organic carbon and nitrogen content, phytopigment concentrations and carbonate concentration). Pore water pH_T profiles were significantly altered by pH and temperature manipulations and the combination of elevated temperature and reduced pH intensified the already more acidic porewater below the oxic zone. Meiofauna community composition was significantly affected by the combination of reduced pH and elevated temperature resulting in increased densities of predatory Platyhelminthes, reduced densities of Copepoda and Nauplii and complete absence of Gastrotricha compared to the experimental control. Furthermore, nematode staining ratio was elevated when seawater pH was reduced pointing towards reduced degradation rates of dead nematode bodies. The observed interactions of pH and temperature on meiobenthic communities and abiotic sediment characteristics underline the importance of multistressor experiments when addressing impacts of global change on the marine environment.

3.1. Introduction

Present day global atmospheric CO_2 concentrations greatly exceed the natural range of the past 800,000 years (IPCC, 2013; Lüthi et al., 2008). As a result of anthropogenic activity CO_2 levels have risen at an unprecedented rate from 280 ppm in the pre-industrial era to 406.05 ppm at present (March 2017, Dlugokencky and Tans, 2017) and a further increase to 421 - 936 ppm by the year 2100 is predicted depending on the emission scenario (IPCC, 2013). Together with the rise in atmospheric methane concentrations that are mainly related to agricultural activities, CO_2 concentrations contribute considerably to the earth's greenhouse effect. As a consequence, average ocean surface temperature are predicted to rise 1.0 – 4.1 °C by the end of this century (Collins et al., 2013). The upper ocean also acts as a true carbon sink and has already absorbed about half of the emissions derived from fossil fuels and cement production, inducing shifts in the oceans carbonate chemistry (Caldeira and Wickett, 2003; Sabine et al., 2004). Depending on the Representative Concentration Pathway (RCP) scenario, this may result in a projected reduction of open ocean surface pH by 0.20 (RCP 6.0) - 0.31 units (RCP 8.5, worst case scenario) by 2100 if no immediate measures are taken to reduce current CO_2 emission rates, a process called ocean acidification (OA) (Gattuso et al., 2015; IPCC, 2013). Changes in ocean temperature and pH have already occurred and past increases in atmospheric CO_2 have led to a temperature rise of 0.25 °C and a pH decrease of 0.1 units in the upper ocean between 1971 and 2010 (Rhein et al., 2013). Further modifications of the upper ocean environment will inevitably disturb marine ecosystems and lead to changes with respect to biodiversity, animal behaviour, trophic interactions and other ecosystem processes (Byrne and Przeslawski, 2013; Fabry et al., 2008; Nagelkerken and Munday, 2016; Orr et al., 2005; Pörtner et al., 2004; Thomas et al., 2004). Ecosystem responses may, however, be greater or smaller than just the sum of the effects from individual stressors (synergistic vs. additive and antagonistic effects)(Darling and Côté, 2008). Depending on the modes of interaction, predictions of climate change effects may therefore be underestimated (if synergy is underlying) or overestimated (in case of antagonistic effects) (Darling and Côté, 2008; Harley et al., 2006; Ong et al., 2017). Consequently investigating the combined effects of multiple stressors is of utmost importance to understand future ocean conditions.

The sensitivity of marine organisms to variations in seawater pH and temperature can be correlated with the activity level, mobility, adaptation capacity, thermal window and developmental stage of the animals and varies greatly among species (Byrne and Przeslawski, 2013; Pörtner et al., 2004; Pörtner, 2008, 2010). Acid-base regulation that involves proton production, transport and consumption as well as buffering of intracellular compartments requires more energy when external pH is

highly variable (Pörtner et al., 2004; Seibel and Walsh, 2001, 2003). Theory predicts that organisms subjected to OA can therefore allocate less energy to other processes resulting in a lower fitness (Fitzer et al., 2012; Sokolova et al., 2012; Stumpp et al., 2011; Thomsen and Melzner, 2010; Wood et al., 2008). Stressor-induced upregulation of the metabolic activities may also lead to biological deficiencies and altered behavior (Nagelkerken and Munday, 2016). The unprecedented rates at which ocean acidification and warming are currently happening may not allow enough time for all species to genetically adapt to the changing environmental conditions in their natural habitat, especially when population turnover rates are slow (Kelly et al., 2012; Sunday et al., 2011). As a result, warming of the surface ocean during the last decades has already lead to a shift in species distributions and facilitated invasion of non-indigenous species causing severe changes in affected ecosystems (Perry et al., 2005; Pörtner, 2008; Stachowicz et al., 2002; Thomas et al., 2004).

Species inhabiting areas that are naturally exposed to high fluctuations in temperature, such as temperate coastal zones and intertidal zones, generally exhibit wider thermal tolerance windows, allowing normal metabolic activity in the specific temperature range of their natural habitat (Pörtner, 2001). Despite this wide thermal tolerance, research demonstrates that these species might be particularly vulnerable to increased temperature and pH fluctuations in future ocean scenarios, as these may surpass the upper limit of the tolerance window (Madeira et al., 2012; Somero, 2010; Spicer and Widdicombe, 2012). Estuarine communities which already experience considerable environmental stress by different factors (e.g. habitat destruction, chemical pollution) are expected to be particularly vulnerable to additional stress imposed by ocean acidification and changes in seawater temperature (Delorenzo, 2015; Kennish, 2002).

Ocean acidification and warming primarily take place in the water column and, more specifically, in the surface layers of the ocean affecting open ocean and coastal species likewise (Rhein et al., 2013). While much research has focused on water column processes and consequences for pelagic and epibenthic communities, less research has aimed to identify responses of endobenthic communities to ocean acidification and warming (Widdicombe and Spicer, 2008). Meiobenthos comprise a very diverse group of small invertebrates defined by their size (38 - 1000 μm), consisting of predators, grazers and decomposers whilst serving as food source for higher trophic levels (Giere, 2009; Moens et al., 2013; Schratzberger and Ingels, 2017). Due to their high relative abundance and ubiquity in soft-sediment communities they are considered an important component of the benthic ecosystem. Studying the effect of ocean acidification and warming on the meiobenthic community can provide insights in the response of benthic

ecosystems to future ocean scenarios since their responses to different environmental changes have proven to be good indicators of ecosystem environmental health (Costa et al., 2016; Zeppilli et al., 2015).

Infaunal organisms are naturally exposed to large fluctuations of pore water pH on temporal and spatial scales, especially in the oxic zone (Widdicombe et al., 2011; Zhu et al., 2006). As such, burrowing activity of benthic organisms and microbial activity results in large pH gradients of up to 2 units (horizontally and vertically) in marine sediments on small spatial scales (Zhu et al., 2006). In experimental studies on subtidal, benthic communities, macrobenthic species (>1000 μm) generally responded more sensitively to changes in seawater pH while dominant meiobenthic taxa (Nematoda, Copepoda) remained unaffected or even increased in abundance (Kurihara et al., 2007; Schade et al., 2016; Widdicombe et al., 2009). This tendency for a low response of dominant, meiobenthic taxa towards seawater acidification together with logistic challenges associated with multi-stressor experiments may explain the very limited number of experimental studies on meiobenthos responses towards combined effects of ocean acidification and warming (Hale et al., 2011; Ingels et al., 2017; Meadows et al., 2015; Sarmento et al., 2017a). So far, experimental studies deployed artificial substrate units on a rocky shore or in a coral reef which were used in laboratory experiments after colonization in the field. Only one recent study investigated effects of temperature and pH on intertidal meiobenthic communities from muddy and sandy sediments (Ingels et al., 2017). In contrast, reports on the effects of reduced pH as single stressor on meiobenthos are well represented (e.g. Barry et al., 2004; Carman et al., 2004; Dashfield et al., 2008; Ishida et al., 2005, 2013; Kurihara et al., 2007; Widdicombe et al., 2009). Many studies, however, focus on one hand on the possible effects of strong pH reduction resulting from liquid CO_2 sequestration in the deep-sea (e.g. Barry et al., 2004; Carman et al., 2004; Fleeger et al., 2010; Thistle et al., 2005) or possible leakage scenarios from CO_2 capture and storage (Schade et al., 2016). In these scenarios, pH reductions are of much greater magnitude compared to those in an ocean acidification context. The relatively high resistance of nematodes, one of the most commonly reported meiobenthos taxa, to pH changes (Dashfield et al., 2008; Kurihara et al., 2007; Schade et al., 2016; Widdicombe et al., 2009) may not be valid for other meiobenthic organisms that possibly respond more sensitively, e.g. copepods and their nauplii, gastrotrichs or ostracods (Meadows et al., 2015; Sarmento et al., 2015, 2017a; Schade et al., 2016). To evaluate the effect of varying abiotic stressors on the entire meiobenthic assemblage all taxa and their biotic interactions should be taken into consideration. Therefore, identifying the effects of ocean acidification in terms of community composition and consequences for biotic interactions are increasingly relevant.

In this study, the results of a microcosm experiment are presented in which we exposed a natural meiobenthos community from intertidal sandy sediments to varying levels of temperature (10 °C, ambient and 13 °C, elevated) and seawater pH (7.9, ambient and 7.5, reduced), in a fully crossed design. Before and after the experiment duration of 8 weeks sediment variables (pore water pH profiles, grain size, total organic matter content, carbon and nitrogen content, phytopigment concentration, carbonate concentration) and meiobenthos community structure as well as nematode staining ratio, as a proxy for mortality, were assessed. Mortality is a clear indication of conditions exceeding viable thresholds and represents the worst case scenario for a species. For meiobenthos, mortality assessment is, however, challenging and was usually conducted on species from lab-cultures. Several authors now acknowledge the difficulty of distinguishing living from dead organisms in *in-situ* and microcosm experiments when using conventional staining methods after fixation (Austen and McEvoy, 1997; Barry et al., 2004; Carman et al., 2004; Fleeger et al., 2006; Grego et al., 2013). The stain Trypan Blue is commonly used in cell viability assessments and cells or tissues that take up Trypan Blue, i.e. that are stained blue, are considered dead. Therefore, we adopted this staining technique that allowed us to compare nematode staining ratios between treatments in the present experiment and serve as a proxy for nematode mortality.

We hypothesized that reduced pH and elevated temperature will negatively affect the meiobenthos community and that combined stressor effects are greater than the sum of their individual effects, i.e. synergistic effects.

3.2. Material and Methods

3.2.1. Sampling

Sediments were collected on March 10th 2015 in the mid-intertidal zone of the Paulina tidal flat, which is located along the southern shore of the polyhaline zone of the Westerschelde Estuary (SW Netherlands, 51° 20' 55.4" N, 3° 43' 20.4" E). Plexiglas cores of 10 cm in diameter were used to collect intact sediments to a depth of 15 cm for the laboratory incubation (n= 24). In addition, 3.5 cm inner diameter Plexiglas cores were collected to determine the in-situ meiobenthos community (n= 6, see below). To maintain a stable temperature in both sets of cores during transport, 32 µm-sieved seawater from the sampling site was gently added on top of the sediments in each core while they were partly immersed in buckets filled with seawater. Upon arrival at the research facility, the sediment cores were incubated in a climate room at in situ temperature (10°C) and aerated. The cores were allowed to acclimatize to those laboratory conditions for one week prior to the start of the experiment.

For analysis of the sediment variables in the field, fifteen 10 mL syringes with cut-off tip were collected to determine phytopigment concentrations and another fifteen syringes (10 mL) were collected to determine total organic carbon and total nitrogen contents (TOC/TN). Additionally, fifteen 20 mL syringes with cut-off tip were collected for granulometry. The sediment in each syringe was sectioned in 0.5 cm layers down to 2 cm and from 2 to 3 cm. From each layer three replicates were pooled and homogenized resulting in 5 pooled replicate samples per variable and depth layer. Within 1.5 hours after collection, these samples were stored frozen at -80°C until subsequent analysis.

3.2.2. Experimental set-up

The 24 Plexiglas cores were randomly allocated to 8 small, rectangular aquaria (3 cores per aquarium, 42.5 cm x 15 cm x 30 cm) and were partly submerged in seawater. Every pair of aquaria was part of a recirculation system that connected a main tank (250L) with 3 big, rectangular aquaria (41 cm x 31 cm x 40 cm) via rubber tubing. The big aquaria were part of an experiment that was run in parallel with this study and were partly (i.e. 15 cm height) filled with sediments from the same sampling site and individuals of the common cockle *Cerastoderma edule*. Seawater recirculation in each system was controlled by a peristaltic pump (Watson-Marlow 520S) that pumped seawater from the main tank to the big aquaria and water originating from the big aquaria was added to the cores from above at a low, constant flow rate and exited by overflowing. Each main tank contained a stirrer and oxygen pumps to ensure seawater homogenization and oxygen saturation. A quarter of the seawater in each main tank was replaced weekly with fresh seawater in order to maintain a constant salinity of 33. All aquaria were subjected to a 12:12 hour light regime. There was one recirculation system per experimental treatment for each of the four combinations of the factors temperature (10 and 13 °C) and pH (7.9 and 7.5). The experiment was terminated after 8 weeks.

At the start of the experiment, temperature of the seawater in two of the circulation systems (for treatments with elevated temperature) was increased with 1°C per day over 3 days to achieve the target temperature of 13 °C. Temperature in all systems was manipulated by Teco chiller heaters (Teco refrigeration technologies, model: TK200H) and monitored with HOBO Pendant temperature loggers (model: UA-002-08) that were placed in each of the big aquaria and recorded seawater temperature every 10 minutes for 8 weeks.

The pH of the seawater in the main tanks that were allocated to the reduced pH treatments was lowered and maintained at -0.4 pH units. Due to logistic constraints at the start of the incubation this setting was only maintained constant

during the last 3 weeks of the experiment. ProMinent Dulcometers coupled with Hamilton glass pH electrodes (S/N: 16458 and 16451) were used to control the seawater pH offset through the bubbling of CO_2 in the main tanks. Seawater pH values were logged every 10 minutes in one aquarium per treatment using pH electrodes (Consort, model: SP10B-50) and a Consort data logger (model: C3040). All pH electrodes were calibrated weekly with Hanna instruments buffer solution (pH 4.01 and 7.01) and seawater pH, temperature and salinity in all big aquaria and main tanks were monitored weekly to ensure similar conditions over the circulation system.

3.2.3. pH profile measurements

pH profiles were measured 2 days before finalizing the experiment from 3 random cores per treatment. The pH microsensor (tip size 500 mm, Unisense) was two-point calibrated with pH buffers (Hanna Instruments) 4.01 and 7.01 and pH profiles were started at 0.5 cm above the sediment surface and measured to a depth of ~3 cm in the sediment (depth intervals of 500 μm). The pH values and depth positions of the micromanipulator were logged with the SensorTrace Profiling software (Unisense).

3.2.4. Sediment processing

At the end of the experiment (T_E) all six cores per treatment were sectioned in 0.5 cm layers down to 2 cm and from 2 to 3 cm. Each layer was homogenized by careful stirring with a spoon and subsampled with 10 and 20 mL syringes with cut-off tip for meiobenthos (13 cm^2), pigments (1.8 cm^2), TOC/TN (1.8 cm^2) and granulometry (6.6 cm^2). To obtain sufficient sediment volume for the pigment, TOC/TN and granulometric analyses, the subsamples of 2 replicate cores were pooled (final n = 3). These samples were stored frozen at -80°C until subsequent analysis.

3.2.5. Trypan Blue staining test[9]

The 6 sediment cores taken in the field were processed one day after collection. To quantify the efficiency of the staining technique, a test was conducted comparing the staining of 3 alive samples (Live) and 3 samples where the fauna was heat killed

[9] Trypan Blue is a stain that is commonly used for viability assessment in tissues or cells. Due to its chemical structure, the stain does not pass intact/living cell membranes but enters the cells when they are dead, therefore, stained tissues are an indication of mortality. Thomas and Lana (2008) were the first to test different stains, including trypan blue, on nematodes, however, this stain did not fulfil the authors purposes (keeping the stained animals alive for a long time, >180 min). The concentrations and protocol used in this study are derived from standard cell counting protocols (typical concentrations vary between 0.04 % and 4 %, cfr. Perry et al., 1997) and own, preliminary tests on nematodes.

by exposure to hot tap water (~80°C) in a waterbath for about 15 minutes prior to the application of the Trypan Blue (TB) stain (Dead).[10] All cores were sectioned in 0.5 cm layers down to 2 cm and from 2 to 3 cm and a 0.4 % Trypan Blue staining solution (prepared with 32 μm filtered, natural seawater) was added in a 1:1 (TB:sediment volume) ratio to each sediment layer. Subsequently, all samples were exposed to the TB solution for 2 hours in the dark. Afterwards, the sediments were washed on a 32 μm mesh sieve, stored in a buffered formaldehyde solution with a final concentration of 4 % and analyzed as described in the following section.

3.2.6. Meiobenthos analysis

Meiobenthos sediment samples from 3 cores per treatment were extracted with density gradient centrifugation using colloidal silica Ludox HS40 Dupont (specific gravity: 1.18, Heip et al. (1985)) after sieving over two stacked sieves of 32 μm (lower limit) and 1 mm (upper limit) mesh size. Centrifugation (3000 rpm, 12 min) was done three times and after each round, the supernatant was sieved (32 μm) to retain the meiobenthos. Finally, meiobenthos was stained with trypan blue as explained above, fixed on 4% buffered formaldehyde and subsequently analyzed under stereomicroscopes. Metazoan meiobenthos (hereafter referred to as meiobenthos) was identified to higher taxonomic level consulting e.g. Higgins and Thiel (1988) and counted. Furthermore, nematodes were grouped as stained (blue color) and not stained (transparent).

3.2.7. Laboratory analyses

Sediment granulometry was measured by laser diffraction with a Malvern Mastersize 2000 particle analyzer (Malvern Instruments, UK). The 0.06 - 1000 mm sediment fractions were classified according to Wentworth (1922) in volume percentage (vol%). Carbonate ($CaCO_3$) concentration in the first centimeter of sediment (2 depth layers) was analyzed by means of coulometry with a CM140 coulometer (UIC Inc., USA) after homogenization of the sediment. Pigment concentrations (Chlorophyl *a*, Pheophytin, Pyropheophytin) were determined by HPLC (Agilent) analysis according to Wright and Jeffrey (1997). For total organic C and N content samples were lyophilized, homogenized and acidified with 1% HCl before analysis with an Element Analyser Flash 2000 (Thermo Fisher Scientific). Total organic matter (TOM) was determined as the mass loss observed upon combustion of the dried sample (48h at 60°C) at 500°C for 2h.

[10] The time between heat killing and staining was approximately 15 min.

3.2.8. Data analysis

The pH values measured on the NBS scale (pH_{NBS}) were converted to total scale (pH_T) via conversion described in Zeebe and Wolf-Gladrow (2001) using the Matlab program CO2SYS (van Heuven, et al., 2011) and taking into account the measured seawater temperature and salinity. The conversion between pH_{NBS} and pH_T equals $pH_T = pH_{NBS}$-C with C=0.1083 at 10 °C and C=0.1142 at 13 °C at a salinity of 33.

The comparison of pH profiles was done based on a data matrix where each depth horizon was considered to be a "variable" and each pH measurement as a measure of "abundance" (Widdicombe et al., 2013). The PERMANOVA+ add-on package for the PRIMER 6 software (Anderson et al., 2008; Clarke and Gorley, 2006) was used to perform a 2-way test for main effects (temperature and pH) and interactions on the Euclidean distance similarity matrix that was constructed on untransformed data. The SIMPER analysis was used to determine in which depth layers any identified differences occurred. To balance the dataset, only data from 0 - 24.5 mm depth layers could be included due to missing data beyond that depth in several samples.

Meiobenthos densities were standardized to the number of individuals 10 cm^{-2} and were square root transformed to down-weight the importance of dominant groups without losing too much information. The Shannon index, Simpson index and Pielou's eveness index were calculated as measure of meiobenthos diversity in the cores. A two-way analysis of variance (ANOVA) was carried out on univariate data (total meiobenthos densities, diversity indices, nematode staining ratios, nematode densities, flatworm densities, whole core median grain size, chlorophyll *a* concentration, pheophytin concentration, pyropheophytin concentration, TOM, TN) when assumptions of normality and homogeneity of variances were met. Tukey HSD pairwise tests were performed when significant differences between factors were revealed by the ANOVA. Permutational ANOVA (Permanova) based on a Euclidean distance resemblance matrix was used as a non-parametric alternative to a 2-way ANOVA when data was not normally distributed (TOC, Copepoda densities, Gastrotricha densities) as explained by Bakker et al. (2012).

Multivariate data was analysed with Permanova using sum of squares type III, permutations of residuals under the reduced model and 9999 permutations. For comparisons between field situation and experimental control unrestricted permutation of raw data was chosen as only one factor (Time) was tested. A Bray-Curtis resemblance matrix was used for meiobenthos data while the resemblance matrix for environmental data was based on Euclidean distances. When testing for differences between depth layers, next to the factor depth an extra factor

(Replicate number) was added and nested in the factors pH and temperature when experimental treatments were compared and in the factor Time in case of comparison between field samples and the experimental control. If the main test yielded significant results, a pairwise Permanova test was carried out. If the number of possible permutations in the pairwise tests was lower than 100, Monte Carlo tests were applied to estimate p-values (p(MC)) with increased accuracy. Homogeneity of multivariate dispersion was ensured with a PermDisp test for any of the tested factors and reported if significant. A significant result could mean that observed differences can be the result of both, the effects of the tested factor or data dispersion effects.

In order to test correlations between the set of environmental data and the variability of meiobenthos community composition a distance based linear model (DistLM) was built by stepwise selection of variables and an adjusted R^2 selection criterion. Multi-collinearity between environmental variables was assessed based on Draftsman Plots and redundant terms (with correlation $|r| \geq 0.8$) were dropped which was not the case for any of the tested variables. The results were visualized with distance-based redundancy analysis (dbRDA) plots.

3.3. Results

3.3.1. Temperature and pH monitoring and sediment pH profiles

After some fluctuations in the first week, temperature remained relatively stable at the set treatment temperatures of 10 °C and 13 °C throughout the experiment (Fig. 3.1). During the first half of the experiment, average daily pH_T of all treatments varied between 7.54 and 7.98 with a mean pH_T of 7.74 ± 0.09 due to problems in the experimental setup (mean ± standard deviation (SD)). After 34 days pH_T stabilized around the set treatment values of 7.89 ± 0.05 and 7.50 ± 0.05 (mean ± SD) for the pH_T 7.9 treatments and reduced pH treatments, respectively (Fig. 3.1). Seawater carbonate chemistry in this period is presented in Tab. S3.1 (supplementary material). The 10-year (2005 - 2015) average pH_{NBS} (NBS scale, see Provoost et al., 2010) at the Westerschelde estuary (Terneuzen boei 20) is 8.02 ± 0.15 (mean ± SD) with a maximum of 8.64 and a minimum of 7.87 (data by the Dutch water management agency Rijkswaterstaat (RWS) obtained from the publicly accessible website http://live.waterbase.nl). Average pH_T variation in the first half of the experiment fell in the range of natural variation measured in the estuary while treatments with reduced pH_T were situated outside of this range from day 34 onwards.

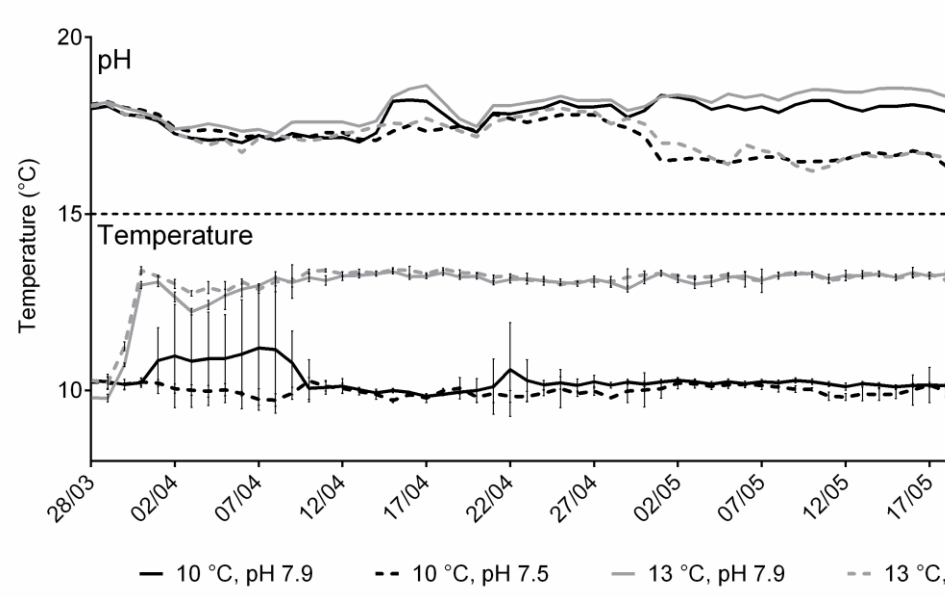

Figure 3.1 Daily average seawater temperature (± standard deviation) and pH_T measured throughout the experiment duration. The dashed line is a visual help to separate the plotted variables.

Seawater temperature (Pseudo-F= 4.4206, $p_{(perm)}$= 0.0178) and pH (Pseudo-F= 30.436, $p_{(perm)}$= 0.0001, PermDisp= 0.0001) both affected the shape of the sediment pH profiles at the end of the experiment (Fig. 3.2). SIMPER indicated a 34.32 % contribution of the upper 6 mm layers to the observed differences between treatments of pH_T 7.5 and pH_T 7.9. In contrast, differences between temperature treatments were predominantly caused by the 1.5-4.5 mm layers (16,71 %) and the deeper layers between 19.0 and 24.0 mm (27.61 %) caused by a pH decrease in the deeper layers at 13 °C compared to 10 °C (Fig. 3.2).

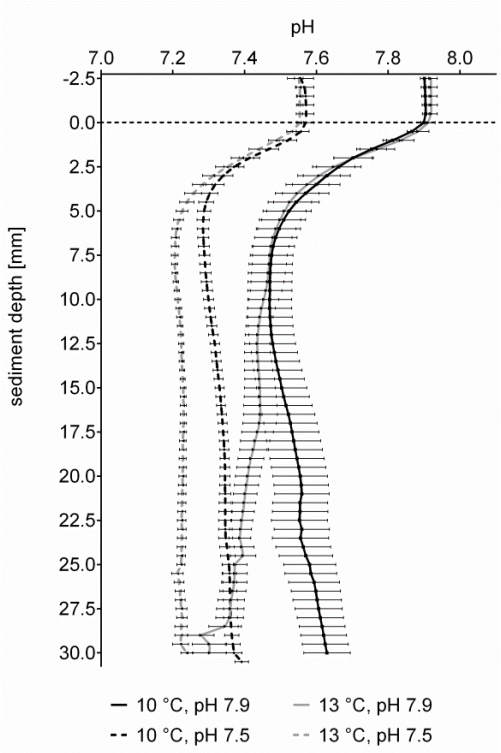

Figure 3.2 Sediment pH_T profiles (mean ± SE) of all treatments at T_E.

3.3.2. Meiobenthos analysis

Out of the 10 meiobenthos groups found in all samples, Nematoda was the most abundant group with 90.74 ± 1.26 % (mean ± SE) followed by Copepoda (5.08 ± 1.10 %), Platyhelminthes (2.22 ± 0.68 %) and Gastrotricha (1.13 ± 0.33 %). All other groups (Polychaeta, Oligochaeta, Amphipoda, Ostracoda, Nauplii, Cumacea) contributed less than 0.3 % to the community composition.

3.3.3. Meiobenthos community composition Field vs. Control (T_E)

Total meiobenthos densities were 901.59 ± 17.15 ind. 10 cm^{-2} (mean ± SE) in the field samples (Live samples only) and 944.46 ± 172.16 ind. 10 cm^{-2} in the experimental Control (T_E). Total meiobenthos density, whole core meiobenthos community composition and diversity indices did not differ between the field and the experimental Control at T_E. Similarly, when taking depth layers into account, no difference was found in community composition between the field samples and Control (T_E) but meiobenthos community differed between depth layers. Meiobenthos communities in the first 0.5 cm differed from all other depth layers >1 cm (Pairwise test (Depth), $p_{(perm)}$<0.0291) and the 0.5-1 cm layer differed from all layers >1.5 cm (Pairwise test (Depth), $p_{(perm)}$<0.0499, borderline significant).

3.3.3.1. Effects of pH and Temperature on meiobenthos density and community composition

Total meiobenthos densities ranged from 757.74 ± 87.05 ind. 10 cm^{-2} (mean ± SE) in the treatment with elevated temperature and reduced pH to 1291.41 ± 84.08 ind. 10 cm^{-2} in the treatment with reduced pH, only. Total densities were lower in treatments with elevated temperature compared to ambient temperature ($F_{(1)}$=8.6233, p=0.0188).

The interaction of pH and temperature significantly altered meiobenthos community composition (Pseudo-F=4.8628, $p_{(perm)}$=0.0171). When pH was reduced, communities differed between ambient and elevated temperature treatments (Pairwise test (Temperature x pH), t=3.3922, $p_{(MC)}$=0.0103) this difference was predominantly caused by a decrease in Nematoda, Copepoda and Nauplii (SIMPER: 42.32 %, 15.35 % and 13.05 %, respectively) and an increase in Platyhelminthes (SIMPER: 11.92 %) at 13 °C compared to 10 °C (Fig. 3.3). When temperature was elevated, community composition differed between pH 7.5 and pH 7.9 (Pairwise test (Temperature x pH), t=2.955, $p_{(MC)}$=0.0133) which was caused by a decrease in Copepoda and Gastrotricha (SIMPER: 29.05 % and 16.31

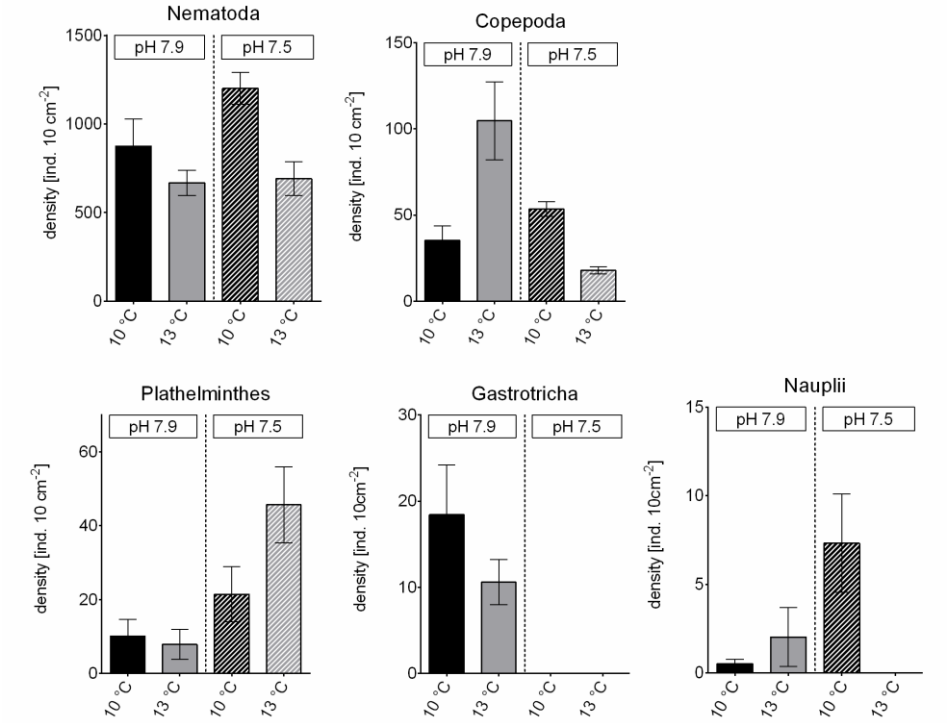

Figure 3.3 Densities (mean ± SE) of meiobenthos groups responsible for observed community changes in the different treatments of ambient / reduced pH (7.9 / 7.5) and ambient / elevated temperature (10/13 °C).

%, respectively) and an increase in Platyhelminthes (SIMPER: 20.63 %) at pH 7.5 compared to pH 7.9 (Fig. 3.3). Furthermore, Shannon diversity and Pielou's evenness index were significantly higher at 13 °C (0.48 ± 0.06 and 0.27 ± 0.02, respectively) compared to 10 °C (0.34 ± 0.03 and 0.18 ± 0.01, respectively) (Shannon diversity: $F_{(1)}$=11.9211, p=0.0087, Pielou's evenness index: $F_{(1)}$=6.5722, p=0.0335) but did not differ between pH levels or an interaction of pH and temperature.

The four dominant groups showed very different responses to pH and temperature treatments (Fig. 3.3). A change in pH had a very clear effect on Platyhelminthes with increased densities in the reduced pH treatments ($F_{(1)}$=12.1481, p=0.0082). In contrast, Gastrotricha completely disappeared from the samples when pH was reduced (Pseudo-F=70.701, $p_{(perm)}$=0.0021). At pH 7.9 copepod densities increased at 13 °C (Pairwise test (Temperature x pH), t=3.0192, p_{MC}=0.0379, PermDisp$_{(Temperature)}$=0.0123) but the opposite was true at pH 7.5 where densities decreased at elevated temperature (Pairwise test (Temperature x pH), t=8.0532, p_{MC}=0.001). At elevated temperature copepod densities were lower

at pH 7.5 compared to pH 7.9 (Pairwise test (Temperature x pH), t=4.8708, p_{MC}=0.0061). Responses of nematodes are presented in section 3.3.

When taking depth layers into account, PERMANOVA indicates a significant interaction term of the factors pH, Temperature and Depth. Meiobenthos community composition reveals a pronounced vertical zonation only in the treatment with reduced pH and elevated temperature where the 0-0.5 and the 0.5-1 cm layers differ from the deeper layers (Tab. S3.2, supplementary material). In this treatment densities of Copepoda, Platyhelminthes and Ostracoda drop considerably from the surface to the 1 cm layer and further decrease in the deeper layers.

3.3.4. Nematode analysis

3.3.4.1. Trypan Blue staining test

Total nematode density was similar in both treatments (dead, alive) but the proportion of stained nematodes over all depth layers was significantly higher in the dead treatment compared to the alive treatments (Pairwise test (Treatment x Time), p_{adj}<0.0001). In alive samples, 13.41 ± 1.76 % (mean ± SE) of the nematodes were stained, while in dead samples 54.59 ± 3.89 % of the nematodes were stained. PERMANOVA analysis including depth layers revealed a significant treatment (dead vs. alive) effect (Pseudo-F= 52.062, p_{Perm}= 0.0016) and, thereby, confirmed the significant effect on the relative abundance of stained versus unstained nematodes.

3.3.4.2. Nematode staining ratios Field vs. Control (T_E)

Whole core nematode density and staining ratios, as indication of mortality, did not differ between field samples (staining ratio= 12.58 ± 2.17 %, alive only) and the experimental Control at T_E (staining ratio= 14.25 ± 3.17 %). Similarly, no differences were found between depth layers.

3.3.4.3. Effects of pH and Temperature on nematode density and staining ratio

Whole core nematode densities were lower in treatments with elevated temperature, regardless of pH ($F_{(1)}$=11.317, p=0.0099, Fig. 3.3). Similarly, when taking depth layers into account, nematode densities differed based on temperature but also with depth in the sediment (Pseudo-F=5.358, p_{Perm}=0.0476 and Pseudo-F=6.8120, p_{Perm}=0.0001, respectively) (Fig. 3.4). Nematode densities in the upper 1 cm were significantly higher compared to the 1-1.5 cm and 1.5-2 cm layer, but not compared to the deepest layer (2-3 cm) (Tab. S3.3, supplementary material).

Nematode staining ratio was significantly higher when pH was reduced (Pseudo-F=20.265, p_{Perm}=0.0005). When taking depth layers into account, a significant interaction between pH and depth layers was found (Pseudo-F=2.3405, p_{Perm}= 0.0194) (Fig. 3.4). Nematode staining ratio at pH 7.5 was higher in all depth layers except for the deepest layer when compared to pH 7.9 (Pairwise test (pH x Depth), p_{Perm}<0.0407).

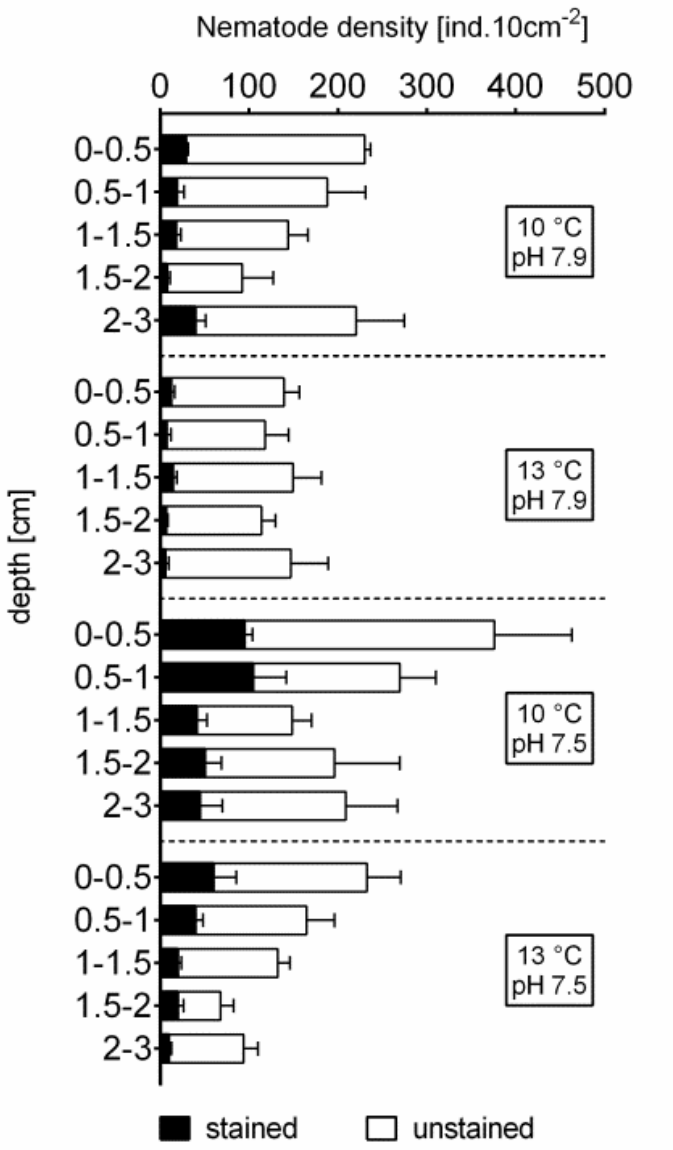

Figure 3.4 Cumulative densities of stained (black bars) and unstained (white bars) nematodes per depth layer in the different temperature and acidification treatments.

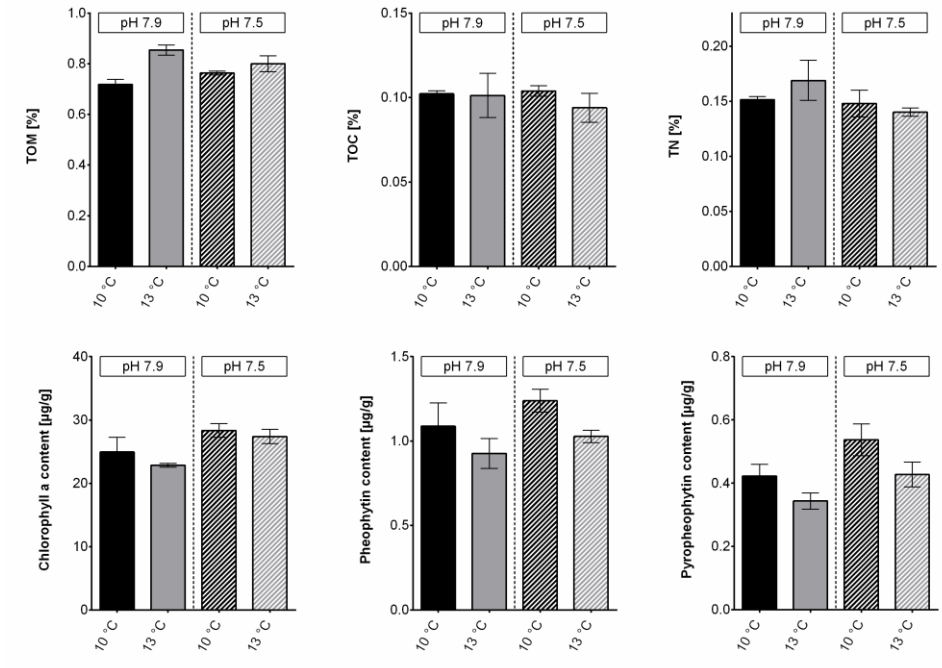

Figure 3.5 Mean (± standard error) values of the different sediment characteristics measured at the end (T_E) of the experiment.

3.3.5. Sediment characteristics

Chlorophyll *a* concentration and total organic carbon (TOC) decreased in the experimental Control (T_E) compared to the field situation ($t_{(2.18)}$=-14.22, p=0.0034 and Pseudo-F=38.719, p_{MC}=0.0041) while pyropheophytin concentration increased ($t_{(2.18)}$=4.34, p=0.042). Pheophytin concentration, total organic matter (TOM) content and total nitrogen (TN) content did not differ among the field situation and the experimental Control (T_E). A decrease in seawater pH led to an increase in chlorophyll *a* and pyropheophytin concentration ($F_{(1)}$=7.99, p=0.0223 and $F_{(1)}$=6.44, p=0.0348) while an increased temperature resulted in a reduced pyropheophytin concentration ($F_{(1)}$=5.80, p=0.0427) and increased TOM content ($F_{(1)}$=15.5962, p=0.0042) in the upper 3 cm of the sediment (Fig. 3.5). TOC and TN contents remained similar in all treatments (Fig. 3.5).

Carbonate concentration varied between 1.485 % and 2.546 % in the uppermost centimetre and did not differ between the field situation and experimental control (T_E). At T_E carbonate concentration in the first half centimetre was lower compared to the 0.5-1 cm layer (Pseudo-F=8.2215, p_{Perm}=0.0202) but did not differ between treatments (Fig. 3.6).

The sediment in the upper 3 cm of all cores was predominantly composed of fine and medium sand (56.74 ± 1.68 % and 39.24 ± 1.89 %, mean ± SD) with small fractions of very fine sand (3.72 ± 0.62 %), coarse sand (0.26 ± 0.156 %), silt (0.03 ± 0.09 %) and clay (0.01 ± 0.02 %). Whole core median grain size and grain size composition did not vary between the field situation and experimental Control (T_E). Surprisingly, whole core median grain size differed between treatments at T_E with an interaction of the factors Temperature and pH ($F_{(1)}$=17.9425, p=0.0029, Fig. 3.6). A higher median grain size was observed in treatments at 13°C compared to 10°C when pH was reduced (Pairwise test (Temperature x pH), p_{MC} =0.0352) and median grain size was also higher at pH 7.5 compared to pH 7.9 in treatments at 13 °C (Pairwise test (Temperature x pH), p_{MC} =0.0074). The same differences were reflected in the grain size composition with differing grain size composition between 10 °C and 13 °C when pH was reduced (Pairwise test (Temperature x pH), p_{MC} =0.0135) and between pH 7.9 and 7.5 when temperature was elevated (Pairwise test (Temperature x pH), p_{MC} =0.0137).

When taking depth layers into account there was a significant interaction between the factors pH, Temperature and Sediment depth. The 0-0.5 cm layer differed

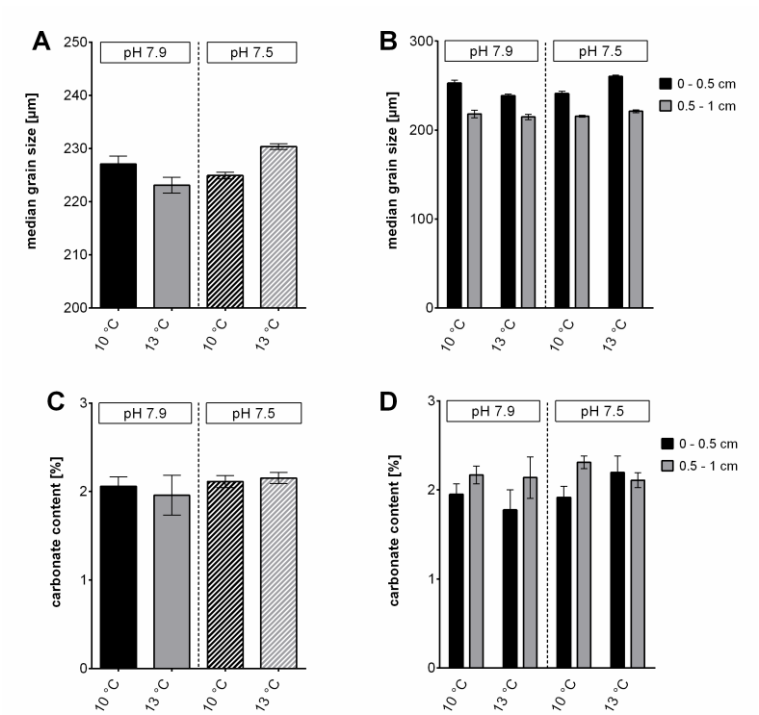

Figure 3.6 A) Average median grain size (± standard error) at T_E of the 0-3 cm layer; B) median grain size (± standard error) at T_E of the 0-0.5 cm layer; C) average carbonate concentration (± standard error) of the first centimetre of sediment and D) carbonate concentration (± standard error) per depth layer.

from all other layers in all treatments and median grain size in the uppermost sediment layer was higher than the whole core average but reflected the same pattern (Tab. S3.4, supplementary material, Fig. 3.6). Grain size was lower in treatments at 13 °C compared to 10 °C at pH 7.9 (Pairwise test(Temperature x pH x Depth), p_{MC}=0.0177) and showed the opposite trend at pH 7.5 (Pairwise test(Temperature x pH x Depth), p_{MC}=0.0019). Differences between pH were only apparent at elevated temperature (Pairwise test(Temperature x pH x Depth), p_{MC}=0.0008). The PermDisp test for the factor Depth was significant (PermDisp, p=0.0001).

When correlating variability of meiobenthos community composition (based on densities) with the environmental data, five of the nine environmental variables (pH, Temperature, median grain size, TOM, TOC, TN, Pheophytin, Pyropheophytin and Chlorophyll *a*) explained 82.74% of the total variation. Those 5 variables were pH (31.05%), Median grain size (19.46%), Temperature (13.98%),

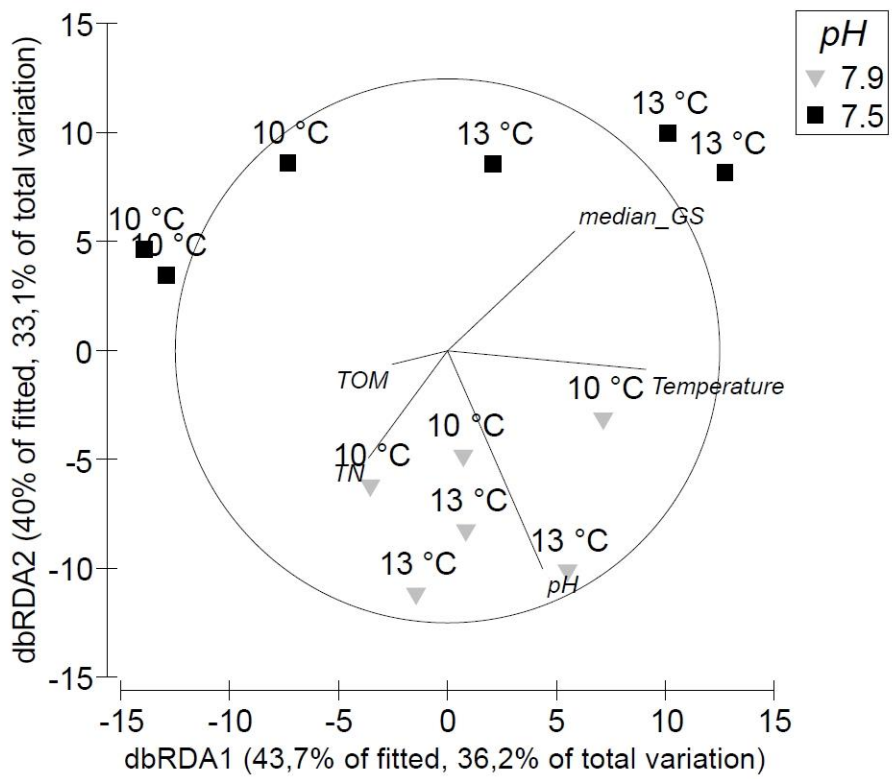

Figure 3.7 Distance-based redundancy analysis (dbRDA) visualizing the DistLM model of the correlation between environmental variables and meiobenthos community composition based on densities in multivariate space (2D). Lines indicate the strength and direction of vectors of the fitted environmental variables.

TOM (13.76%) and TN (4.49 %). Of the total variation 69.31% can be explained by the first two axes in multivariate space (dbRDA1 and dbRDA2, Fig. 3.7). When considered alone, only pH (Pseudo-F= 4.5037, p=0.007), Pyropheophytin (Pseudo-F= 3.333, p=0.019) and median grain size (Pseudo-F= 3.1002, p=0.0277) had a significant correlation with the meiobenthos multivariate data cloud.

3.4. Discussion

The fluctuating seawater pH in the first half of the experiment is an unfortunate limitation of our experiment and requires a careful interpretation of the findings. Nevertheless, our results show that ocean acidification (ΔpH: -0.4 units) and warming (ΔT: +3 °C) can have a significant effect on the meiobenthos community in sandy, intertidal sediments. Temperature and pH occur and change in combination, and together with the use of predicted near-future scenarios, this multi-stressor approach increases the ecological relevance of this research (Caldeira and Wickett, 2003; Hofmann et al., 2011). Coastal environments already experience high pCO_2 levels sometimes exceeding predicted values for the end of this century (Hofmann et al., 2011), therefore, environmental conditions simulated in this study may occur on an even shorter time scale. While daily fluctuations in seawater pH may be relatively low (Wootton et al., 2008), the pH inside intertidal, muddy sediments may show stronger fluctuations (Jansen et al., 2009) which potentially affect the sensitivity of infaunal organisms. Unfortunately, there is no information available on daily or seasonal pH variations in intertidal, sandy sediments that are comparable to our study site. Therefore it is difficult to estimate the difference in pH exposure the meiofauna underwent in this experiment and whether meiofauna tolerates or adapts to the induced pH shift. Though pH and temperature may vary considerably on temporal and local scales in marine sediments (Zhu et al., 2006), our results suggest that pH and temperature changes exerted substantial pressure on the meiobenthic communities.

The generation time of meiofauna is variable, but for most taxa it lies in the range of weeks to months (Coull, 1999; Gerlach, 1971), therefore the experimental duration of 8 weeks sufficed to allow the development of multiple generations increasing the relevance of this experiment with regard to the temporal scale of OAW impacts. We observed a shift in meiobenthos community composition based on differential responses of several meiobenthos groups such as Platyhelminthes, Copepoda and Gastrotricha. Interestingly, pH and temperature explained a considerable amount of variation in the meiobenthos community composition.

Divergent responses of meiofaunal groups to cumulative effects of reduced pH and elevated temperature have also been reported by Sarmento et al. (2017a). Similar to our study, the authors reported declining densities of harpacticoid copepods and nauplii in two scenarios incorporating pH reductions of 0.3 and 0.7 and temperature increases of 2 and 3 °C, respectively, while densities of nematodes increased in those scenarios compared to the control (Sarmento et al., 2017a). Furthermore, Meadows et al. (2015) reported a shift in meiobenthos community composition in artificial substrate units colonized by intertidal communities as a response to increased temperature (12 versus 16°C) and reduced seawater pH (nominal pH of 8.0 (ambient) vs. 7.7, 7.3 and 6.7). Copepod densities declined at reduced pH and increased temperature while nematodes density increased in response to the lowest pH (6.7). As part of a bigger microcosm experiment the decline of macrofauna densities inside the experimental units was thought to stimulate nematode population growth by reducing competition and predation pressure (Hale et al., 2011; Meadows et al., 2015). In contrast to our study, a pH reduction by 0.3 units (8.0 vs 7.7) did not evoke strong responses in most meiobenthos groups present in the artificial substrates regardless of the temperature. Similarly, Ingels et al. (2017) reported only slight decreases in meiobenthos densities of most taxa in muddy sediments, as a result of elevated pCO_2. Analysis of the microbiota revealed possible indirect interaction effects with the meiofauna as microbial density showed the same decreasing trend as the meiobenthos which was not the case for the sandy community (Ingels et al., 2017).

Inside the sediment, a pH minimum was reached at a sediment depth of 5 mm. These subsurface minima are common in many sediment types and predominantly caused by DIC production from the aerobic mineralization of organic matter (Brenner et al., 2016; Jourabchi et al., 2005; Silburn et al., 2017) and therefore often correspond with the lower boundary of the oxic and suboxic zone (Brenner et al., 2016; Preisler et al., 2007). Below this depth, the reduction of iron and manganese oxyhydroxides generally result in an increase of pH in the suboxic zone and in even deeper layers, sulfate reduction and alkalinity production determine the sediment pH (Jourabchi et al., 2005). The discrepancy in the pH profiles below 19 mm depth between the elevated and ambient temperature treatments in our experiment may be explained by an increased rate of anaerobic organic matter degradation through microbial sulphate reduction at elevated temperatures, which results in a net production of protons depending on the local pH (Jourabchi et al., 2005), resulting in a more acidified pore water. Therefore, a temperature increase of 3 degree has led to even more acidic conditions in the deeper layers in treatments where pH was already decreased. In other words, the combination of both factors imposes more extreme conditions on sediment dwelling organisms.

This may explain the different vertical distribution of meiobenthos groups in the treatment of elevated temperature and reduced pH where most organisms stayed in the uppermost centimetre of the sediment avoiding those unfavourable pore water conditions.

Sediment grain size, oxygen penetration depth and organic matter availability are important factors governing the distribution of meiobenthos taxa in the surface layers of the sediment (Coull, 1999; Higgins and Thiel, 1988; Jansson, 1967; Moodley et al., 2000; Steyaert et al., 2003). Interestingly, differences in median sediment grain size were found between the different treatments in our experiment while carbonate concentrations remained comparable. Therefore, this change in median grain size is unlikely to be caused by the dissolution of sedimentary carbonates which may result from ocean acidification (Andersson et al., 2007). Furthermore, correlations between median grain size and other abiotic variables were absent, rendering e.g. flocculation effects due to high organic matter content unlikely. Although significant differences have been found we would like to stress that median grain size only differed by $< 10\ \mu m$ between treatments which is relatively small compared to the median grain size of ~225 μm. The change in pH together with the differences in TOM and sediment grain size were found to be the main abiotic drivers of the community change observed in the sandy intertidal sediments in this experiment (Fig. 3.7).

Moderately increased seawater temperature and reduced pH have a positive effect on primary producer productivity under favourable nutrient and light conditions (Doney et al., 2009; Gao et al., 2012), and CO_2 enrichment has been shown to increase microphytobenthos abundance and chlorophyll *a* concentration in natural CO_2 seeps (Johnson et al., 2013). Chlorophyll *a* concentration is a reliable proxy for photosynthetic standing stock in marine sediments (Underwood, 1984) and showed a significant increase in treatments with reduced pH in our study which corroborates with above mentioned findings. However, chlorophyll *a* concentration did not seem to exert a strong influence on the community structure of the meiobenthos.

Interestingly, the experimental set-up and incubation in the laboratory did not have a large impact on whole core meiobenthos. Similarly, sediment characteristics remained comparable with the exception that primary production (chlorophyll *a* concentration) and organic matter content were reduced at the end of the experiment, possibly caused by a lower nutrient input from the water column and shorter daylight periods and light intensities compared to field situation. Furthermore, the absence of tidal variation may have led to the stronger vertical distribution pattern of meiobenthos at the end of the experimental period with

more animals present in the upper sediment layers. Previous studies indeed reported a significant effect of tidal cycles on the vertical position of meiobenthos in the sediment (J. P. Mclachlan, 1977; Steyaert et al., 2001; Walters and Bell, 1986). Walters and Bell (1986) found that a significant amount of harpacticoid copepods emerged from the sediment into the water column when tides were high and, similarly, Steyaert et al. (2001) reported nematode species specific responses with some species migrating to higher parts in the sediment at high tide and others showing opposite trends.

3.4.1. Meiobenthos group specific responses

Our experiment revealed that responses of particular meiobenthos groups to elevated temperature and reduced pH may differ considerably and induce changes in community composition.

Densities of nematodes, the most abundant group in our study, remained largely unaffected by reduced pH but declined at elevated temperature. This result is in contrast with what has been found in laboratory studies on the life-history traits of marine nematodes under elevated temperatures. Generally, an increase of temperature within the thermal window of the species is related with shorter life cycles, increased population growth, faster development time, higher egg production and increased food assimilation (Moens and Vincx, 2000; Tietjen and Lee, 1972; Van Campenhout et al., 2014; Warwick, 1981). These species-specific responses may have been revealed if the nematode community composition was analysed to a lower taxonomic level, which should be considered in future research. However, also other abiotic and biotic factors such as changes in biotic interactions or other indirect environmental changes resulting from increased seawater temperatures may have affected nematode densities in high temperature treatments.

Although densities remained unaffected by the reduction in seawater pH, nematode mortality, evidenced by higher stain ratios, was increased at reduced pH and ambient temperature and also, to a lesser extent, at elevated temperature. Most ocean acidification studies on meiobenthos from shallow water environments suggest a high tolerance of nematodes to changes in seawater pH as densities remained unaffected or even increased at low pH (Dashfield et al., 2008; Kurihara et al., 2007; Meadows et al., 2015; Schade et al., 2016; Widdicombe et al., 2009). Impacts on nematode survival only became apparent when seawater pH was considerably reduced (pH<7.0) (Takeuchi et al., 1997). Similar results have been found in deep-sea studies when assessing the impact of deep-sea CO_2 sequestration on meiobenthos (Barry et al., 2004; Carman et al., 2004; Fleeger et al., 2006, 2010). High nematode mortality has been reported inside corrals filled

with liquid CO_2 (pH at sediment surface: 5.4) while nematode densities near corrals which experienced less acidic pH conditions (reduction of approx. -0.75 units) remained unaffected (Fleeger et al., 2006). However, supported by biometric evidence, Fleeger et al. (2010, 2006) argue that a large proportion of nematodes in the near-corral samples had died prior to sampling but, due to slow decomposition rates, remained intact suggesting that even "moderate" pH changes (pH 7.0 for 30 days) may induce high mortality rates. In a similar experiment, Barry et al. (2004) used a DNA stain to assess mortality and found that 63% of the nematodes were dead near CO_2 corrals (experiencing pH perturbations of up to -1.6 units for 30 days) compared to only 16% near control corrals. At the same time, the authors measured reduced biovolumes of flagellates and amoebae, which are important decomposers of nematodes (Tietjen, 1967), suggesting that periodic seawater pH changes may hamper the efficiency of the decomposer community.

Although our study was done under very different conditions, the increased proportions of dead nematodes in reduced pH treatments may be explained by reduced decomposition rates due to direct or indirect (decomposing community) effects of pH. The lower nematode densities in the elevated temperature treatment and the lower percentage of dead nematodes at elevated temperature and reduced pH may be explained by a higher activity of decomposing microbiota at elevated temperatures. Our findings underline the importance of mortality assessments in relatively short-term experiments and acidification studies. Stable or even elevated densities of nematodes may point to reduced nematode decomposition, possibly masking more severe impacts of ocean acidification. The degree to which decomposition is affected possibly depends on the resilience of the decomposing community to changes in the environment. While decomposition was slow in previously mentioned studies, deep-sea nematode densities in a low pH (pCO_2: 20 000 ppm) experiment by Ishida et al. (2005) remained unaffected during a short period of three days but decreased significantly after two weeks indicating that mortality and decomposition of nematodes occurred relatively fast.

Although our staining technique was successful in revealing trends in mortality across the different treatments, it was not fully efficient in staining the dead sample since only 55% of the killed nematodes were stained. We noticed that certain nematode genera such as *Metachromadora* usually remain unstained, possibly due to their very thick cuticle or other morphological features. Therefore, we acknowledge that higher proportions of stained nematodes may also arise from a shift in nematode community composition.

Copepods responded sensitively to the induced changes in seawater pH and temperature. While under a pH of 7.9 copepod densities increased at higher

temperatures, the opposite trend was observed at reduced pH suggesting a combined effect of temperature and pH on copepod densities. In an experiment conducted by Sarmento et al. (2017b) the authors could show that responses of harpacticoid copepods to changes in seawater pH (ΔpH=-0.3 to -1.3) and temperature (Δ°C=+4 C) were species-specific with some species benefitting from the changing conditions. This indicates that density shifts may also be accompanied by considerable changes in copepod community composition. A more detailed analysis of the copepod community in our study could therefore have helped to unravel these changes in community structure in response to temperature and pH changes. Differences between responses of adult and pre-adult copepod and nauplii may be explained by a higher sensitivity of nauplius stages or by altered reproductive output of adult individuals. Single-species experiments on copepods (Harpacticoida as well as Calanoida) reported developmental deficiencies resulting from moderate seawater pH changes with reduced egg production, hatching rate and nauplius growth rate but also reported increased nauplius mortality (Fitzer et al., 2012; Kurihara et al., 2004; Kurihara and Ishimatsu, 2008).

The importance of Platyhelminthes, which are among the larger meiobenthos organisms, and their role as predators in meiobenthic communities has been shown in several studies (Boaden, 1995; Maghsoud et al., 2014; Martens and Schockaert, 1986). While few species feed on diatoms, most others are predatory animals feeding on polychaetes, amphipods and other meiobenthos such as nematodes, copepods, gastrotrichs or ostracods (Boaden, 2001; Jennings, 1957; Maghsoud et al., 2014; Martens and Schockaert, 1986; Menn and Armonies, 1999; Watzin, 1985) and may put considerable predation pressure on their prey species (Janiak et al., 2017). It is worth noting that, in our study, density changes of gastrotrichs and copepods show a direct opposing trend from flatworm densities. We hypothesize that this group, which increased in density at elevated temperature and reduced pH in our experiment, exerted an increased predation pressure on other meiobenthic organisms explaining the reduction in copepod density and disappearance of Gastrotricha. In addition, the nematode community in sandy sediments at the Paulina intertidal flat is typically composed of predatory nematodes at the time of sampling (Gallucci et al., 2005). The predacious activity of nematodes is dependent on prey density, light regime and temperature (Moens et al. 2000). As the most dominant component in the meiofauna community in our experiment, differing responses of predatory nematodes to the varying pH and temperature conditions may also have played a major role in the structuring of the meiobenthos community of the different treatments.

Most ocean acidification and warming research focusses on responses of single species in isolations and species-interactions are largely neglected while they may play a significant role in determining the resilience of benthic communities to environmental change (Harvey et al., 2013). Indirect effects by changes in species interactions may be a stronger driver of community shifts than direct effects of environmental stressors on individual level (Kordas et al., 2011) and community-based research including multiple stressors has been recommended to complement research on individual species responses to abiotic stressors (Kroeker et al., 2017). The physiological response of marine organisms to hypercapnia and temperature stress is manifold (Pörtner, 2008) and evidence exists that, among others, predator-prey interactions can be significantly altered under future climate scenarios (Allan et al., 2013; Watson et al., 2017).

As discussed above, meiobenthos represents a multitrophic group composed of grazers, detritivores and predators with a tight network of biotic interactions within this benthic size class but also with other benthic compartments (Maghsoud et al., 2014; Schmid-Araya et al., 2002). Meiobenthic organisms generally exhibit a high activity, short life spans and high turnover rates and, therefore, significantly contribute to organic matter remineralization and biomass production in soft-bottom sediments (Coull, 1999; Evrard et al., 2010; Gerlach, 1971; Moens et al., 2013). Meiobenthic organisms compete for food with macrofauna (Alongi and Tenore, 1985) while their presence can also enhance bacterial productivity (De Mesel et al., 2006; Gerlach, 1978) and influence macrobenthic species interactions (Piot et al., 2013; Watzin, 1983). Vice versa, the presence of particular macro- or megafauna may induce changes in meiobenthos densities and community structure (Dashfield et al., 2008; Ingels et al., 2014; Van Colen et al., 2009, 2015). It is apparent that changes in meiobenthic communities may have repercussions on ecosystem functioning either directly (e.g. biomass production, sediment reworking) or indirectly by altering the structure of other benthic size classes (see review by Schratzberger and Ingels, 2017).

In our case the combined effect of elevated temperature and reduced pH resulted in a strong increase of predatory meiobenthos (Platyhelminthes), disappearance of Gastrotricha and reduced densities of Copepoda indicating a shift in the balance between predators and grazers. This in turn may entail cascading effects on microbiota that play a crucial role in coastal nitrogen cycling (Gilbertson et al., 2012; Herbert, 1999). As such, two experimental studies reported a stimulating effect of meiobenthos abundance and diversity on denitrification processes in marine sediments (Bonaglia et al., 2014; Stock et al., 2014). Nutrient cycling is a key function of benthic ecosystems and estuaries in particular fulfil a crucial role in nutrient movement and biological production as they are among the most

productive and most valuable biomes globally (Costanza et al., 1993, 1997; Meire et al., 2005). As a global phenomenon, ocean acidification and seawater temperature rise may therefore significantly alter benthic communities and ecosystem functioning of valuable coastal zones on large spatial scales.

3.4.2. Conclusion

Our experimental study demonstrated synergistic impacts of elevated temperature and reduced seawater pH on the composition of the meiobenthos in intertidal, sandy sediments. Effects were strongest on the densities of less dominant groups, potentially caused by shifts in biotic interactions, while densities of nematodes were less affected. Though not reflected in terms of density, impacts of elevated temperature and reduced pH on nematodes were observed in differences in the proportion of dead organisms in the sediment. Since mortality and degradation of meiobenthos organisms are often neglected effects of ocean acidification and warming, this study demonstrates that this parameter should be considered in future research to prevent any underestimation of effects. Overall, the combined effects of seawater acidification and elevated temperature as predicted by the end of this century may exert a stronger impact on meiobenthic community structure and vertical distribution in the sediment as compared to the single stressor response by changing pore water biochemistry in deeper sediment layers. Our study underlines the importance of multi-stressor experiments that try to unravel additive or synergistic effects and the use of multi-species assemblages to include biotic interactions enabling more realistic predictions of future climate change effects.

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3.5. Supplementary material

Table S3.1 Average carbonate chemistry of seawater in four treatments during the last 3 weeks of incubation: Temperature (Temp in ° C), pHT, salinity, total alkalinity (TA in µmol kg^{-1}), partial pressure of carbon dioxide (pCO^2 in µatm), concentration of bicarbonate and carbonate ion (HCO^{3-} and CO_3^{2-} in µmol kg^{-1}). The ± values represent standard deviation.

Treatment	Temperature	pH_T	Salinity	TA	pCO_2	HCO_3^-	CO_3^{2-}
10 °C, pH 7.9	10.6	7.85	33	3541	448	2942	260
	± 0.46	*± 0.04*	*± 0*	*± 83*	*± 30*	*± 66*	*± 18*
10 °C, pH 7.5	10.8	7.50	33	3559	1240	3281	120
	± 0.50	*± 0.03*	*± 0*	*± 90*	*± 210*	*± 110*	*± 14*
13 °C, pH 7.9	13.4	7.93	33	3632	466	2961	293
	± 0.57	*± 0.03*	*± 0*	*± 61*	*± 37*	*± 68*	*± 15*
13 °C, pH 7.5	13.3	7.50	33	3712	1542	3435	121
	± 0.08	*± 0.07*	*± 0*	*± 87*	*± 283*	*± 98*	*± 16*

Table S3.2 Pairwise comparisons (Permanova) for the factors pH, Temperature and Sediment depth of meiobenthos community compositions.

	pH 7.9				pH 7.5			
	10 °C		13 °C		10 °C		13 °C	
Depth	t	*p*(MC)	t	*p*(MC)	t	*p*(MC)	t	*p*(MC)
0-0.5 vs. 0.5-1	1.294	0.282	2.795	0.061	2.644	0.077	1.517	0.238
0-0.5 vs. 1-1.5	4.539	**0.021**	2.838	0.053	4.966	**0.023**	4.105	**0.026**
0-0.5 vs. 1.5-2	2.539	0.076	2.704	0.057	2.205	0.096	5.632	**0.013**
0-0.5 vs. 2-3	2.977	0.050	1.953	0.116	2.575	0.081	5.028	**0.017**
0.5-1 vs. 1-1.5	1.919	0.131	1.492	0.213	4.284	**0.027**	Negative	
0.5-1 vs. 1.5-2	1.995	0.114	1.667	0.197	1.826	0.123	3.621	**0.037**
0.5-1 vs. 2-3	3.571	0.055	0.992	0.454	2.034	0.130	4.240	**0.020**
1-1.5 vs. 1.5-2	1.444	0.239	2.738	0.070	1.479	0.224	2.631	0.080
1-1.5 vs. 2-3	2.476	0.120	0.742	0.621	1.365	0.273	2.841	0.065
1.5-2 vs. 2-3	1.674	0.171	0.573	0.770	Negative		2.249	0.103

Table S3.3 Pairwise comparison (Permanova) of nematode densities between different depth layers of all experiment treatments.

	Nematode densities	
Depth	t	$p_{(Perm)}$
0-0.5 vs. 0.5-1	2.207	0.053
0-0.5 vs. 1-1.5	3.916	**0.004**
0-0.5 vs. 1.5-2	3.685	**0.004**
0-0.5 vs. 2-3	2.031	0.069
0.5-1 vs. 1-1.5	2.604	**0.028**
0.5-1 vs. 1.5-2	3.505	**0.003**
0.5-1 vs. 2-3	0.953	0.374
1-1.5 vs. 1.5-2	2.543	**0.025**
1-1.5 vs. 2-3	0.551	0.632
1.5-2 vs. 2-3	2.031	0.061

Table S3.4 Results of the pairwise comparisons (Permanova) for the factors pH, Temperature and Sediment depth of median grain size data.

	pH 7.9				**pH 7.5**			
	10 °C		**13 °C**		**10 °C**		**13 °C**	
Groups	t	*p*(MC)	t	*p*(MC)	t	*p*(MC)	t	*p*(MC)
0-0.5, 0.5-1	29.582	**0.001**	12.811	**0.006**	13.932	**0.007**	13.006	**0.006**
0-0.5, 1-1.5	10.466	**0.009**	4.276	0.052	6.159	**0.023**	30.954	**0.002**
0-0.5, 1.5-2	7.778	**0.017**	7.586	**0.017**	5.335	**0.033**	23.707	**0.002**
0-0.5, 2-3	8.614	**0.014**	5.467	**0.029**	7.348	**0.018**	19.882	**0.003**
0.5-1, 1-1.5	0.121	0.914	0.849	0.479	3.541	0.068	0.599	0.609
0.5-1, 1.5-2	0.870	0.475	2.000	0.181	5.667	**0.028**	1.961	0.191
0.5-1, 2-3	1.185	0.362	2.053	0.174	9.974	**0.011**	2.966	0.099
1-1.5, 1.5-2	4.269	0.053	0.867	0.478	2.518	0.130	4.969	**0.038**
1-1.5, 2-3	9.322	**0.011**	1.393	0.302	1.260	0.332	4.489	**0.044**
1.5-2, 2-3	0.885	0.467	1.551	0.270	1.301	0.316	3.589	0.074

Chapter 4 Impaired short-term functioning of a benthic community from a deep Norwegian fjord following deposition of mine tailings and sediments

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Abstract

The extraction of minerals from land-based mines necessitates the disposal of large amounts of mine tailings. Dumping and storage of tailings into the marine environment, such as fjords, is currently being performed without knowing the potential ecological consequences. This study investigated the effect of short-term exposure to different deposition depths of inert iron ore tailings (0.1, 0.5 and 3 cm) and dead subsurface sediment (0.5 and 3 cm) on a deep water (200 m) fjord benthic assemblage in a microcosm experiment. Biotic and abiotic variables were measured to determine structural and functional changes of the benthic community following an 11 and 16 day exposure with tailings and dead sediment, respectively. Structural changes of macrofauna, meiofauna and bacteria were measured in terms of biomass, density, community composition and mortality while measures of oxygen penetration depth, sediment community oxygen consumption and ^{13}C-uptake and processing by biota revealed changes in the functioning of the system. Burial with mine tailings and natural sediments modified the structure and functioning of the benthic community albeit in a different way. Mine tailings deposition of 0.1 cm and more resulted in a reduced capacity of the benthic community to remineralize fresh ^{13}C-labelled algal material, as evidenced by the reduced sediment community oxygen consumption and uptake rates in all biological compartments. At 3 cm of tailings deposition, it was evident that nematode mortality was higher inside the tailings layer, likely caused by reduced food availability. In contrast, dead sediment addition led to an increase in oxygen consumption and bacterial carbon uptake comparable to control conditions, thereby leaving deeper sediment layers anoxic and in turn causing nematode mortality at 3 cm deposition. This study clearly shows that even small levels (0.1 cm) of instantaneous burial by mine tailings may significantly reduce benthic ecosystem functioning on the short term. Furthermore, it reveals the importance of substrate characteristics and origin when studying the effects of substrate addition on marine benthic fauna. Our findings should alert decision makers when considering approval of new deep-sea tailings placement sites as this technique will have major negative impacts on benthic ecosystem functioning over large areas.

4.1. Introduction

The extraction of mineral resources on land produces large amounts of fine waste material known as mine tailings (Jamieson, 2011). About 60% (iron) to 99.99% (gold) of the ore processed in mines is discarded as non-economic by-product resulting in an annual waste production of 14 billion tons of fine tailings worldwide (Jones and Boger, 2012; Vogt, 2013). While many solutions for the recycling of tailings have been proposed (Adiansyah et al., 2015; Bian et al., 2012), the vast majority is discarded in landfills, lakes, riverine systems and the marine environment. The environmental and socio-economic consequences of tailings disposal can be devastating so that a proper management and sustainable use of mine tailings requires more attention (Adiansyah et al., 2015; Bian et al., 2012; Franks et al., 2011). For reasons of risk reduction on land as well as economic and esthetical considerations (Kvassnes et al., 2009) the disposal of inert tailings material into streams and the marine environment known as riverine tailings disposal (RTD), submarine tailings placement (STP) and deep-sea tailings placement (DSTP) have been proposed and implemented (Vogt, 2013). But, due to the irreversible environmental impacts resulting from direct tailings discharge into riverine systems the use of this approach has seized and RTD is no longer implemented (Vogt, 2013). Submarine tailings placement, however, is allowed and applied at 14 mining sites worldwide (status in 2013) while new sites are still being targeted (Vogt, 2013). Currently, 0.6% of all industrial-sized mines discharge their tailings into the marine environment with Norway as main contributor (Vogt, 2013). In 2013, Norway, the country with most STP sites worldwide, had 7 operational STP sites and 2 sites in a planning stage (Kvassnes and Iversen, 2013).

STP occurs mainly at continental margins at depths between 30 and 1000 m or deeper including highly productive ecosystems such as fjords and canyons (de Leo et al., 2010; Thurber et al., 2014; Vogt, 2013). Continental margins are significant contributors to biodiversity and productivity and fulfil an important role for the provision of ecosystem services (Walsh, 1991). These include a wide range of regulatory services such as nutrient cycling, natural carbon sequestration and primary and secondary production, but also direct provisioning services such as fisheries and mineral or genetic resources (Armstrong et al., 2012; Thurber et al., 2014). Despite this key role, only few studies have assessed the impacts of mine tailings disposal on the functioning of bathyal benthic communities. The environmental impacts of STP can be manifold, including hyper-sedimentation, changes in grain size, smothering of benthic fauna and toxicity by the release of heavy metals or chemicals (Ramirez-Llodra et al., 2015). To prevent irreversible impacts on the environment it is of particular interest to get an understanding of

the risks of submarine tailings placement and the magnitude of its impact on marine biota and ecosystem functioning. A large amount of scientific data is available in the 'grey literature' from monitoring programs accompanying STP operations (Ramirez-Llodra et al., 2015). However, many of these monitoring studies only report impacts on one or few aspects of the ecosystem and often good baseline studies are lacking (Ramirez-Llodra et al., 2015). Furthermore, to be able to give recommendations for future environmental management with regard to land-based mining but also regarding possible future marine mining scenarios, it is crucial to investigate threshold values for tailings deposition. And although monitoring studies have incorporated the impact of tailings disposal at various distances from the outfall (Olsgard and Hasle, 1993) it is difficult to infer threshold values for the directly impacted communities as benthic fauna might naturally vary with depth and distance from the outfall due to changes in grain size or food availability. Especially the composition of meiofauna taxa and nematode species in particular and their vertical distribution in the sediment are strongly determined by abiotic factors such as grain size and sediment oxygenation (Coull, 1999; Higgins and Thiel, 1988; Jansson, 1967; Moodley et al., 2000a) making this group particularly vulnerable to sediment burial and disturbance.

Some important ecosystem functions e.g. organic matter remineralization or primary and secondary production are strongly driven by biotic factors such as biomass, diversity or bioturbation (Braeckman et al., 2010; Danovaro et al., 2008; Lohrer et al., 2004). Moreover, different trophic levels and functional groups can exhibit tight interactions, and changes in the structure of one taxon can have strong repercussions on others which in turn influences ecosystem functioning (Gilbertson et al., 2012; Piot et al., 2013). Therefore, to fully understand the complexity of the processes shaping the structure and functioning of benthic ecosystems under various stressors, well-designed, controlled experiments resembling natural conditions as close as possible, including multiple trophic levels and community-based approaches, are needed (Hale et al., 2011; Wernberg et al., 2012). Oxygen consumption is a good proxy for the depth-integrated overall remineralization of organic matter by benthic communities through aerobic and anaerobic processes (Glud, 2008; Middelburg et al., 2005; Moodley et al., 1998) and has been proven a very useful indicator in various impact studies (e.g. Sweetman et al., 2014, 2010; Vanaverbeke et al., 2008). Furthermore, the processing of organic matter can be traced through different trophic levels by introducing labile organic material with isotopically enriched carbon signatures (Boschker and Middelburg, 2002; Middelburg et al., 2000; van Oevelen et al., 2006a). In combination with estimates of biomass and density it is also possible to assess the relative

contribution of each biotic component to the observed functional changes (van Oevelen et al., 2006b).

To understand the potential environmental impacts of deep-sea tailings placement on the structure and functioning of soft-sediment communities and to assess threshold levels for tailings deposition, we conducted a microcosm experiment with soft-bottom fauna from a deep Norwegian fjord. For this purpose, natural, undisturbed sediments were incubated in the laboratory under in-situ conditions and subjected to three different levels of deposition with mine tailings. Structural changes of the macrofaunal, meiofaunal and bacterial communities were assessed using measures of biomass, densities and diversity. Furthermore, mortality was assessed in macrofauna by "life-checking" and in meiobenthic nematodes by using a staining technique with Trypan Blue. Next to these structural impacts we investigated ecosystem functioning responses such as oxygen consumption dynamics and phytodetritus processing by the different biotic compartments making use of stable isotope tracing techniques. To isolate the effect of the tailings from deposition with natural sediment an additional experimental treatment (i.e. the deposition of dead sediment) was included in the experimental setup.

We hypothesized that I.) Exposure to burial with mine tailings will alter the benthic community structure on the short term due to mortality and changes in vertical community distribution; II.) Changes in benthic community structure due to tailings disposal will cause a reduced processing of organic matter as assessed from O_2 consumption, ^{13}C-labelled phytodetritus uptake and production of dissolved inorganic carbon; and III.) The response of benthic organisms to tailings is different than to a deposition event with natural subsurface sediment.

4.2. Material and Methods

4.2.1. Study site and sediment collection

Sediments were collected from 207 m depth in the Norwegian Hadangerfjord (59°43.48'N, 5°24.18'E, SW Norway) on board MS Solvik (October 28, 2014). Three sediment cores (∅ 14 cm) were subsampled from 14 boxcore deployments with a total of 42 subsampled sediment cores. The cores were transported to the laboratory at the International Research Institute of Stavanger (IRIS, Norway) and maintained in the dark at in-situ temperature (8°C) for acclimatization. The cores were continuously supplied with fresh, cooled and sand-filtered seawater (salinity: 33.02) from a nearby fjord by a flow-through system via gravity feed. Twelve cores were randomly assigned to the following treatments (n=4): 0.1 cm, 0.5 cm and 3 cm of mine tailing (MT) addition. Six cores were assigned to the following

treatments (n=3): 0.5 cm and 3 cm dead sediment (DS) addition. Inert, ground up tailings from a Norwegian iron ore mine were used for the MT treatments. For the DS treatment, subsurface sediment (<20 cm) was taken from the boxcores, temporarily frozen (-80 °C for more than 3 days) to kill fauna and thawed prior to application. Four cores served as controls, i.e. no tailings or sediment was added. The remaining cores were used to measure granulometry of the natural sediment, for nematode staining tests and to determine the necessary amount of substrate required to build up to the target deposition thickness. The latter was experimentally determined as follows: Slurries of tailings and sediment were made from a known amount of material with the estimate to build 1 cm and were added to two separate spare cores. After 24 h of settlement the actual sediment height was measured and more tailings/sediment was added until a total build-up of 10 cm. From this information the required amount of tailings/sediment was calculated.

4.2.2. Experiment set-up and incubation procedure

To start the experiment, the respective amounts of substrate were added to the cores and were left for 24 h to allow settlement of particles. The different treatment thicknesses of the tailings addition could be easily distinguished from the Control (Fig. 4.1A) with visual inspection as the mine tailings formed a dense,

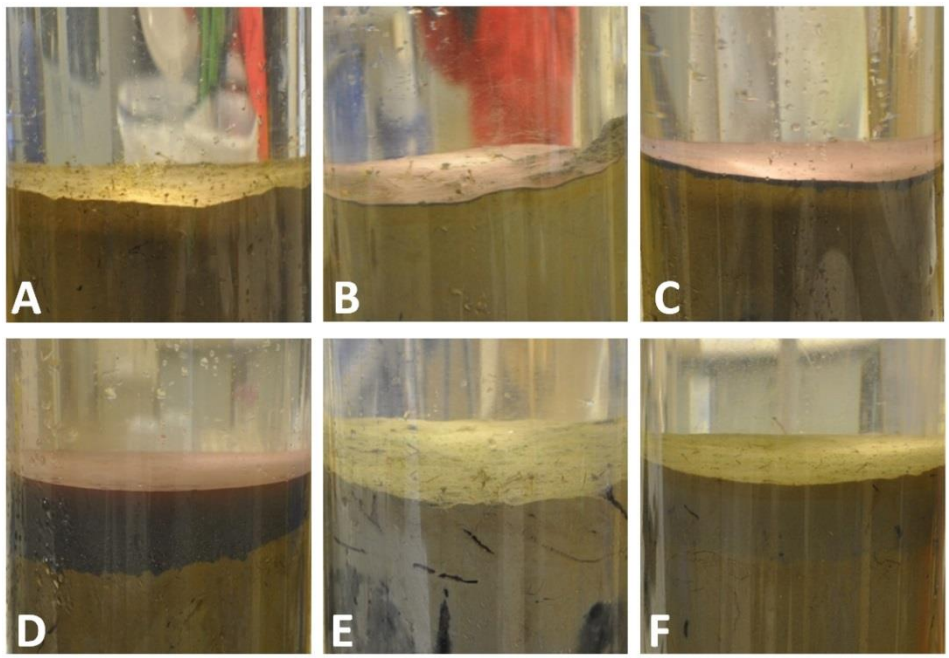

Figure 4.1 Surface layers of the incubation cores 24h after deposition. A) Control B) 0.1 cm mine tailings C) 0.5 cm mine tailings D) 3 cm mine tailings E) 0.5 cm dead sediment F) 3 cm dead sediment.

grey-reddish layer with a separation of fine, light particles on top and coarser, darker particles below (Fig. 4.1, B-D). This distinction was less clear for the dead sediment addition treatments (Fig. 4.1, E-F).

The incubation procedure involved a series of manipulations, measurements and samples obtained at the various time points throughout the incubation duration of 11 and 16 days for the MT and DS treatments, respectively (Fig. 4.2). After substrate addition at T0, particles were allowed to settle for 24 h. One day after tailing addition (T1) the overlying water in the cores was clear indicating that full settlement of particles had taken place and oxygen penetration depth (OPD) in the sediment was determined. At day 8 (T8) and day 13 (T13) for MT and DS, respectively, rates of sediment community oxygen consumption (SCOC) were determined followed by a second OPD measurement. Subsequently, at day 9 (T9) for the MT and day 14 (T14) for the DS treatments, 31 mg (equivalent to 47 mmol C m^{-2}) dried *Skeletonema costatum*, that was enriched in ^{13}C (28% enrichment), was pipetted homogenously on the sediment surface in the cores and SCOC was measured once again. The isotopically enriched algae provided a tool to trace ^{13}C carbon throughout the benthic assemblage and enable quantification of fresh organic carbon processing. At day 10 (T10) for the MT treatments and day 15 (T15) for the DS treatments the experiment was terminated by sampling the sediment for faunal and sediment analyses. The different incubation durations for the two treatments are the result of logistic difficulties.

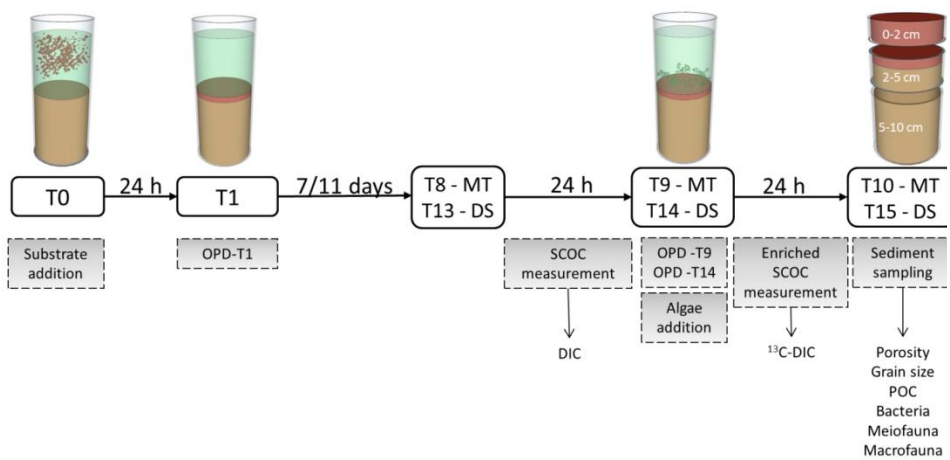

Figure 4.2 Scheme of manipulations and samplings during the experiment. Treatments included the addition of mine tailings (MT) with 0.1 cm, 0.5 cm, and 3 cm thickness and the addition of dead sediment (DS) with 0.5 cm and 3 cm thickness. "T" indicates the days of incubation after substrate addition. Actions and measurements are shown in blue boxes while arrows indicate from which measurements the different variables were obtained. Abbreviations: OPD= Oxygen penetration depth, SCOC= Sediment community oxygen consumption, DIC= Dissolved inorganic carbon, POC= Particulate organic carbon.

The Control followed the same time frame and sampling procedures as the MT treatments, thus, with an incubation time of 11 days.

After the experiment, sediments were sampled by inserting a smaller meiofauna core (∅ 5 cm) into the experimental core and the overlying water in the experimental cores was carefully siphoned off. The sediment surrounding the meiofauna core was sliced in three intervals (0-2, 2-5 and 5-10 cm) measured from the surface of the added substrate. Each layer was homogenized in a bucket and subsamples were taken with 30 mL syringes and immediately stored frozen at -21°C for analysis of sediment characteristics (porosity, total organic carbon, particulate organic carbon) and bacterial specific phospholipid-derived fatty acids (PLFA). Sediment porosity was determined for each treatment and sediment layer by weight loss after freeze-drying. The sediment granulometry of two separate control cores, each sliced in 0.5 cm intervals and of one mine tailings sample were measured by laser diffraction with a Malvern Mastersize 2000 particle analyzer (Malvern Instruments, UK). Grain size classes were determined according to the Wentworth scale (Wentworth, 1922). After acidification, total organic carbon content (TOC) was quantified with a Thermo Flash EA 1112 elemental analyzer (Thermo Fisher Scientific, USA).

4.2.3. Analyses determining structural changes of the benthic community

The remaining sediment from the experimental core was sieved over a 500 and 38 µm sieve. The macrofauna fraction (>500µm) was qualitatively screened under a stereomicroscope ("life-check", see Moodley et al., 1997) before fixation in 4% formaldehyde to avoid abundance overestimates which could result from the lack of decomposition of dead organisms under low temperature conditions in a short-term experiment. In all samples, all encountered macrofauna specimens were found to be alive. The preserved samples were later identified to the lowest taxonomic level.

After siphoning off the overlying water of the meiofauna core, the sediment was sliced in intervals of 0.5 cm starting from the surface of the added substrate down to 2 cm depth of the natural sediment and in 1 cm intervals down to 5 cm depth. After slicing of the meiofauna cores, 5 or 10 mL of 4% Trypan blue solution (see 4.2.4.) were added to the 0.5 cm and 1 cm sediment slices, respectively, and samples were shaken vigorously before incubation for 2 h in sampling vials to ensure sufficient exposure with the stain. Subsequently, the samples were washed with filtered (10 µm) seawater on a 32 µm sieve until most of the stain was washed out and fixed on 4% buffered formaldehyde. Meiofauna was extracted from the

sediment by washing the samples over two stacked sieves of 1 mm and 32 μm. The 32 μm fraction was subjected to density centrifugation with Ludox HS40 (Dupont) at 3000 rpm (specific density of 1.18, (Heip et al., 1985)). Centrifugation was done three times and the supernatant was sieved (32μm) and fixed in 4% buffered formaldehyde. Meiofauna was identified to higher taxon level with a stereomicroscope (50x magnification) and nematodes were categorized in "stained" and "unstained". Copepods and their nauplii were removed from the analysis because those taxa were found after sieving (32 μm) the seawater from the flow through system for 30 minutes. This may point towards the ability of copepods and nauplii to penetrate or inhabit the sand filter. No other meiofauna taxa or larger organisms were found after sieving the water. No meiofauna was found when checking a 30 mL subsample of mine tailings.

Biomass of the three biotic size classes (macrofauna, meiofauna, bacteria) is expressed as organic carbon content per area (mg C m^{-2}) and was directly calculated from the ratio mass spectrometer output (see 4.2.5. for full description).

4.2.4. Nematode staining test

Trypan blue is a dye commonly used in cell viability assessments (Louis and Siegel, 2011) that has already been successfully applied to assess soil nematode mortality (Womersley and Ching, 1989). In order to assess nematode mortality, a new staining protocol with Trypan blue was developed and tested prior to the experiment. To test the protocol, the upper 1.5 cm of two spare cores were sliced in 0.5 cm intervals and three sample vials containing slices of one core were submerged in hot water (± 80° C) for 10 minutes to kill the meiofauna. After 2 hours, all samples (dead and live) were stained with 3 mL of 4% Trypan blue solution (prepared with distilled water) and left to incubate for 2 h. In the live samples 16.42 % ± 6.84 of nematodes were stained while in the dead samples this percentage was 77.75 % ± 7.97. However, a proportion of 7.05 % ± 1.13 and 13.27 % ± 4.77 of the nematodes in live and dead samples, respectively, were left "uncategorized" due to an incomplete staining of the bodies.

4.2.5. Analyses determining functional changes of the benthic community

Oxygen penetration depth (OPD) was determined by means of a microprofiler equipped with oxygen microsensors (50 μm tip; Unisense A.S., Denmark). After 2-point sensor calibration (0% calibration: Na_2SO_3; 100% calibration: air-bubbled seawater), oxygen concentration in each core (1 profile per core) was measured by

penetrating in 100 μm steps into the sediment until oxygen concentration was below detection limit (0.3 μM).

SCOC was measured over a 24 h period in the dark in cores that were sealed off with lids that were fitted with a stirrer and an oxygen optode (PreSens, Germany). During this period the cores were disconnected from the water flow through system. Water samples of 10 mL (filtered through a 0.2 μm filter and conserved by the addition of 10 μL $HgCl_2$) were taken in headspace vials at the beginning and at the end of the incubation for later analysis of the dissolved inorganic carbon (DIC) flux and ^{13}C-DIC measurements. For these analyses a headspace of approximately 1.5 ml was created by injecting Helium gas through a septum. The samples were subsequently acidified with 20 μL concentrated H_3PO_4 to transform DIC into gaseous CO_2. A 500 μL sample of the CO_2 was then injected into a HP 61530 gas chromatograph (Hewlett-Packard/Agilent, USA) connected to a DELTA-Plus Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, USA) to determine DIC concentrations and ^{13}C composition as described by Moodley et al. (2000b). The ^{13}C composition in the freeze-dried, grinded sediment subsamples was quantified with a Thermo Flash EA 1112 elemental analyzer (Thermo Fisher Scientific, USA) coupled with an DELTA V Advantage Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, USA).

The >500 μm macrofaunal fraction was grouped in the following taxonomic groups: Echinodermata, Mollusca, Polychaeta, Sipunculida. Within each sample, each taxonomic group was individually freeze-dried to determine total dry weight. After homogenization, a weighted subsample was taken for isotope and biomass analysis (as described above for sediment, e.g. Moodley et al., 2005). In two samples, one individual or pieces of a sea pen (Pennatulacea) were found which were removed from the stable isotope analysis. The meiofauna fraction (>38 μm) of the experimental core sediment was fixed on 4% buffered formaldehyde for stable isotope analysis of nematodes. For this purpose 130 randomly hand-picked nematodes per sample were transferred to a few drops of Milli-Q water in silver capsules (8x5 mm) that had been pre-combusted for 4 h at 450°C. The nematode samples were dried overnight at 60°C, acidified with 20 μl 2% HCl and dried on a hot plate at 60°C. After acidification the samples were closed and bulk C and N content as well as $\delta^{13}C$ and $\delta^{15}N$ were measured as described for the sediment.

Bacterial tracer uptake and biomass was based on concentrations of bacteria specific PLFA (i14:0, i15:0, a15:0, i16:0 and 18:1ω7c; Middelburg et al., 2000). These PLFAs were extracted from freeze-dried, grinded sediment using the Bligh and Dyer method (Bligh and Dyer, 1959) according to the protocol by Boschker et al. (2004). Subsequently the extracted PLFAs were derivatized to fatty acid methyl

esters (FAME) that were analyzed by GC/C-IRMS (HP 61530 gas chromatographer, Hewlett-Packard/ Agilent, USA; DELTA-Plus Isotope Ratio Mass Spectrometer, Thermo Fisher Scientific, USA) with a polar analytical column (ZB5-5MS; 60 m length, 0.32 mm diameter, 0.25 μm film thickness; Phenomenex, USA).

The incorporation of ^{13}C into macrofaunal, nematode and bacterial biomass was calculated as described by different papers (Guilini et al., 2011; Middelburg et al., 2000; Moodley et al., 2002; van Oevelen et al., 2006a). Total algal tracer uptake is expressed as the quotient of total ^{13}C uptake (I) and the ^{13}C content in *S. costatum* (28%) (see Moodley et al. (2002)).

4.2.6. Statistical analyses

To include the depth dependency of samples, all data sets containing depth information were analyzed using a Permanova analysis with a nested design allowing statistical comparison between treatments and within depth layers (Primer software version 6.1.11 with the Permanova+ add-on, Anderson et al., 2008; Clarke and Gorley, 2006). Tab. 4.1 shows the Permanova designs for the various variables. When the main test yielded significant differences pairwise tests were performed and, if the number of possible permutations was lower than 100, Monte Carlo tests were applied to estimate p-values (p(MC)) with increased accuracy. Graphs were computed using Prism 6 (GraphPad Software, Inc.). Throughout the manuscript, data is reported as mean and standard error, unless mentioned otherwise.

As grain size was measured in one spare core it was not analyzed statistically. Porosity and TOC were tested with Permanova for differences between treatments and the control within each layer.

Standardized densities in individuals per m^2 and individuals per 10 cm^2 were used for macro- and meiofauna analysis, respectively. Total (whole core) meiofauna and macrofauna densities were analyzed using ANOVA in R (R Core Team, 2013) to assess differences between treatments after the assumptions of normality and homogeneity of variances were assured. Pairwise comparisons were conducted by means of the TukeyHSD test. For reasons of better comparison, statistical analyses were done on data from the sediment intervals of 0-2 cm, 2-5 cm and 5-10 cm starting at the added substrate surface for macrofauna and bacteria and on 0-2 cm and 2-5 cm intervals for meiofauna. Nematode distribution and mortality on a high depth interval resolution was analyzed graphically and descriptively. The contribution of specific macro- and meiofauna taxa to differences in their community composition was determined by a similarity of percentages (SIMPER)

analysis. Shannon and Simpson diversity indices and Pielou's evenness index were calculated for meio- and macrofauna densities and tested in terms of whole core diversity (ANOVA) and per depth layer (Permanova).

Due to the repeated measures character of OPD and SCOC data a Permanova analysis was used to test for differences between the factors Treatment and Time (Permanova design, Tab. 4.1). Univariate data of DIC was analyzed using ANOVA

Table 4.1 Permanova designs for the analysis of various parameters.

Parameter	Factor	Nested in	Fixed/Random
Porosity, TOC	Treatment		Fixed
Faunal densities, diversity and community structure	Depth		Fixed
Biomass of macrofauna, meiofauna and bacteria	Core ID	Treatment	Random
Uptake of macrofauna, nematodes and bacteria			
OPD; SCOC	Treatment		Fixed
	Time		Fixed
	Core ID	Treatment	Random

to assess differences between treatments after the assumptions of normality and homogeneity of variances were assured. Total (whole core) tracer uptake of the different biotic compartments was analyzed with Permanova as assumptions for normality and homogeneity of variances were not met. Depth layer information was included with Permanova as described above.

4.3. Results

4.3.1. Effect of substrate addition on abiotic variables

The natural sediment was composed of fine silt characterized by a median grain size of 11.94 ± 0.34 μm in the 0-2 cm layer, 12.26 ± 0.13 μm in the 2-5 cm layer and 12.59 ± 0.56 μm in the 5-10 cm layer. In contrast, the mine tailings were composed of very fine sand with a median grain size of 101.31 μm. The difference in median grain size between the mine tailings and the natural sediment was also reflected in the lower porosity of the added layers of tailings compared to the natural sediment (Permanova, p(MC) ≤ 0.003 Fig. 4.3 A). Additionally, total organic carbon content was lower in the layers with added tailings compared to control cores (Permanova, p(MC) ≤ 0.013, Fig. 4.3 B).

4.3.2. Structural changes of the benthic community

Total macrofauna densities ranged from 12971 ± 3573 ind. m^{-2} in the 3 DS treatment to 20040 ± 2780 ind. m^{-2} in the 0.1 MT treatment. Though total densities did not significantly differ between treatments, a decreasing trend with sediment depth was observed. Within each sediment depth layer community

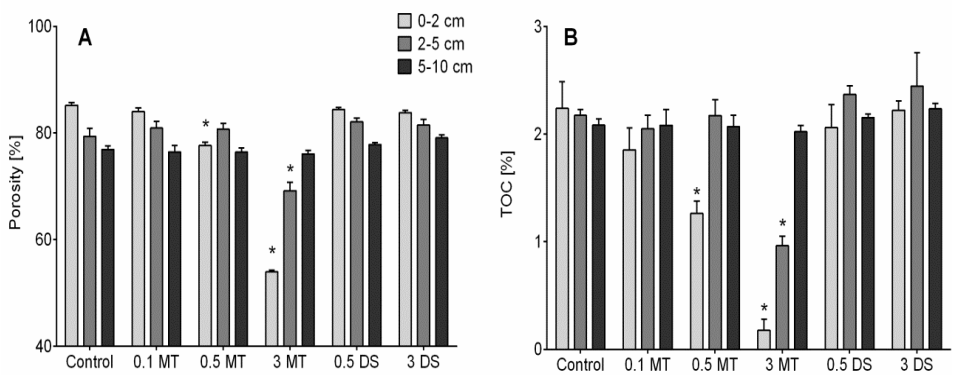

Figure 4.3 A) Porosity and B) total organic carbon (TOC) content of all depth layers per treatment. Error bars denote standard error and an asterisk indicates significant (p<0.05) differences with the control. Abbreviations: 0.1 MT = 0.1 cm mine tailing addition, 0.5 MT = 0.5 cm mine tailing addition, 3 MT = 3 cm mine tailing addition, 0.5 DS = 0.5 cm dead sediment addition, 3 DS = 3 cm dead sediment addition.

composition was similar between treatments and control. Within each mine tailings treatment and the control, the community composition in the 0-2 cm layer was different from the deeper layers (Permanova, p(MC) ≤ 0.0400). Main contributors to the dissimilarities between the 0-2 cm and 2-5 cm and between the 0-2 cm and 5-10 cm layers were *Kelliella militaris* (bivalve, 6.5 %, 7.23 %), *Macrochaeta* sp. (polychaete, 4.11 %, 4.45 %), *Ophelina modesta* (polychaete, 3.39 %, 3.84 %), *Mendicula pygmaea* (bivalve, 3.27%, 3.54%), *Sipuncula* sp. (2.14 %, 4.05 %) and *Nucula nitidosa* (bivalve, 2.83 %, 3.46 %) with much higher densities in the top (0-2 cm) layer and *Paramphinome jeffreysii* (polychaete, 4.26 %, 3.67 %) and *Levinsenia gracilis* (polychaete, 2.27 %, 3.67 %) showing the opposite trend. This depth effect in community composition was not observed in the DS treatments, thus, all organisms were distributed similarly throughout those cores.

Total meiofauna densities (including stained and unstained nematodes) were significantly lower (712 ± 252 ind. 10 cm^{-2}) in the 3 DS treatment compared to the control (2080 ± 93 ind. 10 cm^{-2}, TukeyHSD, p = 0.0115). This difference was attributed to a reduction in nematode densities (TukeyHSD, p = 0.0105) which was the most abundant taxon (94.91 ± 0.34 % of meiofauna community). Nematodes were found in high densities in all surface layers, indicating that the animals were able to move into the added MT and DS substrate (Fig. 4.4). Interestingly, mortality in the MT treatment was substantially higher as compared to the controls (Fig. 4.4), with mortality exceeding 80% in the 0.5 MT and 3 MT treatment. Furthermore, mortality in the 3 DS treatment was high throughout the whole core and not only in the added substrate layer (17.74 % - 63.56 %). The addition of mine tailings had no effect on the whole-core meiofauna community composition (faunal mortality not taken into account). In both DS treatments, however, it differed from the control (Permanova, p(perm) ≤ 0.0073) due to reduced densities of nematodes, oligochaetes and polychaetes (SIMPER).

When taking depth layers into account we observed that all taxa were equally successful in migrating into the mine tailings since community composition remained similar in the Control and MT treatments within the upper 2 cm. Also within the deeper layer (2-5 cm) meiofauna community composition remained similar in all treatments when compared to the Control (Permanova, p(perm) ≤ 0.0162). Interestingly, within each treatment, meiofauna community composition differed between depth layers (0-2 cm and 2-5 cm) for all treatments except the 3 MT and 3 DS treatments (Permanova, p(MC) ≤ 0.049). Differences could be attributed to lower abundances of Kinorhyncha, Ostracoda, Nematoda and Polychaeta in the 2-5 cm layer (SIMPER). Overall meiofauna higher taxon diversity and evenness were low and did not differ between treatments with the

exception that evenness was significantly higher in the upper layer of the 3 DS treatment compared to the control due to reduced nematode densities and, thus, reduced dominance of this taxon. Diversity and evenness did not differ between treatments.

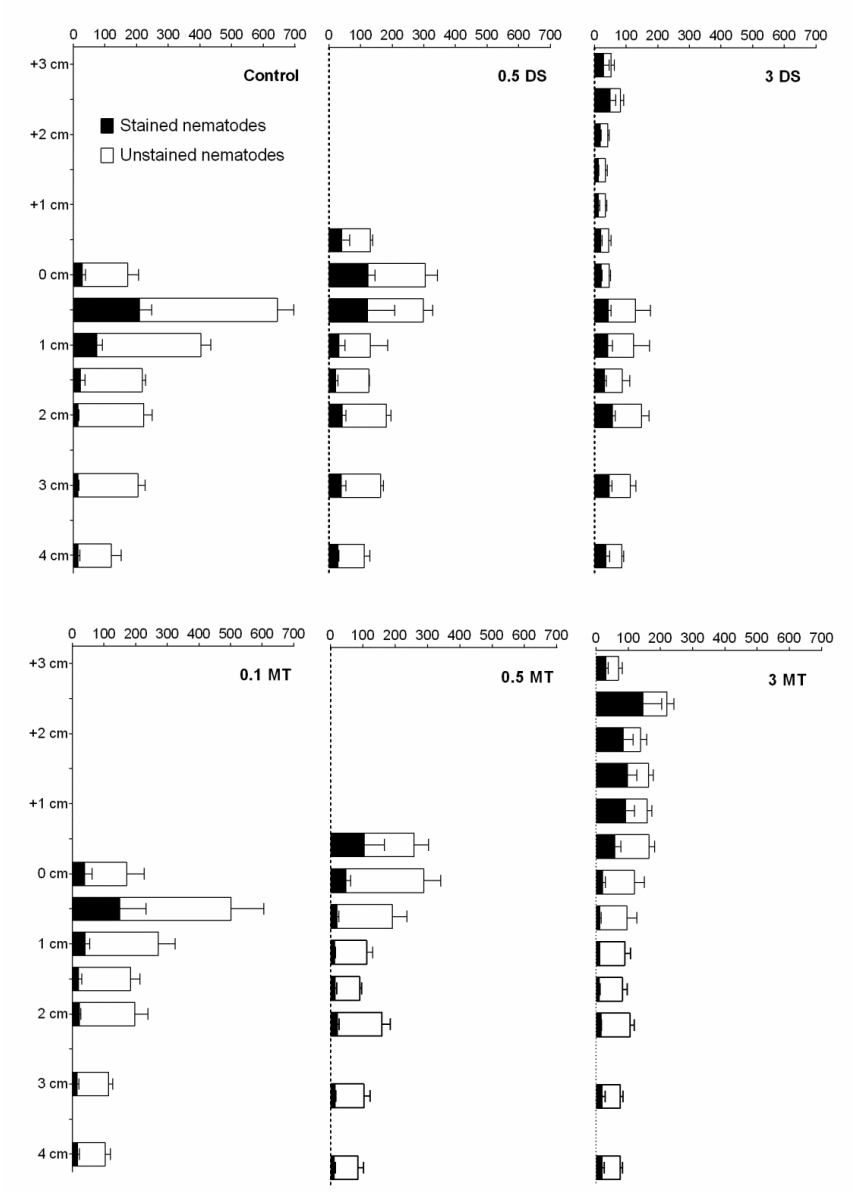

Figure 4.4 Mean abundances (x-axis) of stained (dead) and unstained (alive) nematodes in the different substrate addition treatments. Error bars indicate standard error. Abbreviations: 0.1 MT = 0.1 cm mine tailing addition, 0.5 MT = 0.5 cm mine tailing addition, 3 MT = 3 cm mine tailing addition, 0.5 DS = 0.5 cm dead sediment addition, 3 DS = 3 cm dead sediment addition.

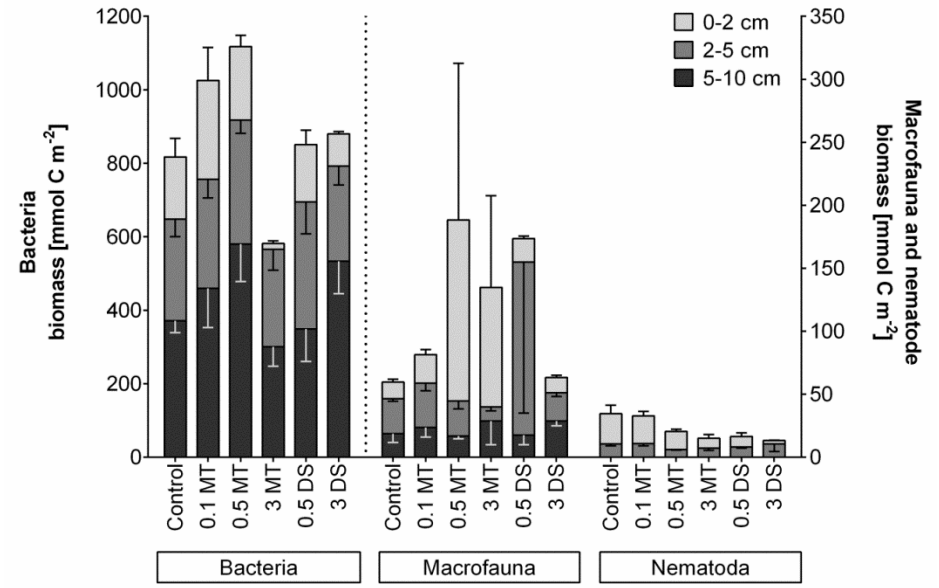

Figure 4.5 Biomass per depth and treatment in the three biological compartments measured: Bacteria (left y-axis), macrofauna and Nematoda (right y-axis). Error bars depict standard errors and point downwards for the 2-5 cm and 5-10 cm data for better visualization. Abbreviations: 0.1 MT = 0.1 cm mine tailing addition, 0.5 MT = 0.5 cm mine tailing addition, 3 MT = 3 cm mine tailing addition, 0.5 DS = 0.5 cm dead sediment addition, 3 DS = 3 cm dead sediment addition.

In the control situation, total biomass was highest for bacteria (9824.93 ± 1503.20 mg C m^{-2}) followed by macrofauna (716.47 ± 109.45 mg C m^{-2}) and nematodes (413.67 ± 90.01 mg C m^{-2}) (Fig. 4.5). The high variability in macrofaunal biomass did not reveal any differences between treatments or depth. For bacteria and nematodes, however, the addition of 3 cm of mine tailings resulted in a reduced biomass in the 0-2 cm layer compared to the control (Permanova, p(MC) ≤ 0.0341). Furthermore, nematode biomass was reduced in the upper layer of the 3 DS treatment and the 2-5 cm layer of the 0.5 MT treatment (Permanova, p(MC) ≤ 0.0197).

4.3.3. Changes in aspects of benthic sediment functioning

Under control conditions oxygen penetrated 1.17 ± 0.11 cm deep into the sediment at the start of the experiment and remained stable throughout the experiment (Fig. 4.6). The OPD of the 3 cm mine tailings treatment was 2.98 ± 0.54 cm, which means that oxygen did not reach beyond the tailings, leaving the natural sediment anoxic. Moreover, the OPD decreased to 1.49 ± 0.18 cm at the end of the experiment (Permanova, p(perm) = 0.0098). The deposition of dead sediment resulted in a shallower OPD compared to the Control (0.61 ± 0.05 cm and 0.57 ± 0.06 cm for 0.5 DS and 3 DS, respectively) after the deposition event

(Permanova, p(MC) ≤ 0.036), but gradually deepened and approached control conditions at the end of the experiment.

After 8 (MT) and 13 (DS) days of incubation, the sediment community oxygen consumption (SCOC) was reduced in the 0.5 and 3 MT treatments compared to the Control (Permanova, p(perm) ≤ 0.0236) while in the 3 DS treatment SCOC increased (Permanova, p(perm) = 0.0342, Fig. 4.7). The SCOC increased in all treatments after addition of the algal tracer (Permanova, p(perm) = 0.0001, Fig. 4.7).

A large fraction of 85.21 ± 1.40 % of the added algal carbon was not processed and remained in the form of particulate organic carbon (POC) in the sediment, especially in the 0-2 cm layer (Fig. 4.8 A). Of the added algal carbon 9.70 ± 1.01

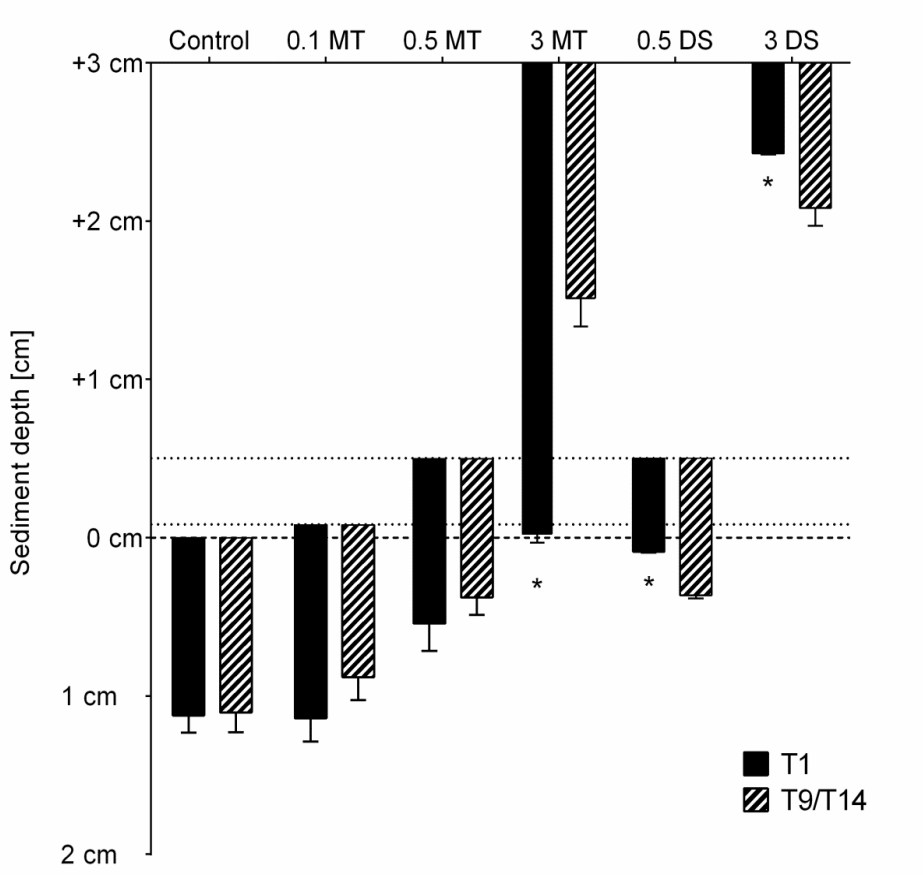

Figure 4.6 Oxygen penetration depth (OPD) in the sediments of the different treatments one day after settlement of the substrate (T1) and at the end of the experiment (T9/T14). Error bars denote standard error and an asterisk indicates significant ($p<0.05$) differences with the control. Abbreviations: 0.1 MT = 0.1 cm mine tailing addition, 0.5 MT = 0.5 cm mine tailing addition, 3 MT = 3 cm mine tailing addition, 0.5 DS = 0.5 cm dead sediment addition, 3 DS = 3 cm dead sediment addition. The dashed line at y=0 represents the natural sediment surface while above this layer the added substrate surface is indicated.

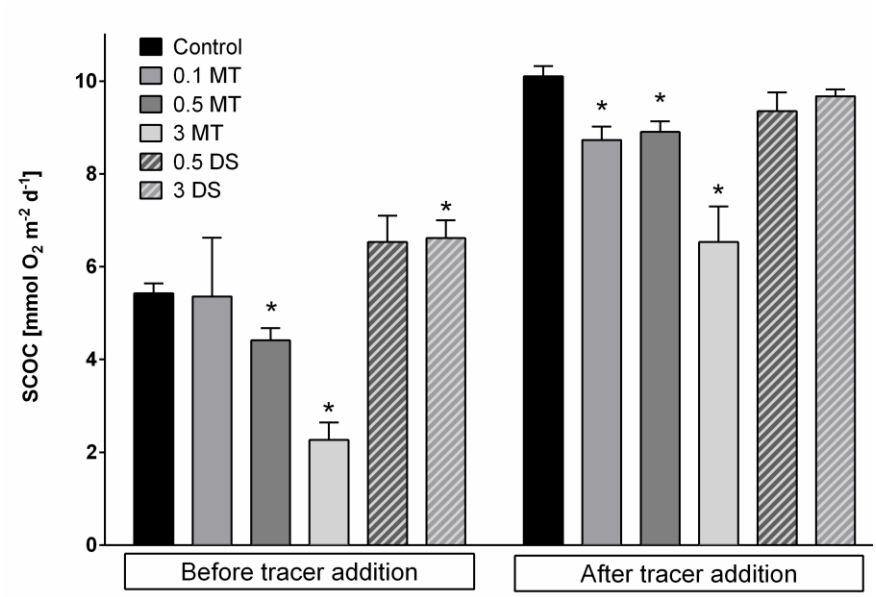

Figure 4.7 Mean sediment community oxygen consumption (SCOC) in mmol O_2 $m^{-2}d^{-1}$ before and after tracer addition for the different substrate addition treatments. Error bars denote standard error and an asterisk indicates significant ($p<0.05$) differences with the control. Abbreviations: 0.1 MT = 0.1 cm mine tailing addition, 0.5 MT = 0.5 cm mine tailing addition, 3 MT = 3 cm mine tailing addition, 0.5 DS = 0.5 cm dead sediment addition, 3 DS = 3 cm dead sediment addition.

% was respired into dissolved inorganic carbon (DIC) (Fig. 4.8 B). No significant differences in total ^{13}C-POC or algal ^{13}C-DIC were found between treatments.

Total Tracer-C uptake was reduced in the 3MT treatment for nematodes and macrofauna (Permanova, $p \leq 0.0038$) and for bacteria (Permanova, $p = 0.0523$, borderline significant, Fig. 4.8 C-E). Additionally, uptake by nematodes was lower for the 0.5 MT, 0.5 DS and 3 DS treatment (Permanova, $p(MC) \leq 0.0365$). When taking depth into account, a significant decrease in tracer uptake by nematodes was observed in the top layer (0-2 cm) of the MT and DS treatments. In general, uptake was highest in the upper 2 cm for bacteria and nematodes while macrofaunal uptake was high in the upper 5cm with significantly lower values in the 5-10 cm layer ($p(MC) < 0.01$).

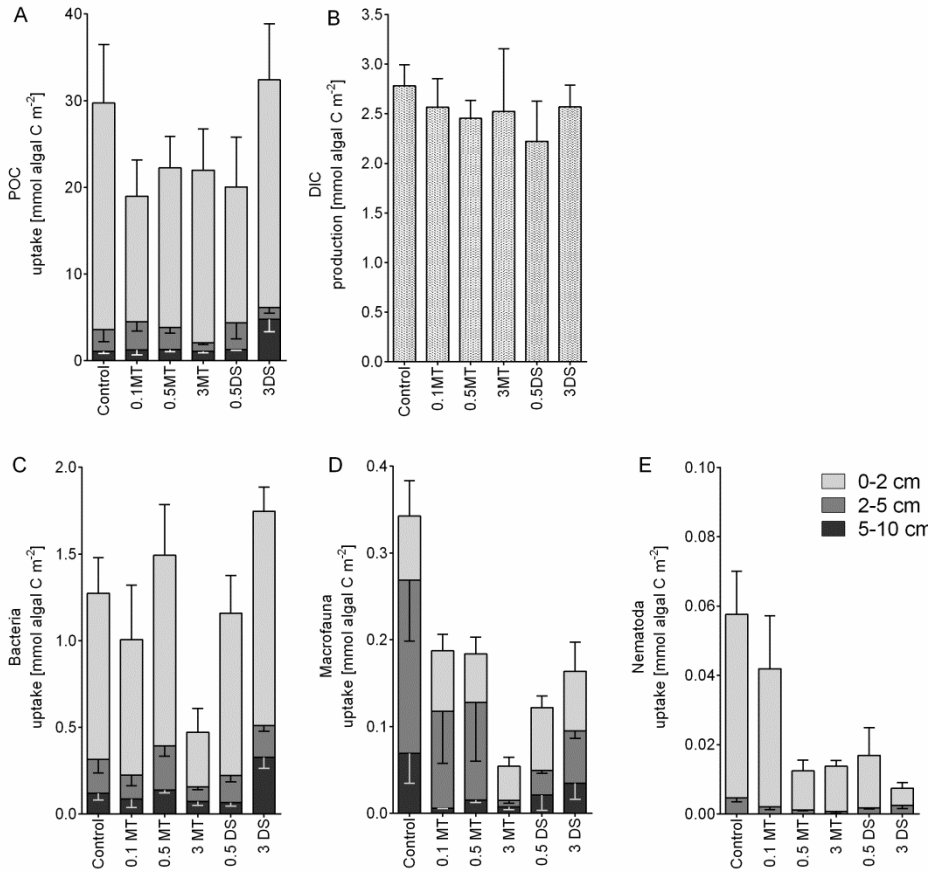

Figure 4.8 A) Particulate organic carbon (POC, A) per treatment and depth; B) dissolved inorganic carbon (DIC, B) per treatment; and uptake of algal ^{13}C per depth and treatment of C) Bacteria, D) Macrofauna and E) Nematoda. Error bars depict standard errors and point downwards for the 2-5 cm and 5-10 cm data for better visualization. Abbreviations: 0.1 MT = 0.1 cm mine tailing addition, 0.5 MT = 0.5 cm mine tailing addition, 3 MT = 3 cm mine tailing addition, 0.5 DS = 0.5 cm dead sediment addition, 3 DS = 3 cm dead sediment addition.

4.4. Discussion

With the continuing STP and prospect of new STP sites from land based mining facilities in many locations, but also with the perspective of deep-sea mineral extraction (e.g. massive sulphides and polymetallic nodules) and associated disposal of waste sediment, there is an urgent need for quantitative assessments of the environmental impact of tailings deposits in marine environments (Levin et al., 2016; Mengerink et al., 2014; Ramirez-Llodra et al., 2015). Due to the slow growth and long life spans of many deep-sea taxa (Young, 2003) they are particularly vulnerable to the impacts associated with tailings placement such as hyper sedimentation, changes in grain size or toxicity effects (Kvassnes et al., 2009; Kvassnes and Iversen, 2013). To our knowledge, this experimental study is the first

of its kind to investigate the impacts of different levels of inert mine tailings and sediment deposition on both, structure and functioning, of upper bathyal soft-bottom communities.

4.4.1. Substrate addition induces structural changes of the benthic community

Sediment characteristics substantially determine the composition of benthic assemblages and infaunal communities are generally structured in response to sediment oxygenation, food availability and biotic interactions (Moodley et al., 2000; Seiderer and Newell, 1999; Snelgrove and Butman, 1994; Vanreusel et al., 1995). In our experiment, the physical modifications from substrate addition were visible in the different properties of the new, added substrates with much coarser grain size, lower porosity and a low total organic carbon content in the mine tailings and relatively high total organic carbon content in the dead sediment. These results confirm the successful addition of both substrates and physical modification of the upper sediment layers.

Furthermore, the addition of each of the two substrates immediately changed oxygen conditions in the natural sediment. The two most extreme treatments (3 MT and 3 DS) resulted in anoxic conditions of the natural sediment throughout the entire experiment duration. The first hypoxia related response of benthic invertebrates is migration to more suitable areas in the sediment, although successful migration strongly depends on the mobility of the individual taxa or species and the properties of the added substrate (e.g. grain size, porosity) (Diaz and Rosenberg, 1995; Hendelberg and Jensen, 1993; Jansson, 1967; Maurer et al., 1986; Wetzel et al., 2001). Accordingly, we observed migration into the added substrate of most metazoan taxa and an upward shift in meio- and macrofauna community composition in all treatments. Successful migration of benthic organisms into added, non-native substrates is well-known (Bolam, 2011; Maurer et al., 1986; Schratzberger et al., 2000), however, the upward shift in our experiment was accompanied by a high nematode-mortality in both substrate additions. Without the applied nematode staining technique this observation would have been missed and the impacts of substrate addition on nematodes would have been underestimated.

In the mine tailings, nematode mortality was possibly linked to a strongly reduced food availability. Compared to other taxa, nematodes are often reported to be able to survive situations of severe stress, such as temporary hypoxia or seawater acidification (Josefson and Widbom, 1988; Schade et al., 2016; Widdicombe et al., 2009) and the capability to change feeding modes to some extent may be advantageous in those situations (Moens and Vincx, 1997). Bacterial growth

strongly depends on the availability of organic matter (Danovaro, 1996; Eiler et al., 2003; Kirchman and Rich, 1997) so despite the fact that the tailings were well oxygenated, the low organic carbon content did not support strong bacterial growth resulting in very low food availability for meio- and macrofauna inside the added layer. The survival of nematodes in low food conditions is very species dependent but generally an incubation time of 10 or more days, as applied here, is sufficient to set the worms at their limit (Ott, 1972; Ott and Schiemer, 1973; Wieser et al., 1974).

In contrast, the mechanism behind the nematode mortality and reduced densities in the DS treatment is likely different from that of the MT treatment. High bacterial biomass and total organic carbon in the added dead sediment, compared to conditions in the control, should have led to higher concentrations of food for the nematodes. Nevertheless, we observed a strong reduction in nematode densities and high proportions of dead nematodes in all layers throughout the core. Here, the cause is likely linked to the anoxic conditions in the sediment produced by the high bacterial activity in terms of carbon uptake and resulting increased oxygen demand. In a laboratory experiment with an intertidal meiofauna community, Steyaert et al. (2007) found that suboxic and anoxic conditions for 14 days led to a decrease by about a third in nematode densities with species specific survival. Similarly, other studies report hypoxia associated mortality of nematode fauna and shifts in meiofauna community composition towards hypoxia adapted species (Hendelberg and Jensen, 1993; Moodley et al., 1997; Van Colen et al., 2009; Wetzel et al., 2001). Low nematode densities in the 3 DS treatment possibly result from a combination of the high mortality and decomposition of dead nematodes, potentially also facilitated by the longer incubation time in the dead sediment treatments.

Macrofaunal responses were less obvious and organisms showed no signs of mortality. Nevertheless, while tailings addition resulted in an upward shift of the entire macrofauna community, dead sediment addition resulted in a less clear distinction between surface and subsurface community composition, compared to the control situation. The stress response of macrofauna is species-specific and depends on their mobility, oxygen requirements and feeding type (Chou et al., 2004; Hinchey et al., 2006; Smit et al., 2008). In a metanalysis, Smit et al. (2008) predicted from marine species sensitivity distributions that instantaneous burial with 5.4 cm of natural sediment would negatively affect about half of the 32 analyzed macrofauna species. Furthermore, burial with 0.63 cm already affected 5% of the tested macrofauna species. Substrate addition and especially tailings addition may lead to emigration or death of certain macrofaunal organisms on a longer term if requirements of oxygen and food availability are not met. While

several studies on shallow water ecosystems report on the effects of burial with sediment following dredging (e.g. Bolam, 2011; Bonvicini pagliai et al., 1985; De Grave and Whitaker, 1999; Thrush and Dayton, 2002) or strong hydrodynamic disturbances (e.g. Dernie et al., 2003; Miller et al., 2002 and citations therein), we need to be cautious in comparing these results with tailings burial. Indeed, as observed in this study, the specific characteristics of tailings in terms of grain size, organic matter content and porosity (disregarding any toxicological properties) compared to the natural sediment led to differing structural responses and community functioning (see 4.2.).

The differential response of meiofauna and macrofauna to low oxygen concentrations and starvation contradicts the general perception that the meiofauna are more resistant to different stressors than macrofauna. Many authors studying the effect of hypoxia on benthic communities report a more negative effect of long hypoxic events on macrofauna, including mortality, whereas meiofauna is generally less affected and can withstand long hypoxic events (Diaz and Rosenberg, 1995; Josefson and Widbom, 1988; Van Colen et al., 2009; Weigelt and Rumohr, 1986). However, these studies focused on hypoxia in the water column which was not the case in our experiment where the overlying water was always well oxygenated. Here, the greater capacity of macrofauna to move vertically in the sediment could have enabled organisms to reach oxygenated layers as well as food-rich surface layers ensuring their survival. The loss of distinction between the macrofaunal community composition in different depth layers in the dead sediment treatments could be a first indication to support this hypothesis. Thus, while meiofauna could not compensate the very rapid occurrence of anoxic conditions in the sediment by vertical migration, macrofauna may have been more successful in doing so.

Despite a comparatively low standing stock, the high activity and life cycle turnover rates of meiobenthic organisms make them a particularly important part of the benthic environment when it comes to biomass production and food consumption (Coull, 1999; Gerlach, 1971). Furthermore, they can exert a strong impact on other benthic organisms by enhancing bacterial productivity (Gerlach, 1978) and influencing macrobenthic species interactions which can result in modified ecosystem properties (Piot et al., 2013). Therefore, the high nematode mortality induced by substrate burial may lead to strong repercussions on other benthic organisms on a longer term.

4.4.2. Community functioning changes as a result of structural changes induced by substrate deposition

In this study we observed strong, negative effects of substrate addition on the benthic community structure in terms of biomass and composition. These changes led to adverse effects of benthic functioning in terms of respiration rates and organic matter processing. Oxygen is a key element in the aerobic respiration and metabolism of organisms and, thus, tightly linked to the mineralization of organic carbon and the activity of benthic organisms. Therefore, OPD and SCOC can provide a reliable indication of organic matter remineralization rates (Moodley et al., 1998) and, combined with stable isotope carbon uptake data of the biota, has proven to be a good tool to assess ecosystem functioning and responses of the benthos to environmental disturbances (Bratton et al., 2003; Sweetman et al., 2016, 2014, 2010).

Immediately after settlement of the added substrates, a shift in oxygen penetration depth occurred in most treatments. OPD did not decrease at 0.1 and 0.5 cm tailings addition but shifted upwards leaving previously oxygenated deeper sediment layers anoxic. Furthermore, despite an increase in OPD at 3 cm of tailings addition compared to the control, the underlying, previously oxygenated sediment became anoxic because oxygen did not penetrate through the mine tailings layer to the natural sediment anymore. Similarly, Cummings et al. (2009) observed an upward shift of the OPD when marine sediments were exposed to very thin layers of terrestrial sediments. In this study OPD (measured throughout 3 days) penetrated ±0.7 mm in the control situation, while after the addition of ±1.1 mm of terrestrial sediment oxygen only penetrated ±0.3 mm into the underlying sediments. Thus, comparable to our study, organisms inhabiting the surface sediment will become exposed to a deterioration of biogeochemical conditions after the addition of a non-native substrate. In our experiment, however, OPD in the tailings treatments became shallower towards the end of the experiment possibly by a higher biogenic activity inside the tailings due to faunal migration. Sediment deposition resulted in a decreased OPD at the start of the experiment that might be linked to strong microbial respiration due to high organic matter contents of the added sediment. Also in this case, underlying sediment layers were exposed to biogeochemical changes negatively affecting structure and functioning of biota. Towards the end of the experiment OPD deepened to values comparable to the control indicating a possible stabilization of the biogeochemical conditions in the dead sediment treatments to a pre-disturbance state.

Values of SCOC in our control incubations were comparable to those reported in other studies with Norwegian fjord fauna (Ishida et al., 2013; Sweetman et al.,

2016, 2014). The SCOC measurements after 8 and 13 days of incubation (MT and DS, respectively) informed about the effect of substrate addition on the SCOC, while the second measurement informed about the response of the sediment community to input of algal detritus. Mine tailings burial reduced oxygen consumption in the 0.5 and 3 cm treatments, but SCOC increased in the dead sediment treatments when compared to the control. Low organic matter content associated with low bacterial biomass and faunal mortality may explain the reduced SCOC measurements in the mine tailings treatments. On the contrary, high organic matter content and an increase in SCOC in the dead sediments may point towards increased bacterial activity as they play a key role in the carbon turnover in marine sediments (Deming and Baross, 1993; Rowe and Deming, 1985). However, in the dead sediment treatments, addition of fresh organic matter in the form of labeled algae did not lead to a pronounced increase in oxygen consumption as it did in the mine tailings treatments and the control. This is possibly due to the already high rate of organic matter processing and strong bacterial respiration resulting from the organic matter input originating from the dead sediment itself. It is important to note that SCOC increased in all treatments after algae addition but, at the same time, the negative effect of tailings addition on the processing of a new food source became more pronounced when compared to the control situation. This way even a deposition of 0.1 cm sufficed to induce a significant reduction in SCOC, illustrating that the benthic community was hampered to process fresh organic matter by as little as 0.1 cm of tailings.

Bacteria dominate deep-sea ecosystems in terms of abundance and biomass and are the main contributors to organic matter remineralization (Danovaro et al., 2014; Wei et al., 2010). Similarly, in our experiment we observed that bacteria had a higher biomass and took up considerably more added algal carbon compared to macro- and meiofauna. Interestingly, at 0.1 cm tailings addition, bacterial tracer uptake and biomass remained close to control conditions while a decreasing trend of tracer uptake was already visible for macro- and meiofauna. Here, the tailings layer may have posed a physical barrier for those organisms to reach the new source of organic matter present on the sediment surface. At 3 cm tailings addition, low bacterial biomass and possibly reduced faunal activity in terms of bioturbation lead to a lower fraction of the added algae being transported to deeper layers and an overall reduced tracer uptake. Bioturbation by infauna strongly influences ecosystem functioning, especially in sediments where disturbances are low, as it provides structure to the sediments and is responsible for irrigation, transport of nutrients and organic matter to deeper layers and providing various microhabitats for meiofauna and bacterial communities (Braeckman et al., 2010; Mermillod-Blondin et al., 2004; Meysman et al., 2006).

Continental margins are responsible of 10-15 % of the global ocean primary production and fulfill an important role in the sequestration of atmospheric carbon and transport to the deep-ocean (Fennel, 2010; Muller-Karger et al., 2005). Furthermore, with high denitrification rates these regions adjacent to land boundaries act as a barrier for nitrogen input from land and atmosphere into the open ocean (Fennel, 2010). This disproportional contribution to the total ocean nutrient cycling is the result of tight biological interactions, biogeochemical transformations facilitated by microorganisms and characteristic hydrodynamics (Hofmann et al., 2011; Renaud et al., 2007). Therefore, disturbing these ecosystems by activities such as mine tailings placement, can have implications on a much larger scale.

4.4.3. The origin of the added substrate results in differential responses

This study clearly illustrated how both substrates used in this experiment resulted in differential responses. Mine tailings addition mainly induced food-limitation for all benthic compartments in the added layers, whereas the high bacterial respiration in the dead sediment layer initially led to oxygen limitation in deeper layers. In the sediment addition treatments, oxygen conditions seemed to return to conditions similar to the control indicating a possible biochemical recovery of the sediments to normal conditions after the 15 day period. With bacterial and macrofaunal biomass and uptake being similar to control conditions it is possible that those taxa might recover relatively fast. Meiofauna, however, suffered most in this scenario with strongly reduced densities, low carbon uptake, and increased mortality. The interconnectedness of the three benthic compartments is widely acknowledged and changes in one taxon can have strong repercussions on the other (Alongi and Tenore, 1985; Evrard et al., 2010; Gerlach, 1978, 1971; Piot et al., 2013), thus we cannot exclude the possibility of adverse effects on the long term. As a naturally occurring phenomenon, marine organisms are to some extent adapted to sedimentation and resuspension, and ecosystems may show increased resilience to sediment disturbance, particularly if they are subjected to a high intensity of natural disturbance (Leduc and Pilditch, 2013; Schratzberger and Warwick, 1998). However, man-made sedimentation events may exceed natural variability in sediment load and frequency and may induce permanent changes in the ecosystem. In the mine tailings treatments no signs of recovery of the benthic community to control conditions were observed within the experiment duration of 11 days. In fact, monitoring studies for submarine tailings placement have shown that after cessation of extensive tailings discharge (up to 4-5 cm tailings addition per year during >20 years) it may take one to four years before the tailings are fully

recolonized while differences in community compositions still prevail (Burd, 2002; Olsgard and Hasle, 1993). However, it remains uncertain if, accompanied with faunal recovery, also ecosystem function will return to normal values. Unfortunately, controlled experiments to determine threshold levels for sediment overburden and tolerated frequencies are largely missing (Miller et al., 2002).

Our study contributes to reducing this knowledge gap since comparing different deposition depths and substrates allows us to gain some information on threshold values and differentiate the effects of substrate characteristics on benthic community structure and functioning. When applying the precautionary principle in a submarine tailings placement scenario, instantaneous depositions with as little as 0.1 cm of tailings over large areas have to be avoided to maintain ecosystem functioning in terms of organic matter remineralization at normal levels. Structural changes of biota with reduced biomass and shifts in vertical distribution become apparent at 0.5 cm burial with tailings and intensify at 3 cm tailings deposition. It remains unclear how fast biological communities can recover from the short-term effects and how repeated burial with tailings will affect species survival on a longer term. Furthermore, macrofauna is often used to assess and monitor environmental impacts, but was actually the most tolerant group in our experiment while the response of meiofauna was much more pronounced. Therefore, monitoring studies should make use of a more integrated approach covering multiple size groups representing different functional traits and trophic levels.

4.5. Conclusion

Our research clearly shows that burial with both, mine tailing or dead sediment, has strong negative effects on the biota and the functioning of benthic communities. However, the processes behind the impacts were different between the two substrate additions.

The most severe effects were observed at 3 cm of tailings deposition with a reduction of bacterial and meiofaunal biomass by more than half, reduced algal carbon uptake of all biological compartments and reduced sediment community oxygen consumption. However, already at 0.1 cm tailings deposition, the ability of the benthic community to process organic matter was significantly reduced while the structure of the community remained largely unaffected at this level. This emphasizes the importance of using multiple trophic levels and an ecosystem-based approach in laboratory experiments including measures of ecosystem functioning. The addition of dead sediment, on the other hand, resulted in an increase in bacterial activity causing severe anoxia in the underlying sediment layers entailing decreased meiofaunal biomass and changes in the vertical distribution of

macrofauna. While productivity in terms of bacterial biomass and carbon turnover are enhanced on the short term, mortality of nematodes and resulting shifts in benthic community composition might induce unforeseen consequences on the longer term. While possibly less obvious in measurements of abundance, physical disturbance and changes in sediment characteristics may substantially influence infauna community composition, particularly that of meiofauna (Leduc and Pilditch, 2013; Schratzberger et al., 2000). Zeppilli et al. (2016) identified a positive exponential relationship between nematode biodiversity and ecosystem functioning and efficiency in different deep-sea habitats. Hence, reductions in biodiversity and changes in community composition may result in decreased ecosystem functioning and a reduced resilience of the system to different additional stressors (Gessner and Hines, 2012; Steudel et al., 2012; Zeppilli et al., 2016).

We need to be aware of the differential response to burial with different substrates when assessing impacts of mine tailings placement on the benthic environment. This study shows that the particular characteristics of the sediment e.g. organic matter content, porosity or grain size strongly influence the biochemistry inside the sediments and the way the ecosystem responses to substrate burial. Therefore, more research is needed using substrate with similar characteristics of the effectively placed tailings. Furthermore, our study indicates that vast areas impacted by low tailings deposition might experience a reduced carbon mineralization capacity, especially over the short-term. While the thickness of mine tailings in the direct surrounding of the deposition site can reach very high values with deposition rates of several m y^{-1}, the seafloor may still be impacted by deposition rates of 1 mm y^{-1} several km off the deposition site (Olsgard and Hasle, 1993). The wider implications will depend on the scale of tailings discharge and the resilience of the targeted ecosystem. If DSTP is implemented in more regions worldwide environmental managers need to be aware that even small deposition rates might negatively impact very large areas disrupting the functioning of important benthic environments.

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Chapter 5 Responses of an abyssal meiobenthic community to short-term burial with crushed nodule particles in the South-East Pacific

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Author contribution statement: AV, AB, KG and LM conceived the study. LM conducted the experimental work and collected the samples. Meiofauna samples were analyzed by LM and KG, nematode copper content was measured and analyzed by BL and LV and sediment metal contents were provided DV and JDG. LM performed the statistical data analysis, LM, BL, KG and AV interpreted the results and LM wrote the manuscript with the assistance of KG and AV.

Abstract

The increasing metal demand due to a rapid technical development drives the exploration and exploitation of deep-sea mineral resources such as polymetallic nodules. To elucidate the potential effects of the deposition of crushed polymetallic nodule particles on abyssal meiofauna communities, an *in situ* experiment at the Peru Basin in the South East Pacific Ocean was conducted. We covered abyssal, soft sediment with 2 cm of crushed nodule particles and sampled the sediment after eleven days of incubation. Meiofauna community composition and vertical distribution in the sediment and in the added substrate, as well as nematode genus composition, were analysed. Additionally, copper burden in a few similar-sized, but randomly selected nematodes was measured by means of μ-X-ray fluorescence. At the end of the experiment, 45.48 ± 0.95 % of the total meiobenthos occurred in the added crushed nodule layer and particularly nematodes, copepods and nauplii from the upper 2 cm of the natural sediment migrated into the added substrate. Densities and community composition in the deeper 2-5 cm layers remained similar. Nematode community composition in the added substrate did not differ from communities in underlying layers or the Control. Nematode copper burden did not indicate elevated copper toxicity resulting from burial with crushed nodule particles. We discuss our results in the light of previous research and identified similar patterns of meiofaunal vertical migration that emerge from substrate burial in different marine environments pointing to an escape response reaction and, therefore, potentially having significant implications for the risk assessment of deep-sea mineral extraction.

5.1. Introduction

The interest in mineral deposits from the deep seafloor commenced in the early 70s, after the discovery of a widespread occurrence of economically valuable polymetallic nodules (Glasby, 2000; Mero, 1977). However, economic restrictions and technological limitations of working in the deep sea at that time hampered further action towards exploitation of those high-grade deposits. The advancements in deep-sea technology and other socio-economic developments have led to a new surge for deep-sea minerals in the past decades and legal frameworks are being developed to manage their extraction in international waters (Lodge et al., 2014). Polymetallic nodules are small, rock-shaped concretions of ore that lie on the surface of abyssal sediments in water depths between 4000 – 6500 m and cover large areas of the Pacific and Indian Ocean (Hein and Koschinsky, 2014). Besides the high content in valuable minerals, nodules exhibit a high porosity, low bulk density and fine grain size with very slow formation and growth rates of < 250 mm My^{-1} (million years) (Jain et al., 1999; Von Stackelberg, 2000). These properties result in very brittle structures that are easily damaged or broken when applying low force (Charewicz et al., 2001; Jain et al., 1999; Thiel et al., 1993; Zenhorst, 2016). Therefore, breakage and abrasion of nodule particles is very likely to occur during a mining operation with heavy gear.

Polymetallic nodule mining is expected to have various direct and indirect environmental impacts such as nodule removal, removal of surface sediment, sediment compaction, sediment suspension and deposition, discharge of tailings material and potential release of toxic amounts of heavy metals (Clark and Smith, 2013; Rolinski et al., 2001; Sharma et al., 2001; Thiel, 2001). Additionally, nodule particles abraded during collection may get mixed with the suspended sediment and redeposit in areas close or further away of the mined site, depending on their sinking rate (Oebius et al., 2001). An economically viable mining operation would cover 300-800 km^2 per year (Smith et al., 2008a) and after 20 years an estimated 8500 km^2 would have been mined per concession area (Madureira et al., 2016). Such a large scale mining operation is expected to directly impact the nodule fauna since polymetallic nodules provide hard substrate for a variety of sessile epifauna such as sponges, corals, crinoids or sea pens, but also to associated mobile fauna including isopods, ophiuroids or octopods (Purser et al., 2016; Vanreusel et al., 2016). However, deposition of sediment and nodule particles on the seafloor resulting from mining activities may also impact the typical abyssal soft-sediment fauna, but knowledge about the direct responses of those organisms to substrate deposition is scarce.

In the abyssal deep sea, meiobenthos (>32 μm), dominated by nematodes, constitute the most dominant metazoan component of infaunal communities in terms of biomass (Rex et al., 2006). Moreover, their ubiquity and high abundance in deep-sea sediments underline the importance of meiobenthos for abyssal ecosystems (Sinniger et al., 2016). Due to their residence inside the sediment, nodule mining will inevitably disturb meiofaunal communities, directly or indirectly. Directly through the removal of the sediment surface layers which cause removal and redistribution of meiofaunal organisms and indirectly through sediment deposition which may have consequences for the survival and vertical structuring of underlying meiobenthic communities. Therefore, this group of organisms is particularly well suited for the assessment of environmental impact related to deep-sea mining activities (Radziejewska, 2014).

Previous research on the effect of nodule mining suggests that abyssal benthic communities have the capacity to recover from small scale sediment disturbances, although full recovery is a long lasting process which may still be incomplete several decades after the disturbance (Gollner et al., 2017). These findings are based on eleven small-scale disturbance scenarios where nodules were removed or ploughed (overview given in Jones et al., 2017). In general, recovery of mobile fauna occurred faster than that of sessile fauna and small organisms tend to recover faster than large organisms (Gollner et al., 2017; Jones et al., 2017). Nevertheless, even meiofaunal nematode communities, comprising small, non-sessile organisms, still deviated from their initial state and from undisturbed sites 26 years after experimental disturbance inside experimentally dredged tracks (Miljutin et al., 2011). Furthermore, meiobenthic densities were significantly reduced immediately following sediment burial resulting from resuspension with a benthic disturbing device, but recovered after 2 years (Kaneko et al., 1997). It was observed that meiofaunal densities remained highest in the top sediment layer suggesting an upward migration of meiobenthic taxa into the deposited sediment (Kaneko et al., 1997). Vertical distribution changes have also been observed in studies where benthic soft-sediment fauna from bathyal and shallow-water environments was buried with native and non-native substrates (Maurer et al., 1986; Mevenkamp et al., 2017a; Schratzberger et al., 2000).

To evaluate the short-term effects of substrate burial on the structure of the meiobenthos community, we deposited a 2 cm layer of crushed nodule substrate on enclosed, undisturbed abyssal sediments in the South-East Pacific, using a remotely operated vehicle (ROV) at 4200 m depth. Density and community structure of the meiobenthos as well as the vertical structuring after eleven days of incubation was assessed in treatments with and without crushed nodule substrate deposition. Furthermore, as nodules contain potentially toxic concentrations of

heavy metals such as copper, we measured individual nematodes using X-ray spectrometry to identify copper content in their body tissue, thereby gaining information about copper bioavailability and uptake in animal body tissues.

5.2. Material and Methods

5.2.1. Experiment set-up and sampling

The substrate burial experiment was performed *in situ* during RV *Sonne* cruise SO242-2 (28.08.2015 - 01.10.2015) at the southern reference site of the DISCOL experimental area in the Peru Basin, Southeast Pacific (7°7.51 S, 88°27.02 W, in 4196 m water depth; Thiel and Schriever, 1989). For this purpose the ROV *Kiel 6000* (GEOMAR, Germany) was used to insert six stainless steel rings (ø = 25 cm, height = 15 cm) into undisturbed sediment avoiding enclosure of nodules or megafauna. The cores were gently pushed 10 cm into the sediment until the collar around the rings touched the sediment surface (Fig. 5.1 A). Subsequently, a substrate distributing device (Fig. S5.1) filled with 250 mL crushed nodule substrate was deployed on three of the steel rings (Burial treatment = SD, Fig. 5.1 B) the other three rings served as an experimental control (Control). Rotation of

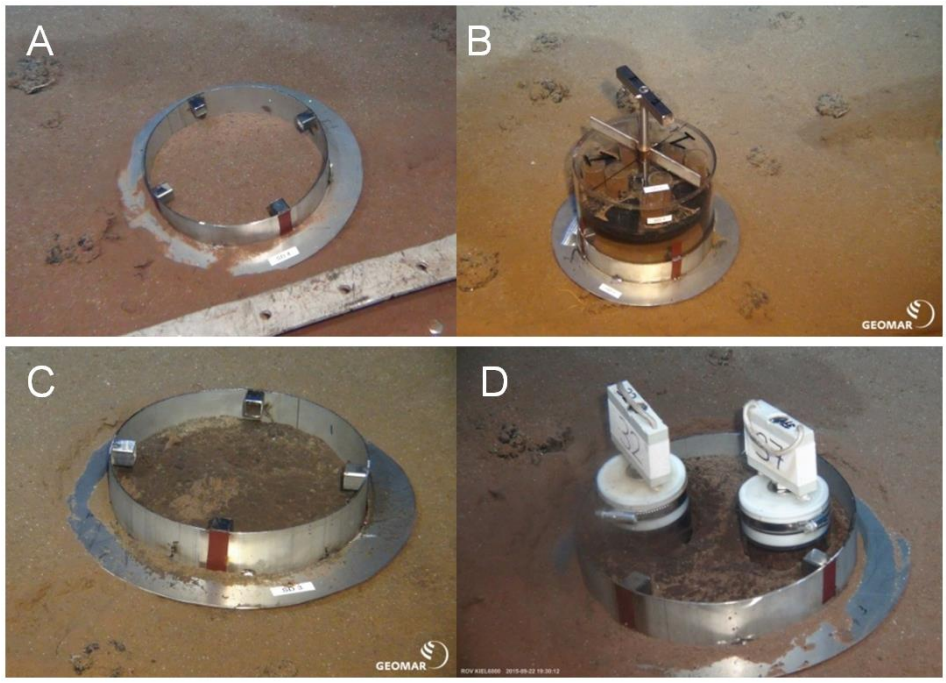

Figure 5.1 Impressions of the deployment and sampling during the *in situ* experiment. A) Stainless steel ring pressed into the abyssal sediment; B) Filled substrate distributing device on top of a stainless steel ring before substrate release; C) Sediment surface after 11 days of incubation; D) Push core sampling at the end of the experiment. Copyright: ROV *Kiel 6000* Team/GEOMAR, Germany

the T-handle activated the release of the substrate that was filled inside the tubes of the device. This resulted in a roughly homogenous distribution of crushed nodule substrate onto the sediment surface over the steel ring area with a thickness of approximately 2 cm (Fig. 5.1 C). After ~20 h, the sediment distributing devices were removed from the steel rings to allow complete settlement of all nodule particles after deployment and to ensure open water exposure during the remaining time of the experiment. After a total incubation time of eleven days, the sediment in each steel ring was subsampled with two push cores (7.4 cm inner diameter, Fig. 5.1 D).

On board, the overlying water in the push cores was carefully siphoned off and sieved (32 µm) to retain any meiobenthos. Subsequently, the sediment was sliced in several depth layers (added substrate layer, 0-1 cm,1-2 cm and 2-5 cm sediment depth) in a climate controlled room at *in situ* temperature (2.9 °C). Sediment of each slice was homogenized and a 5 mL subsample was taken for bulk sediment metal content analysis. Of each set of push cores, one was used for meiobenthos community analysis and slices were fixed in 4% Borax buffered formaldehyde. The retained meiobenthos from the overlying water of that core was added to the sample of the uppermost sediment layer. The second push core served for analysis of sediment characteristics (granulometry, total organic carbon content, total nitrogen content, pigment analysis). Of this push core, a 5 mL subsample of the 0-1 cm layer was taken for pigment analysis and stored at -80 °C; the remaining sediment and the sediment of other depth layers was stored at -20 °C. Unfortunately, one core of the Control treatment was lost during slicing, leaving n = 2 replicates for environmental analyses of the Control.

5.2.2. Meiobenthos analysis

Meiobenthos sediment samples were washed on two stacked sieves of 32 µm (lower sieve) and 1000 µm (upper sieve) and extraction of the 32 µm fraction from the sediment was achieved by density gradient centrifugation with the colloidal silica solution Ludox HS40 (specific gravity of 1.18)(Somerfield et al., 2005). After each of three centrifugation rounds (3000 rpm, 12 min), the meiobenthos in the supernatant was retained on a 32 µm sieve. Subsequently, the sample was fixed in 4% buffered formaldehyde and stained with a few drops of Rose Bengal solution. Meiobenthos was identified to higher taxonomic level using a stereo microscope (50x magnification).

From each sample, approximately 50 nematodes were picked, transferred stepwise to anhydrous glycerine following the formalin-ethanol-glycerol protocol of (De Grisse, 1969) and mounted on paraffin-ring glass slides for microscopic identification. Nematodes were identified with a Leica DMLS compound

microscope (10 x 100 x magnification) to genus level consulting e.g. Guilini et al. (2017) and Platt et al. (1983).

5.2.3. Sediment characteristics and metal contents

Sediment grainsize analysis was done by laser diffraction with a Malvern Mastersizer 2000 particle analyzer (Malvern Instruments, UK) and sediment fractions were classified according to (Wentworth, 1922). Total organic carbon (TOC) and total nitrogen (TN) content in the sediments were analyzed with an Element Analyser Flash 2000 (Thermo Fisher Scientific) after lyophilization, homogenization and acidification with 1 % HCl.

Sediment bulk concentrations of Fe_2O_3 (%), MnO (%), Cu, Ni and Co (ppm) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) following protocol nr. 14869-2:2002(E) of the International Organization for Standardization (2002).

5.2.4. Individual nematode copper content

To determine copper contents in nematodes, respectively 6 and 11 similar-sized and shaped nematodes were taken from one replicate sample of the added crushed nodule layer and the uppermost layer of one background sample. Nematodes were transferred to a drop of water and body length (L, µm, excluding filiform tail) and average width (W, µm, measured at three different positions in the middle body region) were determined under a compound microscope connected to a Leica camera system. These measures were used to estimate nematode wet weight (WW) using an adjusted Andrassy (1956) formula to account for the specific gravity of marine nematodes (i.e. 1.13 g cm^{-1}): µg WW = L x W^2/1,500,000 (as described in Pape et al., 2013).

Nematodes were then transferred to 500 nm thin silicon nitride membranes (Silson Ltd, United Kingdom) by means of a small drop of MilliQ water and left to air-dry. Subsequently, copper contents were assessed by means of micro X-ray fluorescence (µXRF) using the Edax Eagle III (Edax Inc., USA). This instrument is equipped with a 50 W Rh X-ray tube fitted with polycapillary optics which focus the X-ray in a 30 µm spot. A liquid nitrogen cooled Si(Li) detector is employed to capture the fluorescent X-rays. To examine the Cu content of the organisms, small mappings were performed with 30 µm step size; each measurement point of these mapping contains a full XRF spectrum with 10 s live time. These spectra are analysed using AXIL, an iterative least squares algorithm yielding the net intensities for each detectable element present in the sample. The points belonging to the organism are extracted from the XRF element maps using k-means clustering.

Next, the spectra from these data points are summed to obtain the total intensity generated by the nematode during the measurement. The intensities per nematode are normalized using nematode wet weights. Due to the small diameter of the organisms (~ 30 μm) the absorption effects on Cu are negligible, so the normalized intensities of the different scans can be compared directly with each other, in other words, a nematode with more Cu present in its body will yield a higher intensity (counts) per unit body mass (in μg).

5.2.5. Data analysis

Meiofauna densities were expressed as the number of individuals per 10 cm^2 in the different depth layers and over the whole sampled depth (total densities). Due to the unequal thickness of the sampled depth layers, differences in community composition were examined based on relative abundances of the different meiofauna taxa in each depth layer.

K-dominance curves of nematode genera over the whole core were calculated based on untransformed density data (ind. 10 cm^2) and plotted in Primer 6. Additionally, diversity indices (Shannon-Wiener, Pielou's evenness and Simpson) of the whole core community were compared between treatments in univariate analyses. Differences in nematode genus composition between treatments and depth layers was analysed based on relative abundances, only.

Due to the low number of replicates and the unbalanced design of the study with an unequal number of depth layers, statistical differences between treatments and depth layers in multivariate datasets (sediment TOC and TN contents, meiofauna community composition, nematode genus composition) were investigated with a cluster analysis (cluster mode = group average) combined with a similarity profile test (SIMPROF) to indicate statistically significant clusters. For abiotic data, an resemblance matrix based on Euclidean distances was used while biotic data (meiofauna and nematode genera community composition) were analysed based on Bray-Curtis-similarities. Interpretation of the results was further based on a visualization with multidimensional scaling (MDS) plots and on the similarity percentages analysis (SIMPER) of significant cluster groups.

Differences of univariate measures (heavy metal contents, total meiofauna densities) between treatments were tested with a student's t-test in R (R Core Team, 2013) after ensuring normality (Shapiro-Wilk test) and homoscedasticy (Levene's test) of the data or, alternatively, with a Wilcox test as non-parametric test.

An $\alpha = 0.05$ significance level was chosen for all statistical analyses.

5.3. Results

5.3.1. Sediment characteristics and metal contents

The analysis of total organic carbon and nitrogen contents between treatments and depth layers revealed two significant clusters branching at a distance of 0.4 ($\pi = 0.03$, $p = 0.001$). The first cluster was composed of all added substrate layers (NOD) and the second cluster contained all remaining samples. Differences were caused by lower TN and TOC contents in the crushed nodule layer (TN: 0.200 ± 0.004 %, TOC: 0.385 ± 0.002 %) compared to the Control (TN: 0.413 ± 0.045 %, TOC: 0.768 ± 0.018 %) and the underlying sediment layers (TN: 0.399 ± 0.015 %, TOC: 0.706 ± 0.018).

The Control sediment mainly consisted of silt (75.613 ± 0.179 %), clay (12.816 ± 0.165 %) and very fine sand (8.919 ± 0.168 %) with a median grain size of 20.753 ± 0.300 µm, which was similar in the 0-5 cm of the Burial treatment (median grain

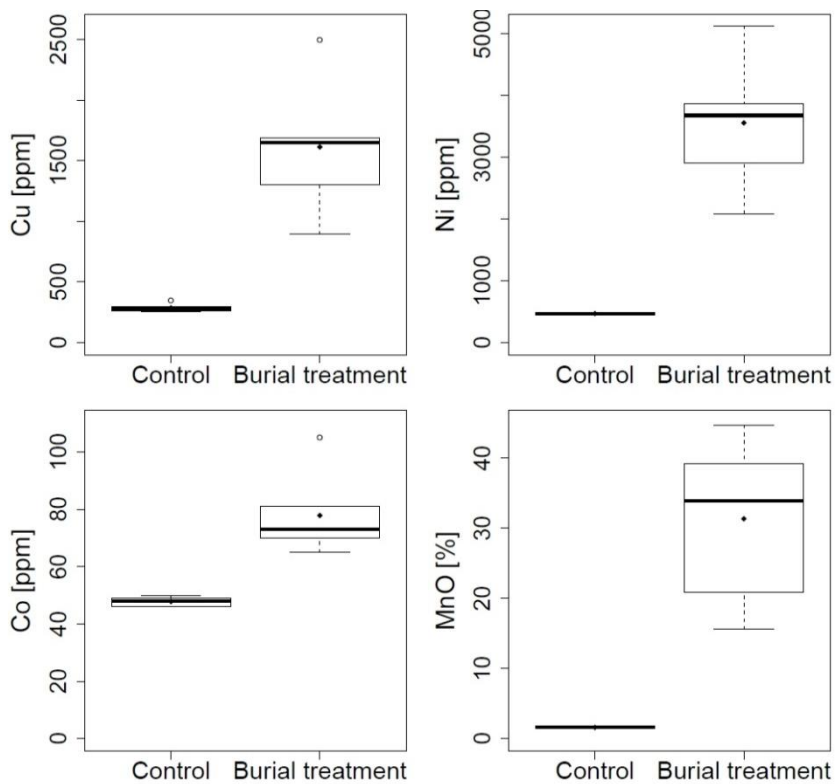

Figure 5.2 Box-Whisker plot of metal contents measured in the 0-1 cm layer of the Control and the added crushed nodule layer of the Burial treatment. Black line depicts the median whereas point indicate the mean of the samples.

size: 22.043 ± 0.302 µm). In contrast, the crushed nodule substrate contained much coarser grain fragments in the mm to cm range (Fig. S5.2).

Concentrations of Cu, Mn and Ni were more than three times higher in the crushed nodule substrate compared to the Control sediments (Fig. 5.2).

5.3.2. Meiobenthic community composition and vertical distribution

After 11 days of incubation, total meiobenthos densities ranged from 275.7 ± 9.7 ind. 10 cm^{-2} (mean ± standard error (SE)) in the Burial treatment to 302.65 ± 24.1 ind. 10 cm^{-2} in the Control and did not differ between both treatments (Fig. 5.3A). Overall, nematodes dominated the meiobenthos community (91.0 ± 1.1 %) followed by harpacticoid copepods (4.4 ± 0.6 %), nauplii (3.2 ± 0.7 %) and polychaetes (0.6 ± 0.1 %, Fig. 5.3B). All other taxa (Ostracoda, Tardigrada, Gastrotricha, Isopoda, Mollusca, Tantulocarida and Loricifera) contributed less than 0.5 % to the meiobenthos community.

In the Control, meiobenthos proportions were similar across all depth layers with 40.2 ± 3.0 % of the meiobenthos occurring in the 0-1 cm layer, 27.7 ± 4.7 % in the 1-2 cm layer and 32.1 ± 4.4 % in the 2-5 cm layer. This vertical profile changed in the Burial treatment with 45.5 ± 0.9 % of meiobenthos occurring in the added crushed nodule layer, 13.2 ± 0.9 % in the 0-1 cm layer, 9.9 ± 0.5 % in the 1-2 cm layer and 31.5 ± 1.7 % in the 2-5 cm layer (note the greater sediment depth of the 2-5 cm layer, Fig. 5.3A). While at the end of the experiment 43.4 ± 0.7 % of nematodes over all depth layers were found in the added crushed nodule layer, this percentage was much higher for copepods (71.0 ± 5.5 %), nauplii (60.6 ± 9.4 %) and polychaetes (72.8 ± 13.6 %).

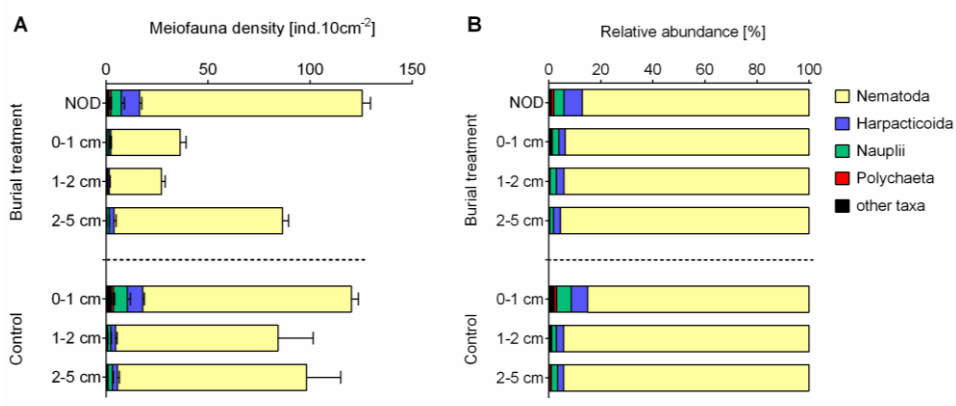

Figure 5.3 Vertical profile of the meiobenthos community in the Control and Burial treatment with a 2 cm layer of crushed nodule substrate (NOD). A: Average densities (ind. 10 cm^{-2}, + standard error) and B) relative abundances of meiobenthic taxa are shown per depth layer in the respective treatments.

Table 5.1 Results of the SIMPER analysis between the significantly different clusters identified in the dataset of relative meiofauna abundances in different depth layers. Av.Abund= average abundance, Av.Diss= average dissimilarity, Diss/SD= average contribution devided by the standard deviation, Contrib%= Contribution to the dissimilarities, Cum%= Cumulative contribution.

	Cluster A	Cluster B				
Group	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Nematoda	86.65	94.68	4.01	2.76	47.52	47.52
Harpacticoida	6.64	2.4	2.12	2.84	25.14	72.66
Nauplii	4.46	2.06	1.3	1.35	15.41	88.07
Polychaeta	0.94	0.39	0.33	1.29	3.88	91.95

Meiobenthos community composition based on relative densities, was similar over the whole depth profile. However, when taking depth layers into account, two significant clusters were revealed branching at 91.55 % similarity ($\pi = 0.99$, $p = 0.001$). The first cluster (Cluster A) was composed of all crushed nodule layers, all 0-1 layers of the Control and one sample of the 1-2 layer of the Burial treatment (Fig. 5.4). While the second cluster (Cluster B) was composed of all remaining samples. Similarities between both clusters were caused by lower abundances of nematodes and higher abundance of harpacticoids, nauplii and polychaetes in Cluster A compared to Cluster B (SIMPER contributions: 47.52 %, 25.14%, 15.41 and 3.88, respectively; Tab. 5.1).

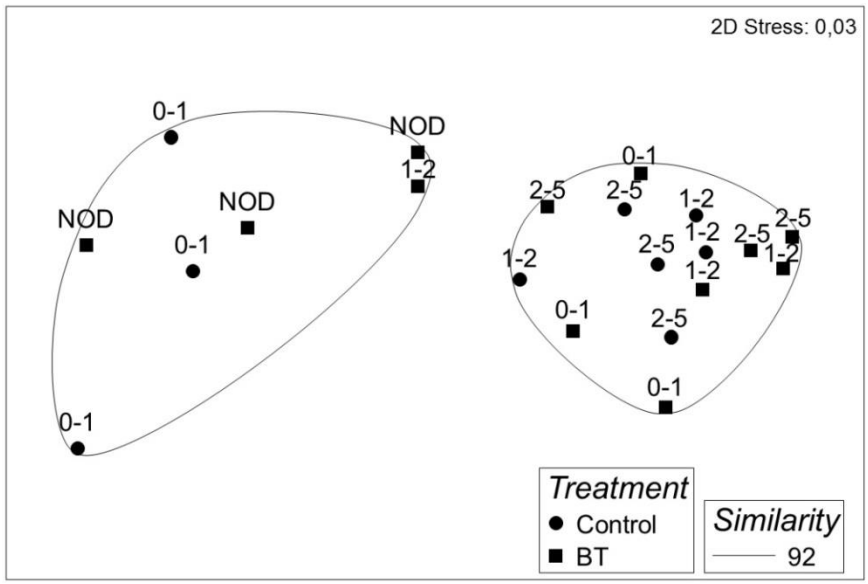

Figure 5.4 MDS plot of the meiobenthos community in each sample of the Control and Burial treatment (BT) per sediment depth layer with overlying contours of significant (SIMPROF test) clusters at an 92 % similarity level. NOD = crushed nodule layer

5.3.3. Nematode generic community composition

Combining all samples, the nematode community, was composed of 96 genera from 33 families (Tab. S5.1). The most dominant genera included *Acantholaimus* (14.3 ± 1.3 %), *Monhystrella* (11.5 ± 1.1 %), *Viscosia* (8.3 ±3.3 %) and *Thalassomonhystera* (5.0 ± 0.6 %), other genera contributed less than 5 % to the overall nematode community. Evenness of nematode genera was higher in the Burial Treatment (0.86 ± 0.01) compared to the Control (0.81 ± 0.01; $t_{2.73} = -3.373$, $p = 0.0499$, borderline significant). Diversity indices were not significantly different between the Burial treatment (Shannon: 3.23 ± 0.06, Simpson: 0.95 ± 0.01) and the Control (Shannon: 3.16 ± 0.08, Simpson: 0.93 ± 0.01). Diversity and evenness are visualized by the k-dominance plots (Fig. 5.5).

The Cluster analysis of relative abundances of nematode genus composition revealed two significant clusters branching at 35.57 % similarity ($\pi = 1.64$, $p = 0.002$, Fig. S5.3). However, due to the low similarity among samples and the lack of clear groupings (e.g. samples of similar depth layers or treatments) within the clusters, we could not assign an ecological meaning to this result.

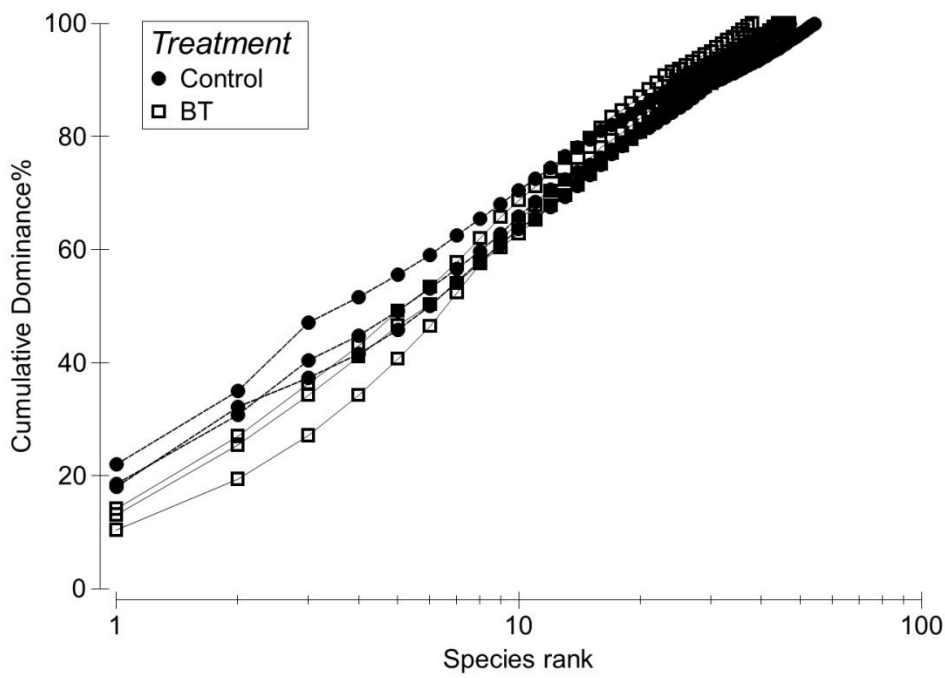

Figure 5.5 K-dominance plot of whole core nematode genera in the Burial Treatment (BT) and the Control.

5.3.4. Copper burden in individual nematodes

Copper contents in nematode bodies could be successfully assessed using micro X-ray fluorescence (Fig. 5.6 A). However, copper burden in the measured nematodes did not differ between treatments (Fig. 5.6 B).

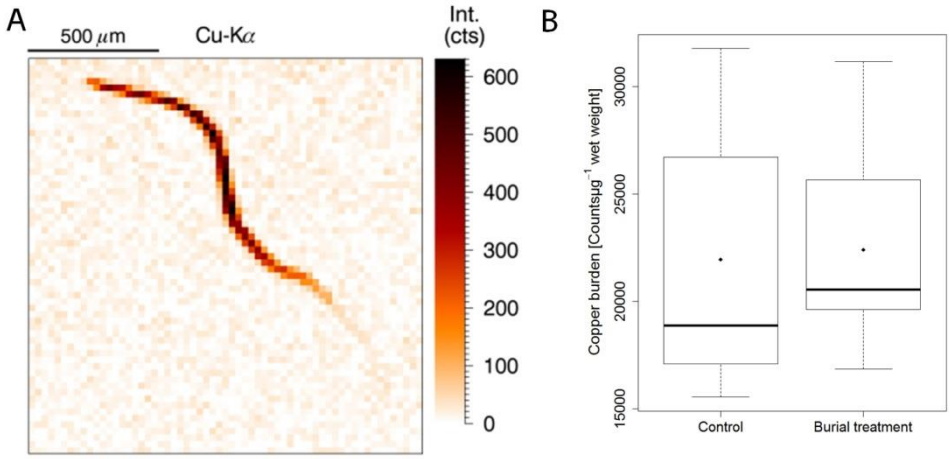

Figure 5.6 A) Example picture of the copper spectrum from a nematode X-ray mapping indicating copper intensity (counts), which is directly correlated to copper concentration. B) Box-whisker plot of the copper burden in nematodes from surface sediment layers of a background sample (n = 11) and from the crushed nodule substrate in the burial treatment (n = 6). Black line depicts the median whereas points indicate the mean of the samples.

5.4. Discussion

5.4.1. Crushed nodule substrate burial induces changes in meiofauna community composition

This research clearly shows how burial with crushed nodule particles changes the vertical distribution of meiobenthos in abyssal sediments in a very short time of eleven days. Almost half of the meiofauna (45.5 ± 0.9 %) was found in the added layer at the end of the experiment and our data suggests that all meiofauna taxa present in the samples were able to migrate into the added substrate. This migration was predominantly seen by fauna from the upper surface layers (0-2 cm) which showed strongly reduced densities compared to the same depth layers of the Control, while meiofauna densities in deeper layers remained comparable between the Control and the Burial treatment. A similar migratory response of meiofauna in artificial sediment has been observed in a different burial experiment conducted during the same sampling campaign to the Peru Basin (Mevenkamp et al., unpublished). Recently, Mevenkamp et al. (2017a) reported the effects of burial with different amounts of mine tailings (0.1, 0.5, 3 cm) and natural sediment (0.5

and 3 cm) on a benthic community of bathyal fjord sediments in a short-term ex-situ experiment. After a comparable experimental duration of 11 days (mine tailings) and 16 days (natural sediment), similar vertical migration patterns were seen, but were associated with considerable nematode mortality in both substrates. While nematode mortality in the mine tailings was attributed to lower food availability, mortality in the sediment deposition treatment was likely caused by hypoxic conditions occurring during the incubation period (Mevenkamp et al., 2017a). Moreover, Mevenkamp et al. (2017a) indicated that burial with 0.1 cm of mine tailings may already reduce the functioning of bathyal, benthic fjord ecosystems in terms of fresh organic carbon remineralization. Especially, nematode uptake of added organic carbon was considerably reduced after burial with 0.5 cm of substrate. Since meiofaunal contribution to the benthic ecosystem in terms of relative abundance and biomass increases with water depth (Rex et al., 2006), it is plausible that the induced changes in meiofaunal distribution -and, possibly, mortality- may entail even stronger effects on the overall functioning of abyssal soft sediments.

The possibility that the migratory response may be accompanied by elevated nematode mortality requires further investigation, especially because re-sedimentation is expected to occur over large areas in a deep-sea mining context (Oebius et al., 2001; Smith et al., 2008a). In this experiment, meiofauna mortality could, however, not be assessed since decompression would cause mortality during sample retrieval from the abyssal seafloor and, therefore, bias the results. Nevertheless, several authors have underlined the importance to assess meiofauna mortality in experimental studies as it may pass unnoticed due to slow decomposition of organic matter in the deep sea (Barry et al., 2004; Fleeger et al., 2006, 2010).

Migratory responses of meiofauna have been widely observed and used in the past to extract meiofauna from sediments (Rzeznik-Orignac et al., 2004; Uhlig et al., 1973). Furthermore, upward migration has also been reported in a shallow-water study investigating the impacts of the disposal of experimental dredging material (Schratzberger et al., 2000). The very consistent findings of changes in vertical structuring of meiofauna from different marine environments give reason to believe that this is an inherent behaviour of upper sediment meiofauna as a response to burial with native and non-native substrates. Interestingly, organisms in the deeper sediment layers of our experiment did not show a migratory response and instead remained at the same depth.

The main drivers of these migratory responses are thought to be oxygen and temperature gradients inside the sediments (Rzeznik-Orignac et al., 2004).

However, the abyssal sediments at our experimental site are very oxygenated with oxygen penetration depths of 5-20 cm (Stummeyer and Marchig, 2001). It is unlikely that the addition of such coarse material as the crushed nodules would have caused anoxic conditions in the underlying sediment or induced a temperature gradient. Therefore, the exact reasons for the observed behaviour remain unknown and require further investigation.

5.4.2. Nematode genera respond similarly to burial with crushed nodule particles

The addition of a new substrate on the undisturbed sediment seemed to evoke stress for the nematode community in the upper sediment layers who showed a clear migratory response. Sedimentation rates in the Peru Basin are generally very low ranging between 0.4 and 2.0 cm ka^{-1} (Haeckel et al., 2001), therefore, the meiofaunal community is naturally not exposed to heavy sediment burial. Interestingly, there was no evidence of opportunistic genera taking advantage of the new situation and being more successful in either inhabiting the new substrate or in remaining in the surface layers of the original sediment and, therefore, being more stress resistant. Generally, the abyssal seafloor is characterized by a very low degree of disturbance and low, relatively constant organic matter input from the euphotic zone, resulting in benthic assemblages that are adapted to a very stable environment. Opportunistic species generally occur under extreme, variable conditions and get outcompeted by less opportunistic species when disturbance is low (Grassle and Sanders, 1973). However, small scale disturbances and habitat heterogeneity in the deep sea may induce a more dynamic environment to allow the persistence of colonizing species (Gallucci et al., 2005). This seems to be supported by the large number of Monhysteridae in our study, which are generally classified as good colonizers at least in shallow water environments (Bongers et al., 1991). Deep-sea monhysterids are characterised by a high local intrageneric diversity not supporting an opportunistic behaviour (Vanreusel et al, 1997). The results of our experiment did not indicate that these nematodes were more successful to inhabit the added substrate.

In their study, Miljutin et al. (2011) revisited a disturbed nodule site where sediment and nodules were removed by dredging 26 years ago and investigated the state of the nematode community. Nematode density, diversity and community structure inside the disturbed track still differed from adjacent non-disturbed areas, largely owing to the strong differences in sediment characteristics with lower porosity in the upper layers of the disturbed track, resulting from sediment removal, compared to the undisturbed sites. This study indicates that changes in nematode communities resulting from shifts in sediment characteristics of the

upper sediment layers may persist for very long time spans. Although the results of our experiment indicated only minor shifts in community composition, stronger impacts due to potential subsequent nematode mortality as observed by Mevenkamp et al. (2017a), are possible and may be long-lasting.

5.4.3. Increased copper concentrations in the added substrate are not reflected in nematode body copper content

The very high concentrations of heavy metals in the crushed nodule substrate raise questions about bioavailability and uptake of these metals in benthic organisms. Previous research has shown that nematodes play an important role in the transfer of heavy metals to and from the benthic food web in harbour communities (Fichet et al., 1999). Similarly, Howell (1982) reported increased zinc uptake in nematodes exposed to pollution, while copper content was very variable and correlations with habitat pollution were less clear. Although concentrations of heavy metals are generally much higher inside marine sediments compared to the water column, this does not necessarily cause a higher toxicity because metal ions may be bound to sediment particles, thus not directly bioavailable, or be present in a non-toxic form. This may explain the similar copper uptake in the nematodes of the Burial treatment and the Background sediments in our study. The abyssal sediment of the Peru Basin is very oxygen rich with an oxic layer of 5 - 20 cm and metal distributions in the sediment are tightly linked to the position of the redox boundary (Stummeyer and Marchig, 2001). In the presence of manganese (Mn) oxyhydroxides, other elements such as the transition metals (e.g. Cu, Ni, Zn) are adsorbed to those phases in the oxic layer (Stummeyer and Marchig, 2001). Under conditions present in the sampled sediment, release of metals during a mining operation will most likely not result in an increased metal toxicity because of the fast oxidation of Mn and absorption of metals. Nevertheless, we strongly advise to integrate a screening of metal uptake by biota into baseline studies and the monitoring of mining activities, as this provides more biologically relevant information on toxicological impacts compared to sediment and pore water measurements. This would enable precise detection of changes in heavy metal burden in benthic organisms due to mining related alterations of the abiotic environment and possible shift of metal speciation inside the sediments.

5.5. Conclusion and recommendations

The brittle character of polymetallic nodules implies that the deposited material following mining will rather be a mixture of natural sediment and nodule particles. In this research we revealed some important insights in the structuring of meiobenthic communities following burial with crushed nodule substrate. Despite

the very differing abiotic conditions in the crushed nodule substrate and the natural sediment in terms of grain size and carbon and nitrogen content, an upward migration of meiobenthic organisms was observed. Comparison with previous research (Maurer et al., 1986; Mevenkamp et al., 2017a; Schratzberger et al., 2000) has shown that meiobenthic organisms from very different habitats and sediment types show upward migration following burial with native and non-native substrate and varying thickness of the deposited layer. Furthermore, this behavioural response was stronger in copepods and their nauplii compared to nematodes resulting in a shift in meiofauna community composition. This may in turn affect biotic interactions inside the sediment.

The effect of vertical meiofauna migration on other benthic size classes and over longer time scales requires further research, especially in a deep-sea mining context where sediment re-deposition is expected over large areas and long time scales (Murphy et al., 2016). Furthermore, we believe that, although it is technically challenging, standardized methods for mortality assessments in deep-sea sediment samples would considerably advance our understanding of short-term environmental impacts on meiofauna.

Acknowledgements

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5.6. Supplementary data

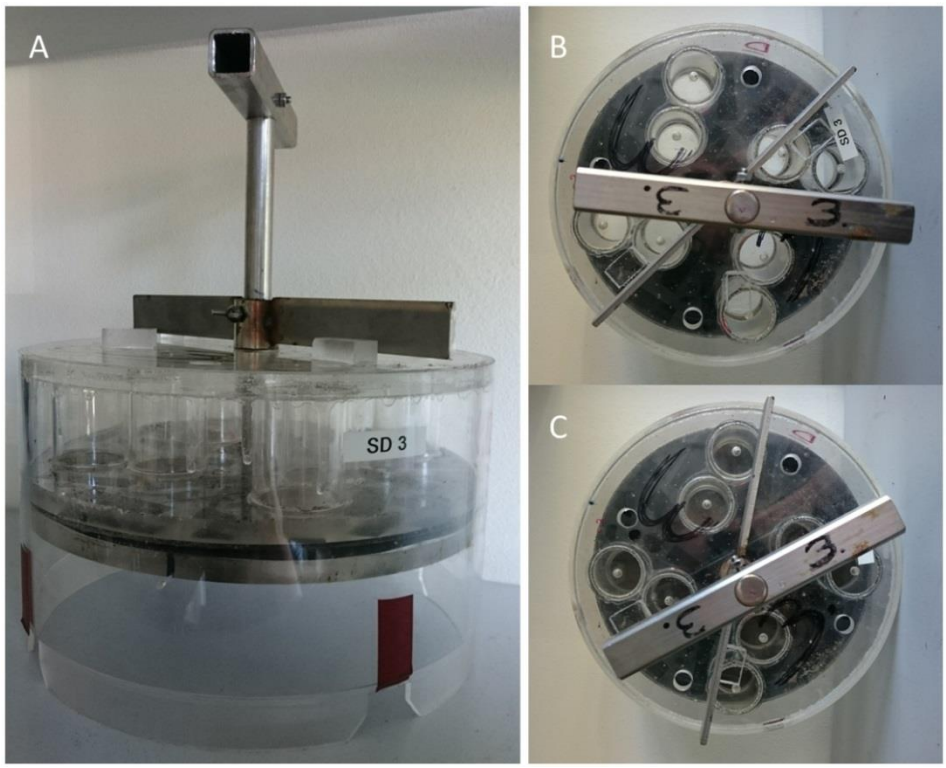

Figure S5.1 A) Front view on the sediment dispensing device, sediment is filled in tubes inside the round plexiglass space B) Top view in open position, tubes are visible as big holes and C) Top view in closed position. Holes in the plexiglass cover ensured escape of all air in the device.

Figure S5.2 Sample of the crushed nodule substrate. Scale in centimetres.

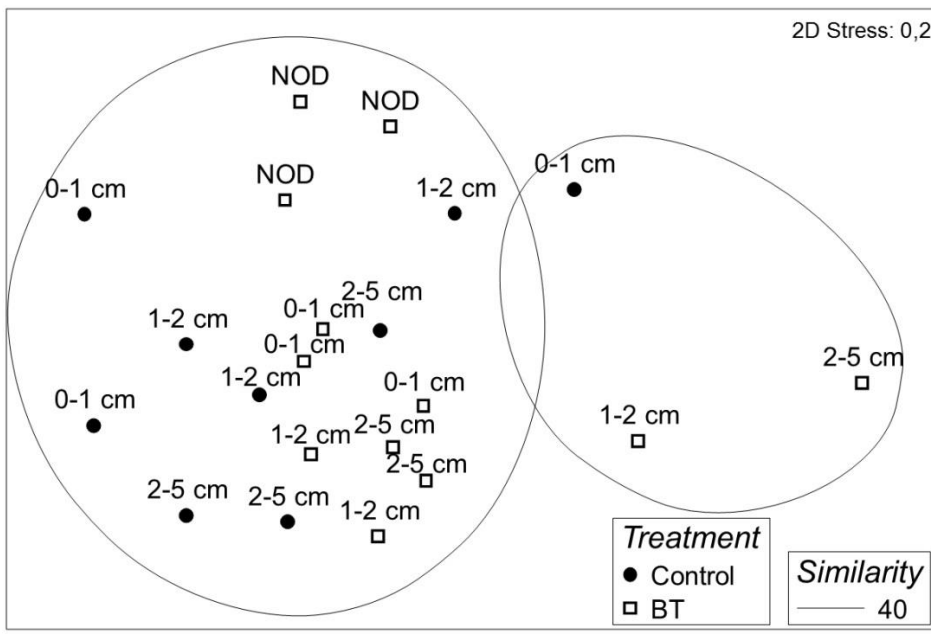

Figure S5.3 MDS plot of the relative nematode genus composition in each sample of the Control and Burial treatment (BT) per sediment depth layer with overlying contours of significant (SIMPROF test) clusters at an 40 % similarity level. NOD = crushed nodule layer

Table S5.1 Mean (± standard error) densities (ind. 10 cm^{-2}) of nematode genera found in all treatments of the experiment combining all depth layers.

Order	Family	Genus	Control	Burial treatment
Araeolaimida	Axonolaimidae	*Ascolaimus*		0.17 ± 0.17
	Comesomatidae	*Cervonema*	3.57 ± 0.81	2.59 ± 0.90
		Minolaimus	0.43 ± 0.43	0.29 ± 0.29
		Pierrickia	0.42 ± 0.21	0.94 ± 0.04
	Coninckiidae	*Coninckia*		0.15 ± 0.15
	Diplopeltidae	*Campylaimus*	0.21 ± 0.21	0.15 ± 0.15
		Diplopeltula	2.29 ± 0.73	1.72 ± 0.17
		Intasia	0.62 ± 0.35	
Chromadorida	Chromadoridae	*Acantholaimus*	12.49 ± 1.13	16.12 ± 2.06
		Actinonema	1.04 ± 0.41	0.78 ± 0.57
		Chromadora	0.62 ± 0.62	0.30 ± 0.15
		Chromadorina	0.62 ± 0.36	0.48 ± 0.27
		Endeolophos		0.47 ± 0.02
		Hypodontolaimus		0.16 ± 0.16
		Prochromadora		0.15 ± 0.15
		Prochromadorella	0.41 ± 0.41	0.47 ± 0.47
		Spilophorella	0.42 ± 0.21	0.17 ± 0.17
	Cyatholaimidae	*Acantonchus*	0.43 ± 0.43	
		Longicyatholaimus	0.20 ± 0.20	
		Marylynnia		0.15 ± 0.15
		Paracantonchus		0.15 ± 0.15
		Paracyatholaimus		0.17 ± 0.17
		Pomponema	0.21 ± 0.21	0.15 ± 0.15
	Neotonchidae	*Gomphionchus*		0.17 ± 0.17
	Selachinematidae	*Synonchiella*	0.22 ± 0.22	0.17 ± 0.17
Desmodorida	Desmodoridae	*Desmodora*	1.70 ± 1.14	1.90 ± 0.34
		Desmodorella	0.20 ± 0.20	
		Metadesmodora	0.21 ± 0.21	
		Molgolaimus	0.85 ± 0.57	1.23 ± 0.40
		Paradesmodora	0.21 ± 0.21	
	Microlaimidae	*Calomicrolaimus*		0.31 ± 0.31
		Microlaimus	1.05 ± 0.55	1.62 ± 0.75
Desmoscolecida	Cyartonematidae	*Cyartonema*	0.20 ± 0.20	0.49 ± 0.29
		Southerniella	0.63 ± 0.36	0.46 ± 0.27
	Desmoscolecidae	*Desmoscolex*	3.31 ± 1.73	2.51 ± 1.06
		Greeffiella	0.62 ± 0.35	0.96 ± 0.54
		Hapalomus		0.31 ± 0.31
		Tricoma	1.24 ± 0.35	1.89 ± 1.19
Enoplida	Anticomidae	*Cephalanticoma*		0.16 ± 0.16
	Enchelidiidae	*Bathyeurystomina*	0.42 ± 0.21	0.45 ± 0.25
		Calyptronema		0.15 ± 0.15
	Enoplidae	*Enoploides*	0.22 ± 0.22	
		Mesacanthion		0.30 ± 0.15
		Paramesacanthion	0.21 ± 0.21	0.17 ± 0.17
	Ironidae	*Syringolaimus*	1.03 ± 0.55	0.94 ± 0.04

	Leptosomatidae	*Anticoma*	1.03	±	0.73	0.47	±	0.02
		Anticomopsis				0.17	±	0.17
	Oncholaimidae	*Adoncholaimus*				0.16	±	0.16
		Metoncholaimus	0.20	±	0.20			
		Meyersia	0.21	±	0.21			
		Oncholaimellus				0.15	±	0.15
		Oncholaimus	0.42	±	0.21			
		Viscosia	4.75	±	3.87	11.81	±	5.37
	Oxystominidae	*Cricohalalaimus*				0.17	±	0.17
		Halalaimus	4.20	±	0.96	4.36	±	0.49
		Litinium				1.25	±	0.32
		Nemanema	0.20	±	0.20			
		Oxystomina	1.48	±	0.58	0.45	±	0.25
	Pelagonematidea	*Anoplostoma*	0.21	±	0.21	0.17	±	0.17
	Phanodermatidae	*Phanodermopsis*				0.29	±	0.29
	Trefusiidae	*Cytolaimium*	0.21	±	0.21			
	Tripyloididae	*Bathylaimus*	0.21	±	0.21			
Monhysterida	Linhomoeidae	*Anticyclus*	0.22	±	0.22			
		Disconema	0.42	±	0.21	0.16	±	0.16
		Eleutherolaimus	0.21	±	0.21	0.17	±	0.17
		Metalinhomoeus	0.21	±	0.21	0.44	±	0.44
		Terschellingia	0.21	±	0.21			
	Monhysteridae	*Monhystrella*	9.77	±	1.58	13.14	±	0.54
		Thalassomonhystera	6.07	±	0.89	3.94	±	0.44
	Siphonolaimidae	*Parastomonema*	0.21	±	0.21			
		Doliolaimus	0.20	±	0.20			
		Metasphaerolaimus	0.00	±	0.00	0.44	±	0.44
		Sphaerolaimus	4.16	±	0.87	2.95	±	0.30
		Subsphaerolaimus	0.82	±	0.41	0.78	±	0.16
	Xyalidae	*Ammotheristus*	0.22	±	0.22	0.15	±	0.15
		Amphimonhystera				0.16	±	0.16
		Amphimonhystrella	0.20	±	0.20	0.78	±	0.32
		Daptonema	5.88	±	1.89	2.77	±	0.95
		Elzalia	0.22	±	0.22			
		Enchonema	0.00	±	0.00	0.15	±	0.15
		Linhystera	1.46	±	0.55	1.69	±	0.36
		Manganonema	3.15	±	0.65	3.46	±	0.47
		Metadesmolaimus	0.43	±	0.43	0.15	±	0.15
		Paramonohystera				0.29	±	0.29
		Rhynchonema	0.22	±	0.22			
		Theristus	3.59	±	1.44	2.29	±	0.83
Plectida	Aegialoalaimidae	*Aegialoalaimus*	1.66	±	1.03	1.26	±	0.44
	Camacolaimidae	*Alaimella*	0.22	±	0.22			
		Camacolaimus	0.63	±	0.01	0.78	±	0.16
	Ceramonematidea	*Ceramonema*	0.20	±	0.20			
		Dasynemoides	0.22	±	0.22			
		Pselionema	1.47	±	0.42	0.29	±	0.29
	Diplopeltoididae	*Diplopeltoides*	5.21	±	2.50	1.76	±	0.70
	Haliplectidae	*Setoplectus*	0.22	±	0.22	0.29	±	0.29

Leptolaimidae	*Antomicron*	0.21 ± 0.21	0.32 ± 0.16
	Leptolaimus	2.49 ± 0.70	0.65 ± 0.45
	Unknown sp. 1	0.22 ± 0.22	
	Unknown sp. 2		0.63 ± 0.16
	Unknown sp. 3		0.47 ± 0.47
	Unknown sp. 4		0.17 ± 0.17
	Unknown sp. 5		0.15 ± 0.15

Chapter 6 Hydrostatic pressure and temperature affect the tolerance of the free-living marine nematode *Halomonhystera disjuncta* to acute copper exposure

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Abstract

Potential deep-sea mineral extraction poses new challenges for ecotoxicological research since little is known about effects of abiotic conditions present in the deep sea on the toxicity of heavy metals. Due to the difficulty of collecting and maintaining deep-sea organisms alive, a first step would be to understand the effects of high hydrostatic pressure and low temperatures on heavy metal toxicity using shallow-water relatives of deep-sea species. Here, we present the results of acute copper toxicity tests on the free-living shallow-water marine nematode *Halomonhystera disjuncta*, which has close phylogenetic and ecological links to the bathyal species *Halomonhystera hermesi*. Copper toxicity (concentrations ranging from 0 – 6 mg Cu^{2+} L^{-1}) was assessed using a semi-liquid gellan gum medium at two levels of hydrostatic pressure (0.1 MPa and 10 MPa) and temperature (10 °C and 20 °C) in a fully crossed design. Mortality of nematodes in each treatment was assessed at 4 time intervals (24 and 48 h for all experiments and additionally 72 and 96 h for experiments run at 10 °C). LC_{50} values ranged between 0.561 and 1.864 mg Cu^{2+} L^{-1} and decreased with incubation time. Exposure to high hydrostatic pressure significantly increased sensitivity of nematodes to copper, whereas lower temperature resulted in an apparent increased copper tolerance, possibly as a result of a slower metabolism under low temperatures. These results indicate that hydrostatic pressure and temperature significantly affect metal toxicity and therefore need to be considered in toxicity assessments for deep-sea species. Any application of pollution limits derived from studies of shallow-water species to the deep-sea mining context must be done cautiously, with consideration of the effects of both stressors.

6.1. Introduction

Economically valuable mineral deposits can be found in a variety of deep-sea habitats such as abyssal plains (polymetallic nodules and deep-sea muds), active and extinct hydrothermal vents (seafloor massive sulphides) or seamounts (ferromanganese crusts) (Hein et al., 2013; Petersen et al., 2016; Sterk and Stein, 2015). The extraction of these mineral deposits may cause significant disturbances of these remote and ecologically valuable habitats, threatening their biological communities (Vanreusel et al., 2016). Despite significant international attention, ecosystems of these areas are poorly studied and mechanisms of resilience and recovery of the benthic fauna are largely unknown (Gollner et al., 2017; Wedding et al., 2015). One major concern is the mobilization and release of elevated concentrations of potentially toxic elements during extraction, transport in riser systems, or after processing of the minerals (e.g. the release of extraction water or tailings to the water column) (Boschen et al., 2013; Koschinsky et al., 2001a, 2001b; Thiel, 2001). Heavy metal concentrations are usually higher within marine sediments than the overlying water column as heavy metals bind to small particles, organic matter and different hydroxides (Pempkowiak et al., 1999). Infaunal organisms are, therefore, particularly vulnerable to metal exposure if conditions in sediment or surrounding seawater change (e.g. pH, oxygen saturation) and bioavailability of those metals increases. The development of appropriate measures to identify risk requires knowledge of the impacts of heavy metal contamination on deep-sea benthic organisms. However, the acquisition and maintenance of deep-sea organisms is challenging, hampering their use in controlled laboratory experiments. As a first step towards understanding heavy metal toxicity in the deep sea, researchers are advised to uncover the effects of abiotic factors such as high hydrostatic pressure and low temperatures on the sensitivity of marine species (Mestre et al., 2014). These two factors play major roles in determining the distribution of marine organisms (Brown and Thatje, 2011; Clarke, 2003; Pörtner, 2002; Pradillon and Gaill, 2007). Knowledge of pressure and temperature effects on metal toxicity would help us to better understand underlying mechanisms and possibly predict potential toxic effects in deep-sea species.

Copper is a trace element that is essential to the health of most organisms (Mertz, 1981). It plays a role in multiple physiological pathways (e.g. in regulating oxidative stress), as a co-factor of several enzymes or structural components and is also associated with biological processes such as responses to hypoxia (Karlin and Tyeklár, 2012; Scheiber et al., 2013). However, an excess of copper can induce severe toxicity leading to metabolic dysfunction and ultimately to the death of an organism (Gaetke and Chow, 2003; Scheiber et al., 2013). Deep-sea minerals contain relatively high concentrations of copper (Hein et al., 2013) and it has been

demonstrated that the potential for copper leaching from deep-sea minerals such as chalcopyrite is high (Fallon et al., 2017; Knight and Roberts, 2016). However, Simpson and Spadaro (2016) have recently reported only limited chalcopyrite-induced mortality in bivalves and amphipods. The relatively high importance of copper in deep-sea mineral extraction and its important role in animal physiology support the need to explore the effects of hydrostatic pressure and temperature on copper toxicity.

Intermediate in size between micro- and macrofauna, metazoan meiobenthos play a major role in the benthic ecosystem as an important component of the benthic food-web, but also through facilitating mineralization and nutrient turnover (Bonaglia et al., 2014; Coull, 1999; Moens et al., 2013). Nematodes are the dominant taxon within this group of organisms and their short life span and high fecundity also make them suitable for laboratory experiments and short-term ecotoxicological research in particular (Beyrem et al., 2011; Kennedy and Jacoby, 1999). The tolerance of nematodes to metal toxicity, hypoxia and changing environmental conditions can be very variable and species dependent (Bongers and Ferris, 1999; Gyedu-Ababio and Baird, 2006). *Halomonhystera disjuncta* is a free-living, bacterivorous shallow-water marine nematode which is known for its tolerance to high concentrations of heavy metals (Vranken et al., 1989, 1988, 1985, 1984). The intertidal, cryptic species *Halomonhystera disjuncta* GD1 (Derycke et al., 2007) is phylogenetically closely related to the species *H. hermesi* (Tchesunov et al., 2014) which inhabits cold-seep ecosystems in the deep sea, e.g. the Nyegga pockmark at 730 m on the Nordic Norwegian margin and the Håkon Mosby mud volcano at 1280 m depth in the Barents Sea (Van Campenhout et al., 2015, 2013; Van Gaever et al., 2006). Interestingly, *H. disjuncta* GD1 also shows higher tolerance towards bathyal seep conditions (high sulphide concentrations, low temperature) than other species in the cryptic species complex (Van Campenhout et al., 2014). The close phylogenetic relationship and *H. disjuncta* GD1's environmental tolerances suggest that *H. disjuncta* and *H. hermesi* share a recent common ancestor (Van Campenhout et al., 2015, 2014, 2013), making *H. disjuncta* a relevant species with which to investigate the effects of bathyal environmental conditions on copper toxicity.

In this study, we performed the first acute copper toxicity tests on the free-living marine nematode *H. disjuncta* incorporating different hydrostatic pressure and temperature regimes. The use of gellan gum as a medium for nematode toxicity testing has been described by Brinke et al. (2011) and was chosen for this study to facilitate the use of pressure chambers under the exclusion of air cavities. In comparison to water, gellan gum provides the advantage that the sediment dwelling nematodes are still able to move through the medium by body

undulations but with lower activity and stress than would result from constant swimming in water. We investigated the acute effects of bathyal pressure on copper toxicity in *H. disjuncta* by including two pressures (0.1 MPa= surface pressure, and 10 MPa ≈ 1000 m water depth) and two temperatures (20°C and 10°C). Here, 20°C represents a standard temperature for toxicity testing that has been applied in previous acute toxicity studies on marine nematodes including *H. disjuncta* (Austen and McEvoy, 1997; Vranken et al., 1984; Vranken and Heip, 1986), whereas 10 °C is at the lower end of the species temperature range whilst allowing normal growth and development (Van Campenhout et al., 2014). With this study we aim to investigate 1) the effect of high hydrostatic pressure on the survival of a shallow-water nematode and 2) the extent to which temperature and hydrostatic pressure affect copper toxicity in the shallow-water nematode.

6.2. Material and Methods

6.2.1. Nematode cultures

Monospecific cultures of *H. disjuncta* cryptic species GD1 were cultivated at 16 °C on petri dishes filled with 0.8 % nutrient:bacto agar in a ratio of 1:7 prepared in artificial seawater (Moens and Vincx, 1998) with a salinity of 25. The cultures were incubated at the respective experimental temperature one week prior to the experiment. An excess of frozen-and-thawed *Escherichia coli* K12 were added as food source. A full description of species acquisition for the cultures is given in Van Campenhout et al. (2014).

6.2.2. Experimental setup

Nematodes of the species *H. disjuncta* GD1 were exposed to five different copper (Cu^{2+}) concentrations at two different temperatures (10 °C and 20 °C) and two different pressures (0.1 MPa and 10 MPa) for 2 time intervals (24 h, 48 h) with 3 replicates per time interval and treatment. In addition, experiments at 10 °C were also run for 72 h and 96 h. Selection of dissolved copper concentrations at 10 °C (0, 0.5, 1, 2, 4, 6 mg Cu^{2+} L^{-1}) and 20 °C (0, 0.2, 0.5, 1, 2, 5 mg Cu^{2+} L^{-1}) were based on preliminary ranging experiments at atmospheric pressure. Survival was the chosen endpoint.

Screw top vials of 5 mL volume with a rubber septum were half filled with Cu^{2+}-contaminated gellan gum and a total of 20 adult and preadult nematodes were placed in the vials. The vials were then filled up with the Cu^{2+}-contaminated gellan gum medium and closed, ensuring that no air bubbles were trapped. One vial (empty control) without nematodes was added to each replicate measurement. Vials were placed in a pressure vessel, acutely pressurised, and incubated at the

respective pressure and temperature for the respective time intervals (24, 48, 72 and 96 hours). A detailed description of the pressure vessels can be found in Mestre et al. (2009). Vials of all treatments, including those at surface pressure, were placed in pressure vessels to avoid any experimental artefacts arising from enclosure in the pressure vessel.

The semi-liquid gellan gum medium was made with 1.5 g L^{-1} gelrite (Merck & Co., Kelco Division) solution prepared in MilliQ water and artificial seawater (Moens and Vincx, 1998) with a salinity of 34. The two components were autoclaved and the gellan gum solution was slowly added to the seawater in a 1:3 ratio under continuous stirring to obtain the required fluidity and salinity of 25. Sufficient volumes of medium were spiked with different dissolved copper (Cu^{2+}) concentrations by adding the appropriate amount of $CuSO_4$ stock solution to the medium under continuous stirring for ~2 minutes. The stock solution was composed of 0.10155 g $CuSO_4$ and 250 mL MilliQ water resulting in a dissolved Cu^{2+} concentration of 259.66 mg L^{-1}.

At the end of each experiment, hydrostatic pressure vessels were immediately depressurised and oxygen levels in the middle of the vials were measured with an oxygen optode connected to a PreSens Microx TX3 array. Nematode mortality was assessed by observing movement through a stereo-microscope and/or response to physical stimulation with a needle.

Unavoidable bacterial contamination of the medium and nematode respiration led to a decrease of oxygen concentrations in the vials, especially at high temperatures. Based on the oxygen measurements we adjusted our experimental setup and only conducted 24 h and 48 h treatments at 20 °C, however, these particular treatment combinations were repeated once with a full set of 3 replicates. Furthermore, data analysis was adjusted by removing treatments where very low oxygen concentrations (<5 %) persisted in most vials at low copper concentrations in combination with an increased mortality of animals in those vials (Tab. S6.1). Potential oxygen-associated background mortality at zero copper concentration was accounted for in the model used for the data analysis (see 6.2.3). Oxygen deficiency and mortality occurred in all replicates of the following treatments: 20 °C, 0.1 MPa, 24 h (first measurement); 20 °C, 10 MPa, 24 h (first measurement); 20 °C, 0.1 MPa, 48 h (second measurement) and 20 °C, 10 MPa, 48 h (second measurement)(Tab. S6.1). Therefore, one set of replicates at each pressure at 24 and 48 h was retained in the analysis.

6.2.3. Data analysis

LC_{50} values and their confidence intervals were estimated from concentration-response curves based on the three replicates of each treatment, fitted with a binomial regression using a probit link and adjusting for background mortality as explained by Proctor et al. (2017). The models of the concentration-response curves were then compared by Analysis of Deviance (chi-squared) which allows comparison of generalized linear models comparable to variance testing in ANOVA (Nelder and Wedderburn, 1972). Additionally, concentration-response curves were fitted with the very similar log-normal function with fixed upper (1) and lower (0) limits (LN.2) using the *drc* package. This package allows for comprehensive visualization of the models. All data were analysed with the statistical software R version 3.4.0 Patched (R Core Team, 2013) and RStudio version 1.0.136 (RStudio Team, 2015) using the packages *LC50* (Proctor and Wotherspoon, 2015) and *drc* (Ritz et al., 2015). A significance level of α=0.05 was chosen for all tests.

6.3. Results

At the end of each experiment, nematode behaviour and motility in treatments without Cu^{2+} contamination appeared unaffected by compression and decompression. Surviving animals from high pressure treatments appeared and behaved similarly to those under atmospheric pressure.

LC_{50} values ranged between 0.561 mg L^{-1} (10 MPa, 10 °C after 72 h) and 1.864 mg L^{-1} (0.1 MPa, 10 °C, after 24 h) and showed a decreasing trend with incubation time (Fig. 6.1). Pressure and temperature significantly affected nematode sensitivity towards copper at the 24 h and 48 h time intervals, without an interaction effect (Tab. 6.1): nematode sensitivity in terms of LC_{50} was greater at 10 MPa than at 0.1 MPa and was greater at 20 °C than at 10 °C (Fig. 6.1). The negative effect of high pressure was still visible after 72 h of incubation but not at 96 h (Tab. 6.1).

In addition to differences in the LC_{50}, the slope of the concentration-response curve was slightly flattened when high hydrostatic pressure was applied (Fig. 6.2, Tab. S6.2). A flattened slope indicates that the range of concentrations that is harmful for part of the population is larger whereas a steep slope is an indication for a sharp threshold level where passing the threshold leads to a strong increase in toxicity producing lower survival rates.

Table 6.1 Results of the Analysis of Deviance comparing concentration-response models of varying factors pressure (0.1 and 10 MPa) and temperature (10 and 20 °C). DF= degrees of freedom, NULL= null model (no variables included), Pr(>Chi)= probability of the model being different from the null model.

		DF	Deviance	Residual DF	Residual Deviance	Pr (>Chi)	
24 h	NULL			63	233.8		
	Temperature	1	66.838	62	166.96	2.95E-16	***
	Pressure	1	24.956	61	142	5.87E-07	***
	Temperature*Pressure	1	0.408	60	141.6	0.5231	
48 h	NULL			63	156.3		
	Temperature	1	30.683	62	125.62	3.04E-08	***
	Pressure	1	12.564	61	113.05	3.93E-04	***
	Temperature*Pressure	1	0	60	113.06	1	
72 h	NULL			31	143.838		
	Pressure	1	79.983	30	63.855	2.20E-16	***
96 h	NULL			31	55.105		
	Pressure	1	1.1235	30	53.982	0.2892	

*** Significant at the p ≤ 0.001 probability level

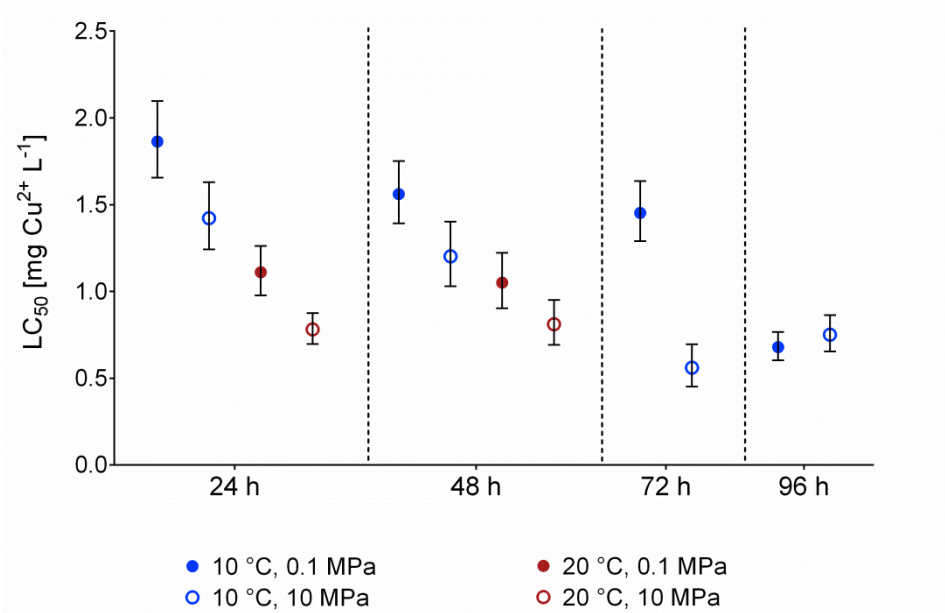

Figure 6.1 Mean LC_{50} values with upper and lower confidence intervals (error bars) for all experiments conducted in a fully crossed design with two varying factors (temperature and pressure), N=3.

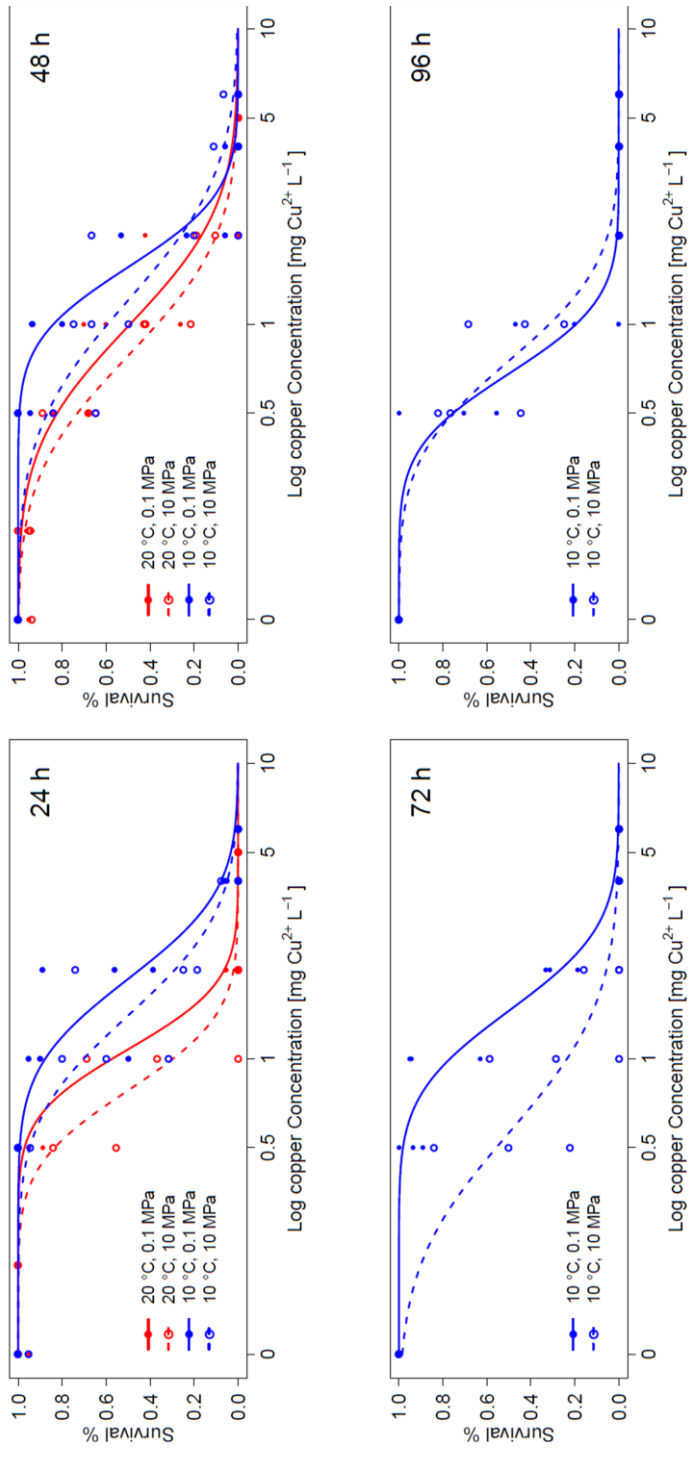

Figure 6.2 Concentration-response curves (log-normal models) of the copper toxicity tests at differing pressure (0.1 and 10 MPa) and temperature (10 and 20 °C) for 4 different time intervals. Symbols represent the individual replicate measurements of nematode survival.

6.4. Discussion

6.4.1. Effects of hydrostatic pressure

Hydrostatic pressure strongly determines the depth distribution of marine species (Brown and Thatje, 2014). This is a result of evolutionary adaptation of marine organisms to the adverse effects of high hydrostatic pressure on molecular interactions, e.g. with regard to electrochemical and hydrophobic interactions in biological systems (Brown and Thatje, 2014; Pradillon and Gaill, 2007). Nevertheless, shallow-water marine invertebrates seem to have a wider tolerance to increased hydrostatic pressure than might be predicted *a priori* (Brown and Thatje, 2014; Mestre et al., 2013, 2009; Smith et al., 2015) and are able to increase their tolerance after a short acclimation period (New et al., 2014). Similarly, in this study, mortality and activity of *H. disjuncta* in treatments without Cu^{2+} contamination remained unaffected by elevated pressure. Therefore, mortality associated with pressurization and depressurization was not an interfering factor in the assessment of copper toxicity. However, while the nematodes were tolerant in the short-term, negative effects of high hydrostatic pressure on the nematodes may arise with longer exposures.

In this study, high hydrostatic pressure reduced the tolerance of *H. disjuncta* to copper exposure at both 10 and 20 °C, as evidenced by lower LC_{50} values and a flattened slope compared to surface pressure (Fig. 6.2). The flattened slope of the concentration-response curves at high hydrostatic pressure suggests that a wider range of concentrations adversely affects part of the population. Therefore, even low copper concentrations led to a weakening of the nematodes and mortality of several individuals at 10 MPa. Interestingly, the effect of pressure was not evident after an incubation time of 96 h which may be caused by interfering factors such as starvation, reducing the organisms' tolerance to additional stress. Research has indicated that starvation effects can become evident after 72-120 h in laboratory experiments with marine nematodes (Ott and Schiemer, 1973; Wieser et al., 1974).

Previous research on copper uptake in nematodes has found a strong association of increased amounts of copper concentrations in the cuticle and hypodermis, supporting an uptake via the body wall rather than the digestive tract (Howell, 1983; Sávoly et al., 2013). Changes in hydrostatic pressure lead to a shift in biochemical reaction rates and in the fluidity of membranes (Brown and Thatje, 2014; Pradillon and Gaill, 2007) which may in turn affect uptake rates of copper from the environment. Copper toxicity is mainly caused by accumulation of oxidative damage resulting from reactive oxygen species in the cells, and organisms may respond to this by enhancing antioxidant enzyme expression (Song et al.,

2014). This, however, will increase basal metabolic rate and may have consequences for the energy allocation of the organisms (Sokolova and Lannig, 2008).

Brown et al. (2017a) assessed the effects of low temperature and high hydrostatic pressure on acute (96 h) lethal and sublethal (respiration rate, antioxidant enzyme activity) copper and cadmium toxicity in the shallow-water shrimp *Palaemon varians*. In that study, the researchers report that, when a pressure of 10 MPa is applied, a significant increase in oxygen consumption and antioxidant enzyme activity (superoxide dismutase, glutathione peroxidase) became evident at lower copper concentrations (100 μg Cu^{2+} L^{-1}) than when at surface pressure (where a significant increase was only evident at 1000 μg Cu^{2+} L^{-1}). The exact mechanisms of the effect that pressure has on copper toxicity (e.g. enhanced copper uptake through membranes, inhibition of gene expression) still remain to be investigated, but the results of Brown et al. (2017a) suggest that enzyme expression and activity were not suppressed by increased hydrostatic pressure.

Deep-sea organisms have been shown to possess a series of adaptations, such as a higher degree of cell membrane fluidity and enzyme stability to high pressure, to counteract the negative effects of hydrostatic pressure on their metabolism (Pradillon and Gaill, 2007; Somero, 2003; Yancey et al., 2004). Nevertheless, it remains to be investigated if these adaptations also enable them to counteract the negative effect of pressure on copper toxicity. This may be answered once our mechanistic understanding of copper toxicity in marine invertebrates and, more specifically in deep-sea invertebrates, improves.

6.4.2. Effects of temperature

Temperature also affected copper tolerance in *H. disjuncta* and survival of nematodes was higher at 10 °C than at 20 °C. *H. disjuncta*'s optimal growth temperature occurs at approximately 16 °C, but most life history traits of the cryptic species *H. disjuncta* GD1, such as life span, egg deposition time and development time, do not vary considerably when temperature is reduced to 10 °C (Van Campenhout et al., 2014). Therefore, a temperature of 10 °C can be considered to lie within the species' thermal window. The metabolism of an organism is an interplay of different biochemical reactions performed by multiple enzymes (Brown et al., 2004). These metabolic reactions obey the laws of thermodynamics and increase exponentially with increasing temperatures inside temperature ranges of normal activity (Brown et al., 2004; Clarke and Fraser, 2004). Therefore, the higher tolerance of *H. disjuncta* towards copper at 10 °C compared to 20 °C may be attributed to a slower metabolism at low temperatures leading to a decreased copper uptake, which delays reaching lethal systemic metal

concentration. This is consistent with previous research stating that in most ectotherms (80 % of N=118 investigated species) an increased temperature enhanced metal toxicity in terms of mortality and uptake (Sokolova and Lannig, 2008; and references therein). Indeed, chromium toxicity in *H. disjuncta* was increased when temperature was high (22 °C) compared to optimal temperature (17 °C) and mortality was reduced at low temperature (12 °C) (Vranken et al., 1989).

Although deep-sea animals have adapted to very stable and low temperatures (typically 4 °C), enzymatic reactions and, consequently, metabolic rate of stenothermal species living at <8 °C is lower compared to eurythermal organisms (Pörtner, 2002). Therefore, the observed lower, acute effects of copper toxicity at low temperature may be similar in deep-sea organisms. However, additional stress responses induced by copper exposure at low, sublethal concentrations may increase basal metabolic maintenance and reduce the amount of energy available for other functions, reducing the organisms' fitness (Brown et al., 2017a; Sokolova et al., 2012). This may especially be of relevance in the food and energy limited abyssal deep sea (Smith et al., 2008b). Chronic effects considerably differ from acute exposures and sublethal copper concentrations have been shown to substantially affect life-cycle characteristics (Bechmann, 1994; Kwok et al., 2008). This, in turn, may have unknown consequences for ecosystem functioning and the assessment of chronic toxicity effects should, therefore, be considered in future studies.

Temperature and hydrostatic pressure have antagonistic properties with regard to kinetics and equilibria in biological systems, e.g. as pressure increases, it reduces the flexibility of lipids and nucleic acids while the opposite is true for temperature increases (Pradillon and Gaill, 2007 and citations therein). However, the results of this study indicate that pressure and temperature act in different ways on the sensitivity of *H. disjuncta* to copper since a simultaneous increase in both, pressure and temperature, lead to an increase in copper sensitivity.

6.4.3. Conclusion

Our research shows that increased hydrostatic pressure and temperature increase the sensitivity of *H. disjuncta* to acute copper exposure in terms of mortality. An integrative approach of laboratory experiments using shallow-water species under controlled conditions in combination with *in situ* deep-sea experiments using related species is, therefore, crucial to fully understand ecotoxicology in the deep sea (Brown et al., 2017b). Nevertheless, the use of shallow-water species helps to elucidate general mechanisms of both factors on copper toxicity in marine nematodes.

As an acute toxicity assessment with only one toxicant tested, these results are not intended to assess the specific risks of deep-sea mineral extraction, but they provide evidence for effects of hydrostatic pressure and temperature on copper toxicity that need to be considered in environmental risk assessment. *In situ* studies including realistic multiple metal exposures are needed to produce environmentally relevant data and enable proper risk assessment. Furthermore, experiments with longer exposures would enable investigations of effects of chronic exposures to toxicants, which may pose greater risks for organisms in the long term and which cannot be assessed in acute toxicity studies (Freitas and Rocha, 2014).

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6.5. Supplementary data

Table S6.1 Mortality and oxygen content in replicate vials of all treatments. Treatments with an asterisk were excluded from the data analysis due to low oxygen concentrations and mortality in treatments with low copper concentrations.

Temperature [°C]	Pressure [Mpa]	Time [h]	Concentration [Cu^{2+} mg L^{-1}]	Mortality [%]			Oxygen concentration [%]			
				Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	
20	0.1	24	0	17.65	0.00	5.26	0.50	1.10		*
20	0.1	24	0.2	66.67	21.43	73.33	0.30	0.60		*
20	0.1	24	0.5	18.18	0.00	0.00	65.50	72.10		*
20	0.1	24	1	66.67	26.32	50.00	76.60	78.40		*
20	0.1	24	2	93.33	72.73	100.00	83.10	83.10		*
20	0.1	24	5	100.00	100.00	100.00	86.20			*
20	0.1	24	0	0.00	4.76	0.00	32.70	29.30	26.20	
20	0.1	24	0.2	0.00	0.00	0.00	40.70	12.00	36.30	
20	0.1	24	0.5	0.00	5.00	11.11	30.40	62.20	14.70	
20	0.1	24	1	50.00	50.00	10.53	54.20	46.90	53.20	
20	0.1	24	2	94.44	94.74	100.00	52.10	46.20	46.90	
20	0.1	24	5	100.00	100.00	100.00	76.50	75.60	68.90	
20	0.1	48	0	4.76	5.00	0.00	6.70	17.60	1.10	
20	0.1	48	0.2	0.00	6.25	3.70	18.00	35.90	37.70	
20	0.1	48	0.5	15.79	31.58	0.00	68.50	62.90	62.40	
20	0.1	48	1	30.00	73.68	40.00	68.50	68.10	77.10	
20	0.1	48	2	94.12	100.00	57.89	69.50	68.00	67.10	
20	0.1	48	5	100.00	100.00	100.00	78.40	75.60	72.50	
20	0.1	48	0	6.25	14.29	6.25	0.00	0.00	0.00	*
20	0.1	48	0.2	31.25	12.50	33.33	0.00	0.00	0.00	*
20	0.1	48	0.5	86.67	66.67	94.12	0.00	0.00	0.00	*
20	0.1	48	1	100.00	81.25	100.00	0.00	0.00	0.00	*
20	0.1	48	2	100.00	100.00	100.00	0.00	0.00	0.00	*
20	0.1	48	5	100.00	100.00	100.00	27.60	42.00	39.80	*

Table S6.1 continued

Temperature [°C]	Pressure [Mpa]	Time [h]	Concentration [Cu^{2+} mg L^{-1}]	Mortality [%]			Oxygen concentration [%]			
				Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	
20	10	24	0	0.00	0.00	0.00	7.60	12.70	20.30	*
20	10	24	0.2	0.00	5.26	0.00	16.00	15.10	8.80	*
20	10	24	0.5	14.29	0.00	14.29	88.10	90.40	92.70	*
20	10	24	1	55.00	10.53	25.00	92.40	90.40	93.80	*
20	10	24	2	70.59	68.75	56.25	94.90	94.00	88.50	*
20	10	24	5	100.00	100.00	100.00	93.40	95.50	88.30	*
20	10	24	0	0.00	0.00	0.00	26.30	64.40	32.40	
20	10	24	0.2	0.00	0.00	0.00	39.60	56.70	43.30	
20	10	24	0.5	0.00	44.44	15.79	19.80	48.70	22.30	
20	10	24	1	31.25	100.00	63.16	81.70	88.90	81.90	
20	10	24	2	100.00	100.00	100.00	76.40	103.10	59.00	
20	10	24	5	100.00	100.00	100.00	73.80	93.70	91.30	
20	10	48	0	0.00	6.25	0.00	1.40	0.40		
20	10	48	0.2	5.56	5.00	0.00	29.60	65.00	24.80	
20	10	48	0.5	35.29	31.82	11.11	44.70	66.20	64.70	
20	10	48	1	78.57	57.14	57.89	45.60	68.00	73.60	
20	10	48	2	89.47	100.00	80.95	55.10	70.40	71.70	
20	10	48	5	100.00	100.00	100.00	39.40	68.80	69.90	
20	10	48	0	6.25	12.50	13.33	0.00	0.00	0.00	*
20	10	48	0.2	5.56	19.05	0.00	1.30	0.00	1.90	*
20	10	48	0.5	100.00	100.00	100.00	0.30	0.00	0.10	*
20	10	48	1	100.00	100.00	100.00	0.00	0.00	1.10	*
20	10	48	2	100.00	100.00	100.00	0.00	0.00	0.00	*
20	10	48	5	100.00	100.00	100.00	43.20	43.80	46.20	*

Table S6.1 continued

Temperature [°C]	Pressure [Mpa]	Time [h]	Concentration [Cu^{2+} mg L^{-1}]	Mortality [%]			Oxygen concentration [%]		
				Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
10	0.1	24	0	0.00	0.00	0.00	119.80	98.30	90.40
10	0.1	24	0.5	0.00	0.00	0.00	116.90	100.70	93.10
10	0.1	24	1	10.00	50.00	4.76	120.30	102.90	94.90
10	0.1	24	2	11.11	61.11	43.75	120.90	99.60	94.30
10	0.1	24	4	93.75	95.00	100.00	118.70	99.70	92.60
10	0.1	24	6	100.00	100.00	100.00	118.90	100.20	96.20
10	0.1	48	0	0.00	0.00	0.00	44.30	29.80	45.00
10	0.1	48	0.5	5.26	0.00	0.00	98.50	100.70	95.40
10	0.1	48	1	6.67	6.25	20.00	100.40	88.00	96.30
10	0.1	48	2	46.67	94.12	76.47	98.80	95.50	61.10
10	0.1	48	4	100.00	94.12	100.00	103.30	100.60	104.10
10	0.1	48	6	100.00	100.00	100.00	102.30	103.10	105.10
10	0.1	72	0	0.00	0.00	0.00	82.60	47.20	80.80
10	0.1	72	0.5	11.11	0.00	6.67	84.80	94.40	78.30
10	0.1	72	1	5.88	5.00	36.84	87.60	79.70	96.40
10	0.1	72	2	68.75	66.67	81.25	85.00	83.40	86.10
10	0.1	72	4	100.00	100.00	100.00	85.00	74.80	90.60
10	0.1	72	6	100.00	100.00	100.00	84.30	78.40	94.70
10	0.1	96	0	0.00	0.00	0.00	20.50	7.70	1.00
10	0.1	96	0.5	29.41	0.00	44.44	82.20	88.40	81.10
10	0.1	96	1	100.00	52.94	80.00	88.50	85.50	100.50
10	0.1	96	2	100.00	100.00	100.00	65.40	73.10	92.40
10	0.1	96	4	100.00	100.00	100.00	78.70	87.90	104.40
10	0.1	96	6	100.00	100.00	100.00	82.20	92.10	108.70

Table S6.1 continued

Temperature [°C]	Pressure [Mpa]	Time [h]	Concentration [Cu^{2+} mg L^{-1}]	Mortality [%]			Oxygen concentration [%]		
				Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
10	10	24	0	4.76	0.00	0.00	108.60	97.50	102.10
10	10	24	0.5	5.56	0.00	0.00	110.10	102.80	115.80
10	10	24	1	68.42	40.00	20.00	109.10	99.00	110.40
10	10	24	2	75.00	81.25	26.09	104.90	103.80	105.00
10	10	24	4	100.00	100.00	92.31	104.90	101.40	107.70
10	10	24	6	100.00	100.00	100.00	117.60	113.60	110.70
10	10	48	0	0.00	0.00	0.00	71.30	87.50	67.10
10	10	48	0.5	0.00	15.79	35.29	128.50	149.00	108.30
10	10	48	1	25.00	50.00	33.33	151.40	151.20	138.00
10	10	48	2	33.33	100.00	80.00	141.80	161.80	128.80
10	10	48	4	88.89	100.00	100.00	143.80	152.00	171.50
10	10	48	6	93.33	100.00	100.00	124.50	148.40	146.90
10	10	72	0	0.00	0.00	0.00			
10	10	72	0.5	50.00	15.79	77.78			
10	10	72	1	100.00	41.18	71.43		no O_2 measurements	
10	10	72	2	100.00	84.21	100.00			
10	10	72	4	100.00	100.00	100.00			
10	10	72	6	100.00	100.00	100.00			
10	10	96	0	0.00	0.00	0.00	46.20	97.60	39.80
10	10	96	0.5	23.53	55.56	17.65	100.50	109.20	121.20
10	10	96	1	75.00	57.14	31.58	108.50	244.70	64.10
10	10	96	2	100.00	100.00	100.00	139.10	144.00	85.50
10	10	96	4	100.00	100.00	100.00	86.50	156.60	61.50
10	10	96	6	100.00	100.00	100.00	59.40	186.50	88.10

Table S6.2 Overview of experiment parameters and estimated LC_{50} values with their respective upper and lower confidence interval limits.

Time [h]	Pressure [Mpa]	Temperature [°C]	Copper concentrations [mg L^{-1}]	LC50 [mg L-1]	95% Upper [mg L-1]	95% Lower [mg L-1]	Rate	95% Upper	95% Lower
24	0.1	20	0, 0.2, 0.5, 1, 2, 5	1.111	1.262	0.978	-2.780	-1.608	-3.952
24	10	20	0, 0.2, 0.5, 1, 2, 5	0.782	0.876	0.697	-2.177	-1.636	-2.718
24	0.1	10	0, 0.5, 1, 2, 4, 6	1.864	2.098	1.656	-1.864	-1.493	-2.236
24	10	10	0, 0.5, 1, 2, 4, 6	1.423	1.629	1.242	-1.596	-1.264	-1.928
48	0.1	20	0, 0.2, 0.5, 1, 2, 5	1.051	1.222	0.904	-1.587	-1.182	-1.992
48	10	20	0, 0.2, 0.5, 1, 2, 5	0.812	0.951	0.692	-1.449	-1.101	-1.798
48	0.1	10	0, 0.5, 1, 2, 4, 6	1.562	1.752	1.392	-2.232	-1.744	-2.720
48	10	10	0, 0.5, 1, 2, 4, 6	1.203	1.403	1.031	-1.266	-1.005	-1.527
72	0.1	10	0, 0.5, 1, 2, 4, 6	1.453	1.636	1.291	-1.958	-1.553	-2.363
72	10	10	0, 0.5, 1, 2, 4, 6	0.561	0.696	0.452	-1.248	-0.881	-1.614
96	0.1	10	0, 0.5, 1, 2, 4, 6	0.680	0.766	0.603	-2.232	-1.593	-2.870
96	10	10	0, 0.5, 1, 2, 4, 6	0.752	0.864	0.654	-1.681	-1.245	-2.116

Chapter 7 Acute effects of burial with copper-spiked sediment on the community structure of abyssal meiofauna and nematode copper uptake

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Author contribution statement: LM, AB and AV conceived the study. LM, AB and LL performed the experiment and collected the samples. LM analysed the meiofauna community structure and did the sample analysis for nematode copper uptake that was analysed by BL and LV. Analysis of sediment metal concentrations was provided by JDG and DV. LM and AV interpreted the data and LM wrote the manuscript.

Abstract

The continuing plans for the implementation of deep-sea mineral extraction raise questions about its potential environmental impacts of e.g. the release or mobilization of toxic amounts of heavy metals in the sediment. For only little is known about deep-sea ecotoxicology, research on the sensitivity of deep-sea organisms to heavy metals is receiving increasingly more attention. Here, we report the effects of a 96 h exposure to a ± 2 cm layer of artificial sediment spiked with 4 different copper concentrations (0, 1, 5, 10 and 20 mg L^{-1} Cu^{2+}) on an abyssal meiobenthic assemblage. The experiment was conducted *in situ* at an abyssal nodule field at a depth of 4200 m in the Peru Basin, South East Pacific. Because of the high total copper content in the natural sediment (288 ± 5.19 ppm), added copper concentrations could not be detected in the bulk sediment analysis. However, as the sediment was spiked with copper in solution, bioavailability may have differed but was not measured. At the end of the experiment, 39.47 ± 12.50 % of the meiofauna was found in the added sediment layer with reduced densities in the underlying natural sediment, indicating upwards migration. The proportion of organisms migrating into the added substrate was highest for mobile taxa such as copepods and nauplius larvae. Meiofauna densities or community composition were not affected by increasing copper concentrations in the added sediment. Interestingly, nematode copper burden, quantified with μ x-ray fluorescence, was lower in nematodes from the artificial sediment that was spiked with 20 mg L^{-1} Cu^{2+} compared to nematodes from the undisturbed sediment. The vertical migration of meiofauna induced by sediment burial indicates a disturbance of the community structure and may have wider implications for the abyssal ecosystem on the long term. Moreover, different copper contents in nematodes raise questions about metal accumulation and detoxification mechanisms that require further research.

7.1. Introduction

Economically valuable mineral deposits can be found in a variety of deep-sea habitats such as abyssal plains (polymetallic nodules), active and extinct hydrothermal vents (seafloor massive sulphides) and seamounts (ferromanganese crusts) (Kato et al., 2011; Petersen et al., 2016). Mining operations of deep-sea hard structures will differ in scale and the used technology but generally, direct disturbances on the benthic environment are inevitable and likely accompanied by indirect impacts with a larger footprint due to e.g. sediment plumes (Gollner et al., 2017; Sharma, 2017; Van Dover et al., 2017). One concern when it comes to deep-sea mining impacts is the mobilization and release of elevated concentrations of potentially toxic elements during extraction, transport or after processing of the minerals by either release of tailings or extraction water (Boschen et al., 2013; Koschinsky et al., 2001a, 2001b; Thiel, 2001).

First assessments have indicated that the extraction water from seafloor massive sulphides mining may be considerably enriched in heavy metals after extraction of the target minerals and would require dilutions of >1000 times to meet protection levels of water quality guidelines (Nautilus Minerals Niugini Limited and Coffey Natural Systems, 2008). Furthermore, in the case of polymetallic nodule mining, recovery of the resources is expected to be incomplete with dilution of part of the ore in adjacent areas (ISA, 2013). Although elevated metal concentrations do not necessarily entail an equally high toxicity to organisms, ecotoxicological studies in a deep-sea context are scarce and urgently needed (Mestre et al., 2014). However, controlled toxicity testing of deep-sea organisms in the laboratory is technically challenging since many organisms only survive collection in specific pressure chambers which are rare and expensive (Mestre et al., 2014). Alternatively, researchers have focussed on understanding the effects of abiotic factors prevailing in the deep sea (high hydrostatic pressure, low temperature) on metal toxicity using shallow-water relatives of deep-sea organisms (Brown et al., 2017a, Mevenkamp et al., 2017b) or have implemented a comparative approach with shallow water and deep-sea species (Brown et al., 2017b).

Bioavailability and toxicity of metals inside sediments strongly depend on the structure and chemical properties of the substrate and surrounding abiotic environment and only little is known about these complex processes (Eggleton and Thomas, 2004; Thiel, 2001). In the marine environment, metals tend to bind to small particles, organic matter and different hydroxides and accumulate at the seafloor resulting in considerably higher concentrations of metals in marine sediments compared to the water column (Pempkowiak et al., 1999). By physically disturbing the sediment, mining activities may result in shifts of the abiotic

conditions (pH, oxygen saturation) inside the sediments which may in turn result in a potential release and dissolution of toxic metal species (Atkinson et al., 2007; Calmano et al., 1993). As such, removal of the top layer of the sediment may expose underlying, anoxic sediment layers or changes in oxygen penetration depth may be induced by sediment deposition. Therefore, impacts of mining activities with regard to metal toxicity will have a considerable impact on infaunal organisms with a low mobility which hence, require special attention.

Meiofauna - metazoan animals permanently or temporarily residing inside the sediment with a size between 32 µm and 1000 µm - are highly abundant and ubiquitous in most marine sedimentary environments (Giere, 2009; Vanreusel et al., 2010). These properties are very advantageous for environmental research, especially in the deep sea where densities and biomass of larger benthic size classes decrease with depth (Rex et al., 2006). Furthermore, meiofauna communities respond rapidly and sensitively to changes in the abiotic environment which makes them good indicators of environmental impacts from different anthropogenic disturbances (Zeppilli et al., 2015). As such, meiofauna has proven very useful in testing effects of pollution with heavy metals and other toxicants in environmental studies (Austen and Somerfield, 1997; Gyedu-Ababio and Baird, 2006; Millward and Grant, 1995). Due to their small size, meiofaunal organisms can exhibit very high abundances and with possibly high turnover rates may also constitute an important component of the benthic ecosystem in terms of secondary production (Giere, 2009; Schratzberger and Ingels, 2017). By facilitating microbial processes and serving as a food source for higher trophic levels, they are tightly linked to the other benthic size classes in the "small benthic food-web" meaning that changes in meiofauna community structure and density may have cascading effects on other benthic organisms (Giere, 2009; Schratzberger and Ingels, 2017).

To understand the effects of potentially toxic sediment plume re-deposition following deep-sea mining activities, we conducted an *in-situ* experiment with an abyssal soft-sediment meiofauna community from a nodule area in the South East Pacific Ocean. For this purpose, artificial sediment was contaminated (spiked) with different copper concentrations ranging from 1 - 20 mg Cu^{2+} L^{-1} and distributed on top of the undisturbed sediment inside corrals resulting in deposition thicknesses of 1.5 – 3 cm. Corrals with uncontaminated sediment and corrals where no sediment was added served as Controls. After 96 h the sediments were sampled with push cores and meiofauna community structure as well as copper content in selected nematodes were analysed. Copper is a naturally occurring metal that is essential to most organisms in low concentration but becomes toxic when concentrations exceed a certain threshold value and may cause oxidative damage and internal acid-base imbalance in marine invertebrates (Boitel and Truchot, 1989;

Gaetke and Chow, 2003). The high concentrations of copper in nodules and in the sediments of our experimental site (Hein and Koschinsky, 2014; Koschinsky, 2001) combined with its strong toxic potential support the choice of this element for the present study.

This experiment was done to test the following null-hypotheses:

$H_{0,1}$: Burial with uncontaminated artificial sediment does not alter meiobenthos density, community composition and vertical structure

$H_{0,2}$: There is no effect of the addition of copper contaminated artificial sediment on meiobenthic density and community structure.

$H_{0,3}$: Experimental addition of copper is not reflected by a higher copper content in the tissue of individual nematodes compared to nematodes from the natural sediment.

7.2. Material and Methods

7.2.1. Experimental site and set-up

During RV Sonne cruise SO242-2 six enclosure corrals (40 cm height, 40*40 cm top and 30*30 cm bottom dimensions, Fig. 7.1A) made of laboratory grade laminate were deployed at 4196 m depth at a southern reference site of the DISCOL (Disturbance and recolonization experiment in a manganese nodule area of the deep South Pacific) experimental area (88° 27.066' W, 7° 7.521' S, Thiel and Schriever, 1989) in the Peru Basin with the use of the remotely operated vehicle (ROV) *Kiel 6000*. Enclosure corrals were pushed ~10 cm into the undisturbed sediment and were assigned to the following treatments: Control without sediment addition (NS), Control with uncontaminated sediment (UnC) and 4 treatments with copper spiked sediment of different concentrations (1, 5, 10 and 20 mg L^{-1} Cu^{2+}, C1 - C4). For each treatment, 500 g artificial sediment, consisting of 85% quartz sand (99.9% <90 μm), 14% kaolin (96% < 45 μm) and 1% peat (100% < 500 μm), were incubated in 250 mL deionized water spiked with the respective copper concentrations by addition of a $CuSO_4 \cdot 5H_2O$ stock solution for 72h prior to deployment, following OECD (Organisation for Economic Co-operation and Development) guidelines (OECD, 2004). Sediment mixtures containing 500 g of artificial sediment were filled in sampling jars of 1 L and were emptied above the enclosure corrals by means of the ROV (Fig. 7.1 B and C) after which the corrals were left untouched for approximately 96 h. Subsequently, three push core samples (7.4 cm inner diameter) were taken from each corral which represents a case of pseudoreplications as the corrals themselves were not replicated. After sampling, the overlying water in the cores was siphoned off and sieved over a 32 μm mesh

Figure 7.1 Impressions of the deployment of the experimental corrals to test toxic sediment plume impacts on an abyssal meiofauna community, A) prior to, B) during and C) after sediment deposition. Copyright: ROV Kiel 6000 Team/GEOMAR Kiel

and the sediment was sliced at the artificial sediment horizon, the underlying 1 cm, 2 cm and 5 cm sediment depth horizons. Material retained on the sieve was added to the sample of the uppermost sediment layer. A 5 mL subsample of each slice was taken and preserved at -20 °C for measurement of bulk sediment metal concentration. Sediment samples were preserved in 4 % borax buffered formaldehyde for meiofauna analysis. The retained meiofauna from the overlying water was added to the sample of the uppermost sediment layer. Additionally, three push cores from the sediments outside the corrals (Background) were taken, sliced at the 1 cm, 2 cm and 5 cm sediment horizon and preserved at -20 °C to determine sediment characteristics of the area (total organic carbon and nitrogen content, granulometry). A subsample of 5 mL was taken from the uppermost sediment layer of each Background core and stored at -80 °C to determine the content of chloroplastic pigment equivalents (CPE, i.e. the sum of chlorophyll a and its degradation products (phaeopigments)) in the sediment.

7.2.2. Sample processing

Granulometry of the dried background samples was determined using a Malvern Mastersizer 2000 particle analyser (Malvern Instruments, UK) and sediment grain sizes were classified according to Wentworth (1922). Total organic carbon (TOC) and total nitrogen (TN) were analysed with a Flash 2000 Organic Elemental Analyser (Thermo Fisher Scientific, USA) after lyophilization, homogenization and acidification with 1 % HCl. Phytopigment measurements were done by fluorimetry with a Hitachi spectrophotometer after pigment extraction with 90 % aceton and cell mill grinding with glass beads, and were kindly provided by the Max-Planck Institute for Marine Microbiology (MPI, Bremen, Germany).

The subsample of the uppermost layer of each sediment was analysed for its copper content by inductively coupled plasma optical emission spectrometry (ICP-OES) with a Varian 720-ES ICP Optical Emission Spectrometer (Agilent Technologies, USA) following protocol nr. 14869-2:2002(E) of the (International Organization for Standardization, 2002).

Meiofauna samples were sieved over two stacked sieves of 1000 and 32 μm and the 32 μm fraction was extracted from the sediment using density gradient centrifugation at 3000 rpm for 12 minutes with Ludox HS40 (Dupont) with a specific density of 1.18 (Heip et al., 1985). The supernatant was sieved (32 μm) after each of three centrifugations after which the meiofauna retained on the sieve was fixed in 4% buffered formaldehyde and stained with a few drops of Rose Bengal. Meiofauna was identified to higher taxon level and counted.

To determine copper contents in nematodes, respectively 7 and 11 similar-sized nematodes were taken from one sample of the added sediment (AS) layer of the C4 treatment and of the 0-1 cm layer of one Background sample. Due to logistic constraints, only a limited number of nematodes could be analysed, which is why nematodes from only two samples were measured. Nematodes were transferred to a drop of water and body length (L, μm, excluding filiform tail) and average width (W, μm, measured at three different positions in the middle body region) were determined under a compound microscope connected to a Leica camera system. These measures were used to estimate nematode wet weight (WW) using an adjusted Andrassy (1956) formula to account for the specific gravity of marine nematodes (i.e. 1.13 g cm^{-1}): μg $WW=L \times W^2/1{,}500{,}000$ (as described in Pape et al. (2013). Subsequently, nematodes were transferred to 500 nm thin silicin nitride membranes (Silson Ltd, United Kingdom) by means of a small drop of MilliQ water and left to air-dry. Finally, copper contents were assessed by means of micro X-ray fluorescence (μXRF) using the Edax Eagle III (Edax Inc., USA). This instrument is equipped with a 50 W Rh X-ray tube fitted with polycapillary optics which focus the X-ray in a 30 μm spot. A liquid nitrogen cooled Si(Li) detector is employed to capture the fluorescent X-rays. To examine the Cu content of the organisms, small mappings were performed with 30 μm step size. Each measurement point of these mappings contains a full XRF spectrum with 10 s live time. These spectra are analysed using AXIL, an iterative least squares algorithm yielding the net intensities for each detectable element present in the sample. The points belonging to the organism are extracted from the XRF element maps using k-means clustering. Next, the spectra from these data points are summed to obtain the total intensity generated by the nematode during the measurement. The intensities per nematode are normalized using nematode wet weights. Due to the small diameter of the organisms (~ 30 μm) the absorption effects on Cu are negligible, so the normalized intensities of the different scans can be compared directly with each other, in other words, a nematode with more Cu present in its body will yield a higher intensity (counts) per unit body mass (in μg).

7.2.3. Data analysis

The distribution of the artificial sediment by the ROV resulted in different deposition heights between 1 and 3 centimetres on the corral surface, but in most of the samples (9 out of 14), the height of the added sediment was 2 cm. Meiofauna densities have been standardized to the number of individuals per 10 cm^2. One replicate of the C1 treatment was excluded from the analysis as an outlier due to exceptionally low densities (16.51 ind. 10 cm^{-2}).

The data analysis has been performed to allow the evaluation of the research hypothesis. For that purpose, we first report the differences between the control without added sediment (NS) and the treatment with uncontaminated sediment (UnC) to investigate the effect of sediment addition on the meiofauna community. Secondly, differences between all treatments where sediment was added (UnC and C1 – C4) were tested to evaluate the effect of copper addition on the meiofauna community. Thirdly, nematode copper concentrations were compared between selected nematodes from the NS and the C4 treatment.

Differences between treatments in bulk sediment copper concentrations were analysed with a one-way analysis of variance (ANOVA) using the R software (R Core Team, 2013) after assumptions of normality (Shapiro-Wilk test) and homoscedasticy (Levene's test) were validated. In case of a significant main test, pairwise Tukey tests were performed.

Differences in total meiofauna densities (expressed as number of ind. cm^{-2}) were analysed with the non-parametric Wicoxon test (NS vs. UnC) and Kruskall-Wallis test (UnC, C1-C4) due to the non-normal distribution of the data (Shapiro-Wilk test) and unequal number of replicates (C1: n=2). Due to the unequal thickness of the sampled depth layers, differences in meiofauna community composition between treatments and depth layers were examined based on the relative abundances of the different meiofauna taxa in the respective depth layers. Differences between treatments or depth layers were examined using a Cluster analysis (group average method), based on a Bray-Curtis resemblance matrix, combined with a SimProf test to reveal significant sample groupings. Significant clusters were visualized in multidimensional scaling (MDS) plots. The relative contribution of each meiofauna group to the differences between significant clusters was evaluated with a Similarity Percentages (SIMPER) test.

7.3. Results

7.3.1. Sediment analysis

With a median grain size of 19.82 ± 0.16 µm the natural sediment was mainly composed of medium silt (Wentworth, 1922). Organic carbon and nitrogen content was 0.63 ± 0.01 % and 0.18 ± 0.01 %, respectively, which is comparable to previous studies in that region (Grupe et al., 2001; Koschinsky et al., 2001b). Chloroplastic pigment equivalents (CPE) ranged between 0.68 and 0.31 µg mL^{-1}.

Copper concentrations of the Background samples and the NS differed significantly from the artificial sediment in the various treatments (Tukey-test, $p < 0.002$) but did not differ from one another (Fig. 7.2). Similarly, sediment copper concentrations did not differ between treatments with added sediment. It is very likely that during slicing of the added sediment layer in the sampled cores, small amounts of natural sediment contaminated the artificial sediment. Therefore, the high copper concentrations in the natural sediment may have masked the comparatively low copper concentrations that were experimentally added. Nevertheless, addition of copper in a soluble form may still have resulted in different bioavailability between naturally present copper and added copper.

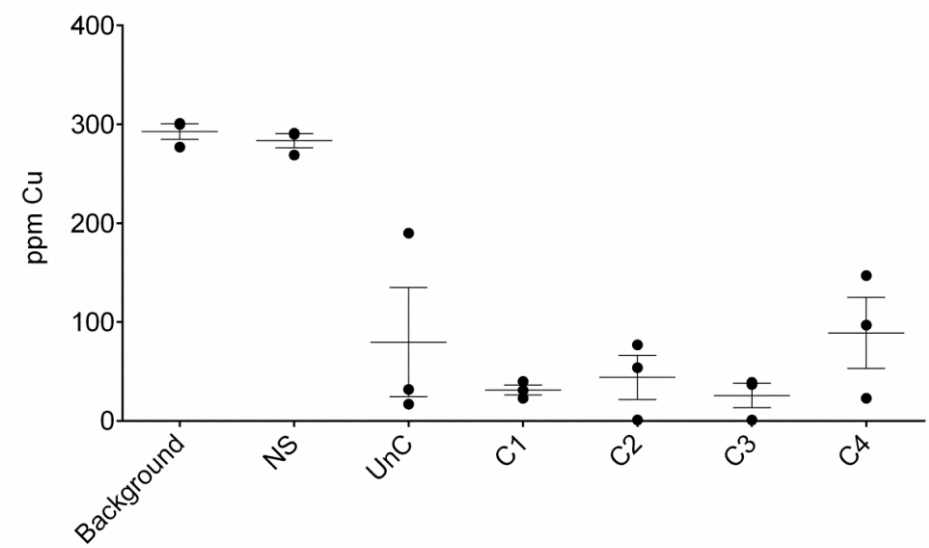

Figure 7.2 Copper concentrations in the uppermost layer of all samples. NS= no sediment addition, UnC= addition with uncontaminated sediment, C1-C4= addition with sediment spiked with 1, 5, 10 and 20 mg L^{-1} Cu^{2+}. All data points are shown, the line and error bars depict mean and standard error, respectively (n=3, except C1 where n=2).

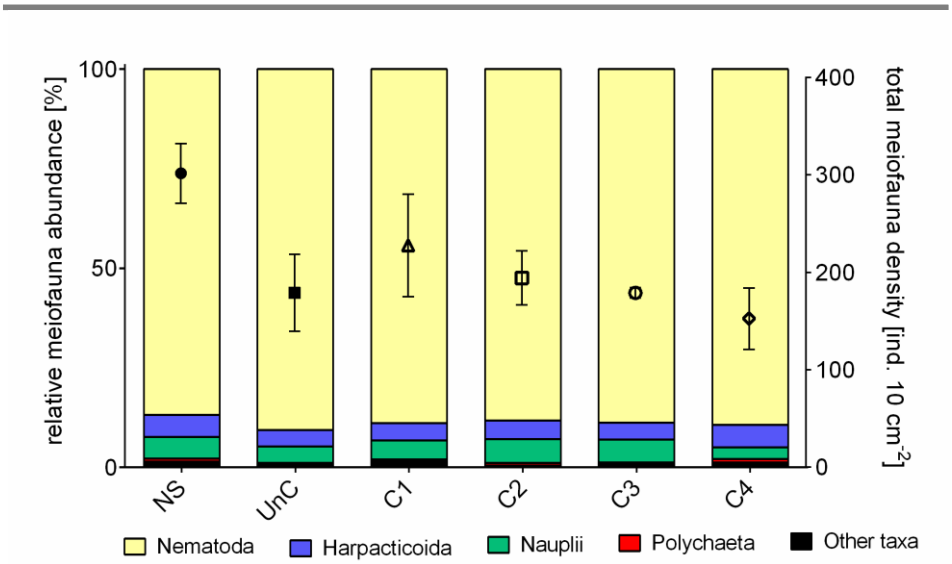

Figure 7.3 Relative abundances of meiofauna taxa in the different treatments (bars, left y-axis) and average, whole core meiofauna densities (in number of individuals per 10 cm^2 ± standard error; symbols and error bars, right y-axis) NS = no sediment, UnC = addition of uncontaminated sediment C1-C4 = addition of copper-spiked sediment (1, 5, 10 and 20 mg L^{-1} Cu^{2+}). N = 3 for all treatments except C1 where N=2.

7.3.2. Meiofauna analysis

Total meiofauna densities ranged from 152.37 ± 31.55 ind. 10 cm^{-2} (mean ± standard error) in the C4 treatment to 301.72 ± 30.66 ind. 10 cm^{-2} in the Control (NS) (Fig. 7.3). The meiofauna community combining all samples was composed of 88.78 % ± 0.66 % Nematoda, 4.80 % ± 0.23 % Harpacticoida, 4.80 % ± 0.49 % Nauplii, 0.51 %± 0.13 % Polychaeta and 1.10 % ± 0.17 % other taxa (Ostracoda, Tardigrada, Gastrotricha, Kinorhyncha, Bivalvia, Tanaidacea, Halacaroidea, Loricifera, Tantulocarida and Isopoda, Fig. 7.3).

7.3.2.1. Meiofauna responses to sediment deposition

Total meiofauna densities were lower in the UnC (178.88 ± 39.40 ind. 10 cm^{-2}, mean ± SE) compared to the NS treatment (301.72 ± 30.66 ind. 10 cm^{-2}) but did not differ significantly (Wilcoxon test, W=8, p=0.2).

In the NS treatment, 37.69 ± 7.76 % of meiobenthos was found in the uppermost centimetre of sediment, 29.50 ± 1.03 % in the 1-2 cm layer and 32.81 ± 8.42 % in the 2-5 cm layer (Fig. 7.3). Whereas in the UnC treatment, most meiobenthos was found in the added sediment (39.53 ± 12.53 %) while 17.65 ± 1.87 %, 17.63 ± 4.63 % and 25.25 ± 9.65 % remained in the 0-1 cm, 1-2 cm and 2-5 cm layer, respectively.

The results of the cluster analysis revealed three significant clusters branching at branching at 85.11 % similarity ($\pi = 1.87$, $p = 0.001$) and 91.87 % similarity ($\pi = 0.71$, $p = 0.001$, Fig. 7.5). The first cluster was composed of two samples of the artificial sediment layer (AS) and all 0-1 cm layers of the NS treatment (Cluster A, Fig. 7.4). The second cluster contained the third AS layer and two samples of the 1-2 cm layer of the NS treatment (Cluster B, Fig. 7.4). The third cluster contained all remaining samples (Cluster C, Fig. 7.4). Differences between cluster A and C and between B and C were mainly caused by higher nematode and lower harpacticoid and nauplii abundances (SIMPER results in Tab. 7.1). Similarly, differences between Cluster A and B were due to higher nematode abundances and lower abundances of harpacticoids and nauplii in samples of cluster B (SIMPER results in Tab. 7.1). This indicates that after addition of the artificial sediment, meiofauna community composition in that added layer resembled the composition of the upper cm layers of the control without sediment addition.

The dominant meiofauna taxa showed a differential migration potential into the added sediment and at the end of the experiment, 36.28 ± 7.14 % of the nematodes were found in the added layer, while this percentage was higher for harpacticoids and nauplii (69.75 ± 3.97 % and 74.68 ± 2.51 %, respectively)..

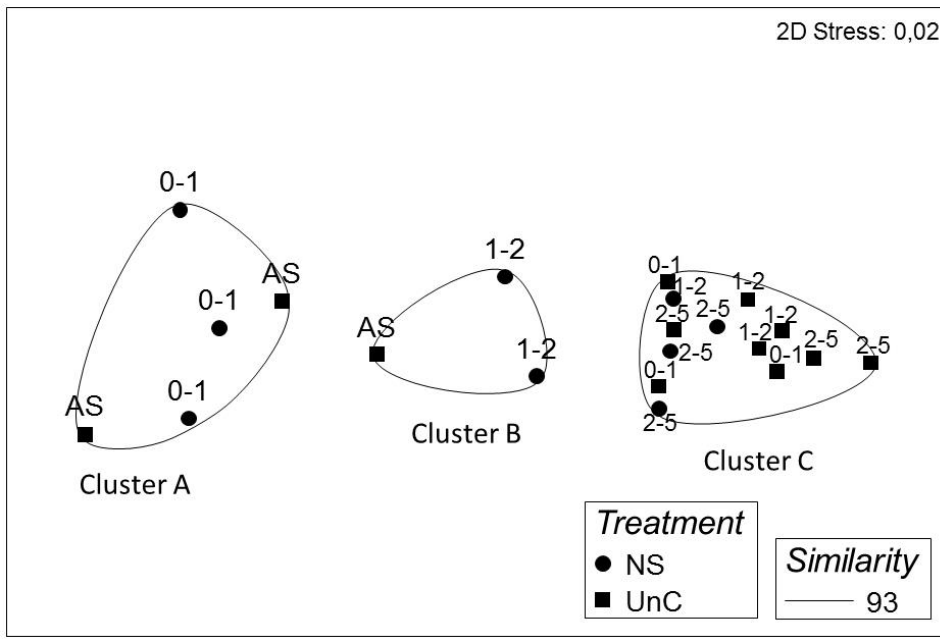

Figure 7.4 Multidimensional scaling of meiofauna community composition per depth layer in the treatment without and with addition of artificial sediment, NS and UnC, respectively. Contours indicate significant clusters (SIMPROF test) at the 93 % similarity level.

Table 7.1 Results of the SIMPER analysis between significantly different clusters identified in the dataset of relative meiofauna abundances in different depth layers of the NS and UnC treatment. Contrib%= Contribution to the dissimilarities,.

	Average abundance		Contrib%
Average dissimilarity = 9.12	*Cluster A*	*Cluster B*	
Nematoda	78.79	87.11	45.61
Nauplii	9.11	5.11	21.91
Harpacticoida	9.28	5.74	19.39
Polychaetes	0.97	0.31	4.29
Average dissimilarity = 16.23	*Cluster A*	*Cluster C*	
Nematoda	78.79	94.70	49.04
Nauplii	9.11	2.01	21.87
Harpacticoida	9.28	2.60	20.58
Average dissimilarity = 8.13	*Cluster B*	*Cluster C*	
Nematoda	87.11	94.70	46.72
Nauplii	5.11	2.01	19.97
Harpacticoida	5.74	2.60	19.75
Ostracoda	0.88	0.06	5.23

7.3.2.2. Responses towards experimentally elevated sediment copper concentrations

Total meiofauna densities were lowest in the C4 treatment (152.37 ± 31.55 ind. 10 cm^{-2}, mean ± SE) and highest in the C2 treatment (194.30 ± 27.66 ind. 10 cm^{-2}) but did not differ significantly between treatments (Kruskall-Wallis test, χ^2= 2.457, p= 0.652).

The cluster analysis of the relative meiofauna composition revealed 5 significant clusters (Fig. 7.5). Cluster A (branching at 76 % similarity, π = 1.88, p= 0.001) and B (branching at 86.06 % similarity, π = 1.52, p = 0.001) contained all but three AS layers (Fig. 7.5). Cluster C (branching at 93.45 % similarity, π = 0.52, p = 0.001) contained the three remaining AS layers and several samples of other depth layers

Table 7.2 Relative abundances of nematodes, harpacticoids and nauplii in the significantly different clusters identified by the cluster analysis based on the meiofauna community composition per depth layer in treatments where uncontaminated (UnC) or copper-spiked sediment (1, 5, 10 and 20 mg L^{-1} Cu^{2+}, C1 – C4) was added.

	Cluster A	Cluster B	Cluster C	Cluster D	Cluster E
Nematoda	68.38	80.61	89.61	93.13	96.17
Copepoda	14.19	8.43	4.34	3.36	1.95
Nauplii	12.49	8.77	4.04	2.98	1.25

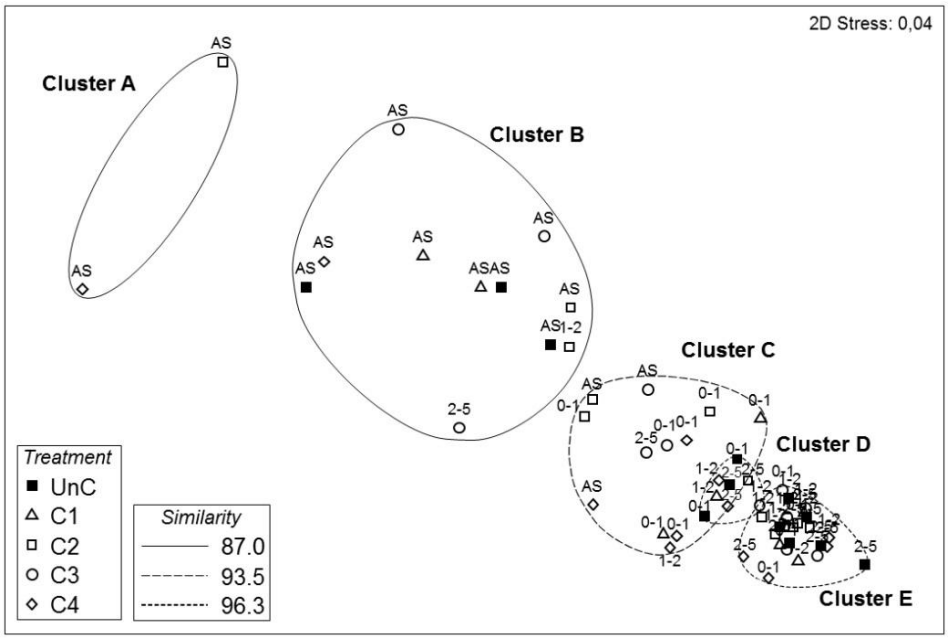

Figure 7.5 Multidimensional scaling of the meiofauna community composition (based on relative abundances) per depth layer in the treatments where uncontaminated (UnC) or copper-spiked sediment (1, 5, 10 and 20 mg L^{-1} Cu^{2+}, C1 – C4) was added. N = 3 for all treatment except C1 where N=2 Contours indicate significant clusters (SIMPROF test).

without any clear grouping according to treatment or depth layer (Fig. 7.5). Similarly, clusters D and E (branching at 96.25 % similarity, $\pi = 0.12$, $p = 0.044$) revealed no clear grouping of samples according to treatment and depth layer (Fig. 7.5). SIMPER analysis between clusters indicated that differences were largely caused by increasing abundances of nematodes (A<B<C<D<E) and decreasing harpacticoid and nauplii abundances (A>B>C>D>E, Tab. 7.2, Tab. S7.1).

7.3.2.3. Nematode copper uptake

Internal copper concentrations of nematodes averaged 5409.64 ± 383.64 ppm in nematodes of the NS treatment and 2791.29 ± 500.95 ppm in the C4 treatment and differed significantly between both treatments ($t_{12.52}$=4.150, p=0.0012, Fig. 7.6).

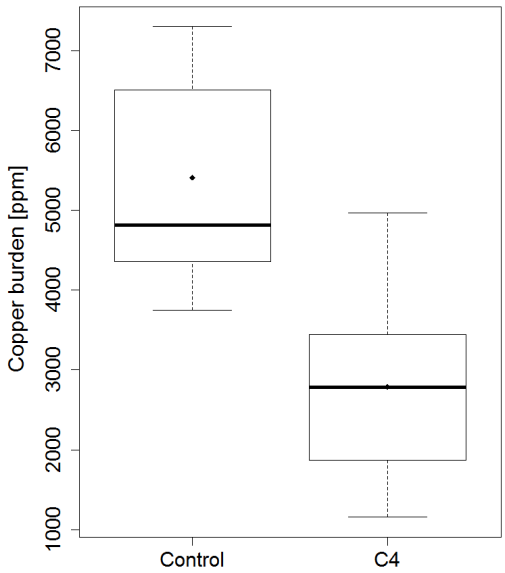

Figure 7.6 Box-whisker plots of nematode copper burden derived from µ-XRF measurements of individual nematodes in the Background samples ("Control", N=11) and the C4 treatment (N=7). Error bars denote standard errors of the mean. Black lines indicate the median, points the mean.

7.4. Discussion

7.4.1. Sediment deposition induces shifts in vertical meiobenthos distribution

One important finding of our study is that sediment deposition, regardless of its copper contamination, resulted in a disturbance of the meiofauna community with upward migration of most taxa. A similar response was observed in an experiment where 2 cm of crushed nodule particles were deposited on sediment in the same experimental area and incubated for a longer time of eleven days (Mevenkamp et al., unpublished). Also in that experiment, the majority of the meiofauna was found in the added substrate at the end of the incubation period and, similarly, relatively mobile taxa such as copepods and nauplii were proportionally more abundant in the added substrate. In a mesocosm experiment with fjord sediment, Mevenkamp et al. (2017a) could furthermore show that the migratory response to burial with inert mine tailings and sediment was associated with elevated nematode mortality in the added substrates, a worrisome finding considering potential long-term effects on the impacted ecosystem.

The observation of relatively more copepods and nauplii in the added substrate may be explained by their higher mobility, as these taxa have been shown to

possess a higher capacity of active migration and emergence from the sediment compared to e.g. nematodes (Armonies, 1988; Boeckner et al., 2009; Commito and Tita, 2002; Mevenkamp et al., 2016). This enables them to colonize new substrates faster than nematodes, but they may get outcompeted by other meiofauna taxa on the long term (Fonsêca-Genevois et al., 2006).

The vertical distribution of meiofauna communities is generally structured in relation to biochemical gradients inside the sediment and one main drivers appears to be sediment oxygenation (Moens et al., 2013; Moodley et al., 2000a). Interestingly, copepods show a higher sensitivity towards low pore water oxygen concentrations, whereas nematodes are more tolerant to such unfavourable conditions (De Troch et al., 2013; Grego et al., 2014; Moodley et al., 1997; Wetzel et al., 2001). This high sensitivity of meiofauna and of copepods in particularly may point towards a flattened oxygen penetration depth (OPD) as a result of sediment burial as has been previously reported in the mesocosm experiment by Mevenkamp et al. (2017a).

Although the sediments in the Peru Basin are generally well-oxygenized with an OPD of more than 6 cm (Haeckel et al., 2001), substrate burial may have induced a flattening of this OPD. Indeed, when looking at the control situation without added sediment, we see copepods and nauplii distributed across the whole depth of the core while their density is strongly reduced in all layers of the natural sediment when artificial sediment was added. Despite the lack of measurements to support this hypothesis, a shifted OPD resulting from sediment deposition would explain the change in copepod and nauplii vertical distribution observed in our experiment.

A lower OPD would in turn have an effect on heavy metal availability inside the sediment. It has been shown that the redox-conditions at the Peru Basin are very stable due to the constant oxygenation of the surface layers, but disturbances of those redox conditions could potentially entail heavy metal release for a short time until a new stable redox-boundary is established (Koschinsky, 2001).

7.4.2. Meiofauna response to burial with copper-spiked sediment

The predominant aim of this study was to evaluate the effect of copper contaminated sediment on the structure of the meiofauna community in abyssal nodule areas. We were not able to detect any differences in the short-term response of meiofauna community structure between treatments and vertical distribution patterns were similar in all samples. This is despite the fact that applied concentrations of copper widely covered lethal limits of shallow-water copepods and nematodes determined under laboratory conditions (Bengtsson and

Bergström, 1987; Hagopian-Schlekat et al., 2001; Verriopoulos and Dimas, 1988; Vranken et al., 1988; Vranken and Heip, 1986). This discrepancy may, however, be explained by the short duration of our experiment. While 96 h is a standard duration of acute toxicity tests, this may not be sufficient for environmental meiofauna research since an endpoint such as mortality may be masked by slow degradation rates of dead bodies resulting in unrecognized mortalities. However, longer incubation times were logistically difficult during the research cruise. As a further limitation, our measurements and analyses did not allow us to infer concentration, availability and speciation of copper inside the artificial sediment.

Copper concentrations in the Control were within the range of previously measured values of ~400 ppm in bulk sediment samples from the Peru Basin (Koschinsky, 2001). With a high content in manganese-oxihydroxides and iron-oxides, the natural sediment possesses a high capacity to absorb the divalent copper ions added with the artificial sediment under oxic conditions, thereby possibly reducing its immediate bioavailability (Koschinsky, 2001). It is, therefore, possible that experimentally introduced copper was absorbed to particles in the upper sediment layer immediately after addition resulting in a low toxic potential. However, in an experiment in the same experimental area that used nearly the same experimental design, Brown et al. (2017b) observed a strong avoidance response of deep-sea Holothuria to the amended copper-spiked sediment after 96 h. This is an indication that addition of copper-spiked sediment resulted in a successful contamination of the water column indicating that experimentally added copper was washed out of the artificial sediment and remained in the water column. The possibly reduced toxic potential or out-washing of copper from the artificial sediment and the short incubation time of the experiment may explain the absence of a meiofauna community response.

7.4.3. Copper uptake by individual nematodes

To our surprise, internal copper concentrations of individual nematodes from the highest copper concentration (20 mg L^{-1}) were lower than those of the upper layer of untreated sediments. This may be an indication of a lower copper availability in the added artificial sediments, corroborating with above mentioned hypotheses of copper removal due to absorption or out-washing. Uptake of heavy metals in nematodes may occur directly through the cuticle or via the digestive tract. Howell (1983) investigated copper content in different tissues of two marine nematodes and found a higher association of copper with the cuticle and the hypodermis. In that experiment, two nematode species from a polluted and unpolluted site were exposed to 0.01 ppm $CuSO_4$ and measured at different time intervals. Copper concentrations in the various dissected tissues ranged between 10 and 900 ppm

after 3 days of exposure. Another study reported copper concentrations of 500 ppm after 120 h exposure to approximately 20 mg L^{-1} $CuSO_4$ in the plant-feeding nematode *Xiphinema vuittenezi* (Sávoly and Záray, 2014). A third study reported individual copper levels of 4.10 and 12.32 ppm in the soil nematode *Caenorhabditis elegans* after 36 h exposure to uncontaminated growth medium and medium contaminated with approx. 13 mg L^{-1} $CuCl_2$ (Gao et al., 2008). It is striking that copper values of the nematodes in our study are much higher than those of previously mentioned studies in both, uncontaminated and contaminated samples. Except for the nematodes from the polluted site in the study of Howell (1983) nematodes in previously listed experiments came from unpolluted sites or cultures. Nematodes from our study are naturally exposed to the high metal concentrations of 300 ppm inside the sediments characteristic for nodules sites (Koschinsky, 2001; Nath et al., 1989) but, as previously discussed, possibly only a fraction of it is present in a bioavailable form. Mechanisms of metal detoxification may include increased production of metal binding metallothioneins (MT) and metallothionein-like-proteins (MTLP) and storage of the detoxified metals but also excretion of metals before or after storage and adjustment of other, more general physiological processes (Wang and Rainbow, 2005). It has been shown that pre-exposure of marine invertebrates moderates bioaccumulation of trace metals potentially leading to an adaptation of exposed populations to elevated metal contents (Wang and Rainbow, 2005). In our study, nematodes from the nodule fields may have been adapted to elevated metal concentrations in such a way that when the organisms moved in the added substrate they were exposed to significantly lower (biologically available) copper concentrations (considering hypothesized reduced concentrations by absorption or washing) and were continuing their detoxification processes resulting in a lower copper burden in nematode bodies.

Acknowledgements

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7.5. Supplementary data

Table S7.1 Results of the SIMPER analysis between significantly different clusters identified in the dataset of relative meiofauna abundances in different depth layers of treatments where uncontaminated (UnC) or copper-spiked sediment (1, 5, 10 and 20 mg L^{-1} Cu^{2+}, C1 – C4) was added. Contrib%= Contribution to the dissimilarities,

	Average abundance		Contrib%
Average dissimilarity = 14.09	*Cluster A*	*Cluster B*	
Nematoda	68.38	80.61	43.39
Harpacticoida	14.19	8.43	21.02
Nauplii	12.49	8.77	17.03
Ostracoda	1.77	0.46	5.56
Polychaetes	1.10	0.67	4.13
Average dissimilarity = 22.49	*Cluster A*	*Cluster C*	
Nematoda	68.38	89.61	47.20
Harpacticoida	14.19	4.34	21.90
Nauplii	12.49	4.04	18.80
Ostracoda	1.77	0.12	3.72
Average dissimilarity = 25.08	*Cluster A*	*Cluster D*	
Nematoda	68.38	93.13	49.35
Harpacticoida	14.19	3.36	21.61
Nauplii	12.49	2.98	18.98
Ostracoda	1.77	0.00	3.52
Average dissimilarity = 28.17	*Cluster A*	*Cluster E*	
Nematoda	68.38	96.17	49.33
Harpacticoida	14.19	1.95	21.74
Nauplii	12.49	1.25	19.96
Average dissimilarity = 10.68	*Cluster B*	*Cluster C*	
Nematoda	80.61	89.61	42.15
Nauplii	8.77	4.04	22.68
Harpacticoida	8.43	4.34	20.68
Tardigrada	0.57	0.90	4.27
Polychaetes	0.67	0.52	3.93
Average dissimilarity = 12.88	*Cluster B*	*Cluster D*	
Nematoda	80.61	93.13	48.63
Nauplii	8.77	2.98	22.54
Harpacticoida	8.43	3.36	19.70
Average dissimilarity = 16.01	*Cluster B*	*Cluster E*	
Nematoda	80.61	96.17	48.61
Nauplii	8.77	1.25	23.49
Harpacticoida	8.43	1.95	20.24

Average dissimilarity = 4.67	*Cluster C*	*Cluster D*	
Nematoda	89.61	93.13	37.70
Harpacticoida	4.34	3.36	20.64
Nauplii	4.04	2.98	19.05
Tardigrada	0.90	0.27	9.74
Polychaetes	0.52	0.21	6.00
Average dissimilarity = 7.20	*Cluster C*	*Cluster E*	
Nematoda	89.61	96.17	45.54
Nauplii	4.04	1.25	19.99
Harpacticoida	4.34	1.95	18.68
Tardigrada	0.90	0.15	6.32
Average dissimilarity = 3.75	*Cluster D*	*Cluster E*	*Contrib%*
Nematoda	93.13	96.17	40.45
Nauplii	2.98	1.25	24.59
Harpacticoida	3.36	1.95	21.41
Polychaetes	0.21	0.30	5.56

Chapter 8 General discussion

The aim of the research presented in this thesis was to investigate responses of meiobenthic communities to different environmental stressors through experimental approaches. The investigated stressors were related to potential impacts associated with atmospheric carbon increase and mineral extraction.

The studies related to the impacts of seawater acidification on the benthos of a shallow, subtidal and an intertidal area are presented in Chapter 2 and 3 (Fig. 8.1). The results of these studies emphasize the species/taxon-specific responses of bivalves and meiobenthos to changes in seawater pH and underline the importance of rare taxa and biotic interactions for the evaluation of ocean acidification effects on subtidal and intertidal communities. Apart from the pH levels predicted by the end of this century, much lower values, that may result from a CO_2 leakage into the marine environment during or after CCS practices, were applied in Chapter 2. Nematodes generally showed a high resistance to changes in pH while other taxa (e.g. bivalves, ostracods, copepods) responded more sensitively (Chapter 2 and 3). In addition to seawater acidification, Chapter 3 also incorporated elevated seawater temperature to investigate cumulative effects of both stressors on an intertidal, meiobenthic community. Indeed, a synergistic effect on sediment pH profiles and meiofauna community composition was evident at high temperature (13 °C) and low pH (7.5) compared to ambient temperature (10 °C) and pH (7.9) and ambient temperature and reduced pH (7.5). Meiobenthos responses were taxon-specific and less dominant taxa showed significant density changes between treatments possibly resulting from a combination of species specific sensitivity to the altered abiotic conditions and biotic interactions.

Both studies advance our understanding of dynamics and interactions of biota and biogeochemistry in sediments of shallow, coastal ecosystems under changing seawater pH and temperature conditions. The results of these studies reveal new patterns that require further investigation, especially with regard to biotic interactions and cumulative effects of multiple stressors.

The subsequent Chapters 4-7 investigated impacts of stressors related to the burial with toxic and non-toxic material resulting from mineral mining on the structure and functioning of bathyal and abyssal communities (Fig. 8.1).

In a short-term mesocosm experiment, burial of a bathyal, soft-sediment community from a Norwegian fjord with different amounts of mine tailings and natural sediment resulted in reduced ecosystem functioning measured in terms of community oxygen consumption and uptake of ^{13}C-labelled algae by biota (Chapter 4). First signs of reduced ecosystem functioning were already visible at 0.1 cm mine tailings deposition and were aggravated at higher amounts of tailings burial. Furthermore, meiofauna showed a vertical migration response, possibly as a

result of reduced oxygen concentrations in the original sediment. Nematode viability assessment with a trypan blue stain suggested elevated mortality in treatments with 3 cm of deposited substrate. Through the use of different deposition depths, this experiment allowed for the evaluation of threshold values regarding tailings disposal in bathyal fjords. The transferability of the results to other situations and environments, however, needs to be done with caution since factors such as surface productivity (Smith et al., 2008b) or the rate of substrate addition (Schratzberger et al., 2000) may influence responses of the benthic community.

In an *in situ* experiment in the abyssal South East Pacific (Chapter 5) a first effort was done to investigate impacts of burial with crushed nodule particles on meiobenthic structure. The similar incubation period of 11 days facilitated comparison of the results from this study with those obtained in Chapter 4. In both experiments, changes in vertical structure of meiobenthos were observed due to migratory responses into the respective added substrate. Apart from the vertical migration, no obvious changes in the overall structure of the abyssal meiofauna assemblage or in nematode community composition were found. In addition to meiofauna community responses, we assessed the uptake of copper in selected nematodes since metal concentrations in the crushed nodule substrate were very high. However, no difference was found between the copper burden in control

Stressors		Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7
pH		X	X				
Temperature			X			X	
Pressure						X	
Copper						X	X
Substrate burial:	fine sediment			X			X
	coarse substrate			X	X		

Analysed responses of meiobenthos		Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7
Meiobenthos community:	Composition	X	X	X	X		X
	Vertical distribution		X	X	X		X
Nematodes:	Genus composition	X			X		
	Mortality		X	X		X	
	Copper uptake				X		X
	Carbon uptake			X			

Temporal scale months — week — days

Figure 8.1 Overview of the different chapters in this thesis in relation to the applied stressors and analysed responses of meiofauna and duration of the exposure.

animals and those found in the crushed nodule substrate, possibly owing to the low metal bioavailability as a result of sorption processes under oxic conditions.

The sensitivity of nematodes to copper under varying conditions of hydrostatic pressure and temperature was tested in a laboratory experiment described in Chapter 6. Through acute toxicity tests we aimed to understand the effect of pressure and temperature on copper toxicity and to unravel potential synergies and general mechanisms of metal toxicity under deep-sea conditions in the shallow-water marine nematode *Halomonhystera disjuncta*. The experiments revealed an increased copper toxicity at high hydrostatic pressure (10 MPa, comparable to 1000 m water depth) and high temperature (20 °C). Pressure and temperature generally exhibit antagonistic effects on metabolic equilibria and kinetics (Pradillon and Gaill, 2007). In contrast, our results indicate cumulative effects of both stressors, not antagonistic responses. This suggests that hydrostatic pressure and temperature act on different metabolic pathways or mechanisms on copper toxicity in the nematode. Our findings are an important step forward to understand metal toxicity in the deep sea and underline the importance of temperature and pressure in deep-sea ecotoxicology.

Laboratory experiments only have limited ecological relevance and should be accompanied by field experiments or be validated by field observations. Chapter 7 describes an experiment where we exposed an abyssal soft-sediment community to burial with copper contaminated artificial sediment over a period of four days under *in situ* conditions. We aimed to not only understand copper toxicity effects on abyssal soft-sediment communities but also to investigate cumulative effects of sediment deposition and metal toxicity. However, due to the short exposure duration and sampling circumstances (retrieval of samples from >2000 m) mortality of meiofauna could not be assessed. Interestingly, internal copper burden revealed lower copper contents in nematodes exposed to the highest contaminated sediment compared to control animals. The vertical migration response of meiofauna taxa observed in previous experiments was also confirmed in this study and occurred in all treatments, regardless the copper contamination of the added sediment. This paper is a valuable addition to the other chapters in this thesis as it confirms previously reported results (vertical migration) and it points out the limitations and challenges of deep-sea experimental research.

In the following sections, we will first elaborate on the limitations and challenges of experimental research in general, and in particular with regard to marine ecology. Some of the patterns found in most experiments in this thesis (taxon specific responses and interactions, cumulative effects) will be discussed in the light of previous research. Thereafter follows an integration of our results in the

larger context of the different topics. We will investigate the potential integration of our research results in outlining management strategies for anthropogenic activities and give recommendations for future experimental research.

8.1. Limitations and general considerations of the experimental approaches

The experiments reported in this thesis covered different modes of organization (from species to ecosystem) and complexity (single stressors and multiple stressors). This enabled us to shed light on different aspects of the capacity of an organism or a community to cope with environmental stress. What follows is a description of the different spatial and temporal scales that need to be considered for the interpretation of our results and a critical evaluation of the experimental approaches.

8.1.1. The problem of scale

In ecology, every process and every organism acts on its own spatio-temporal scale (Levin, 1992). If we try to understand patterns in one group of organisms, in our case meiofauna, we need to look through the eyes of a meiofaunal organism and consider factors that are important for its size (Azovsky, 2000). The small size of meiofaunal organisms makes them e.g. much more susceptible to even weak water currents, that can carry the animals over large distances, in comparison to larger macro- or megafaunal animals (Palmer, 1988). Similarly, when investigating multispecies assemblages we need to adjust the spatio-temporal scale of our experiments to obtain relevant information on all components of the assemblage. For the investigation of meiofauna and macrofauna communities, small mesocosms of several litters volume suffice to capture the effect of environmental changes on these organisms as they interact with their environment on small spatial scales (Murray et al., 2002). These low logistic requirements are very advantageous for experimental research. Nevertheless, the necessary but narrowed approach to scale in experimental work sometimes conflicts with the scale of the investigated topics.

Ocean acidification and warming is a process that acts on a global scale, albeit with profound regional differences, but also occurs gradually and over very long time scales (Rhein et al., 2013). Impacts of mineral extraction, on the other hand, are expected to be comparatively regional and confined, acting on a spatial scale of tens to thousands of km² and impacts may occur instantaneous (e.g. substrate removal, burial or metal toxicity) but may as well persist over time frames of multiple decades (Gollner et al., 2017; Miljutin et al., 2011). Therefore, immediate

responses of the sediment fauna to the disturbances but also impacts over longer time scales are of interest. The design of experimental studies needs to be optimized to capture the spatio-temporal scale of the processes of interest, whilst remaining within the range of technical/logistic feasibility. This can be very challenging and implies that all experimental studies exhibit some kind of limitation.

The experiments presented in this thesis were conducted over a range of temporal scales. While the experiment duration of 24 - 96 h in Chapter 6 was based on existing protocols of standard toxicity testing, the durations of all other experiments was a compromise between practical feasibility of our experimental studies and the relevance for the impact of interest. Due to the large temporal scale relevant in an OAW scenario, but also in the context of mineral mining (e.g. in the assessment of recovery potential), long experimental durations would be preferable and are urgently needed in ecological studies (Hughes et al., 2017). However, constraints in the logistics but also in the funding of long-term projects often limit the scale and duration of experiments which, ultimately, has repercussions on the design of the study and underlying research questions.

In Chapter 2 and 3, experiments were conducted over a longer period of time encompassing several months (Fig. 8.1). The generation time of meiofauna is variable, but for most organisms it lies in the range of weeks to months (Coull, 1999; Gerlach, 1971). Therefore, meiofaunal communities in those experiments had the time to develop over multiple generations which increases the relevance of these experiments with regard to the temporal scale of OAW impacts. Different life-stages of benthic organisms have shown to exhibit differential sensitivity towards environmental stressors in relation to OAW (Harvey et al., 2013; Kroeker et al., 2013) or metal toxicity (Mohammed, 2013). Therefore, changes in community demography are among the potential impacts of these stressors on benthic ecosystems and need to be incorporated in experimental research. As such, the duration of the experiments presented in Chapter 2 and 3 revealed a differential impact of stressors on the nauplius stages compared to adult and pre-adult copepods. Nauplii tended to show stronger density variations between treatments, pointing towards either a higher sensitivity of the early life-stages or variations in the fecundity of the adult animals.

During deep-sea research cruises, time is a limited resource and experimental studies are generally of short durations due to the tight schedule of the research cruise. Often it is not possible to revisit an experimental site and it is difficult to monitor manipulative experiments over longer times. Experiments conducted over a long time in open systems always come with the risk of unnoticed events

happening in the absence of the researchers, interfering with the study and biasing the results. This is of particular concern when researchers aim to isolate effects of one or several specific, manipulated factors on the environment. Due to these challenges but also to ensure comparability between studies, manipulative experiments investigating effects of mineral extraction were performed with short durations encompassing days to weeks (Chapter 4 to 7, Fig. 8.1). As a consequence, only acute effects on the community could be assessed, disregarding effects on organisms life-history traits, population dynamics or adaptation potential.

All of our experiments were conducted in enclosed or semi-enclosed systems preventing or hampering colonization and migration into or out of the experimental set-ups. Guilini et al. (2011) described the colonization of azoic sediment by deep-sea meiofauna via horizontal migration through the sediment revealing the importance of horizontal dispersion of meiofaunal organisms. By limiting horizontal migration in our experiments, we were unable to identify possible vertical migration responses of meiofaunal organisms to stressor exposure. Nevertheless, despite their relatively high mobility given their small size (Boeckner et al., 2009), lateral dispersion is likely not very meaningful for meiofauna when disturbances cover larger spatial scales (>hundreds of metres).

Despite many difficulties, experimental approaches are an important tool to investigate environmental patterns and may lead to the crafting of new theories that can be tested in subsequent experimental research of field studies (Benton et al., 2007). Ultimately, this may lead to the understanding of global or general patterns. The combination and integration of multiple approaches in environmental research, from field experiments to models, is necessary to create a complete picture of the effects of large scale processes (Queirós et al., 2015; Stewart et al., 2013).

8.1.2. Species specific meiofauna responses with special attention for rare taxa

Next to differences in temporal scale, our experiments also covered different modes of complexity. As such, we investigated responses of single organisms (Chapter 6) but also those of multispecies assemblages as they occur in the field (Chapter 2, 3, 4, 5 and 7). The study of multi-species assemblages including different trophic levels, revealed that species interactions play a major role in the assessment of environmental impacts on an ecosystem level, especially with regard to effects of seawater acidification and warming (Guilini et al., 2017; Manríquez et al., 2014; Montoya and Raffaelli, 2010; Petchey et al., 2010).

Similarly, our results suggested differential sensitivity of different meiofaunal groups to changes in abiotic conditions. The dominant meiofauna group, nematodes, was very tolerant to a broad range of pH (7.9 – 6.4) and elevated temperature (Chapter 2 and 3). This has been widely observed in OAW studies of meiofaunal communities from different environments (e.g. Dashfield et al., 2008; Ingels et al., 2017; Meadows et al., 2015; Widdicombe et al., 2009). In contrast, copepods and nauplii showed more sensitive, but also very variable responses. The research of Sarmento et al. (2017) indicated that responses of harpacticoid copepods and their nauplii to seawater acidification are species specific with some species benefiting from acidified conditions, which could explain the variable responses observed in our experiments. Interestingly, Gastrotricha showed opposite responses to reduced seawater pH in Chapter 2 and Chapter 3. In Chapter 2, organisms of this taxon originating from subtidal sediments of the Kiel Fjord (Wetsern Baltic Sea) exhibited highest densities in treatments with the highest pCO_2 level corresponding to a pH of 6.4 compared to treatments with lower pCO_2 values (900 – 12,800 µatm corresponding with pH 7.8 – 6.7). In contrast, intertidal Gastrotricha from the Scheldt Estuary completely disappeared from samples with a reduced pH 7.5 (Chapter 3) compared to samples of the control at pH 7.9. Increased predation by Platyhelminthes that showed elevated densities in low pH samples could explain part of the disappearance of Gastrotricha.

Taxon-specific responses were also observed in our abyssal *in situ* experiments where copepods and nauplius larvae showed a stronger migratory response than the more abundant nematodes (Chapter 5 and 7). This may be attributed to the higher colonization potential of copepods and nauplii compared to nematodes (Boeckner et al., 2009) but it does, nevertheless, indicate a change of the vertical distribution of meiofauna inside the sediment. It remains to be investigated how far this will have repercussions on the whole meiobenthic assemblage or on other, infaunal and epifaunal organisms through e.g. effects on biotic interactions. Given the relatively high importance of meiofauna in abyssal plains (Rex et al., 2006; Wei et al., 2010) and the relatively large predicted scale of sediment-plume impacts during mining (see section 8.3.1) this issue should be closely followed up.

Because of their low abundances, it is difficult to forecast the direct consequences of the disappearance or enhancement of less dominant taxa on the functioning of the impacted ecosystem. It has been shown that the less common taxa of the meiofauna show a less ubiquitous distribution and preference for particular habitats and that differences between study sites in terms of meiofauna community composition become more obvious when dominant taxa were excluded (Bianchelli et al., 2010; Gambi et al., 2010). In that sense, the research of Pusceddu et al.

(2011) showed that the throphic status, i.e. the quality and quantity of sedimentary organic matter, of coastal marine ecosystems is reflected in the taxonomic composition of the rare meiofauna community which may be used to facilitate the assessment of the trophic status of marine benthic environments. Similarly, the abundance and community composition of rare meiofaunal taxa can be used to reveal environmental stress from organic enrichment by fish-farms (Mirto et al., 2010). Based on these findings and our own results, we argue that the specific responses of rare taxa may considerably contribute to the identification of environmental harm in disturbed benthic communities.

8.1.3. Cumulative effects of stressors

In the natural environment, stressors rarely appear in isolation and cumulative effects of multiple stressors need to be considered and taken into account in experimental research. The way in which different stressors interact can be additive, synergistic or antagonistic and may differ between different organizational levels (population, community) and between trophic groups (autotrophs, heterotrophs)(Crain et al., 2008; Kroeker et al., 2017). Furthermore, the interaction effect can vary depending on the number of stressors where addition of one stressor may result in entirely different responses (Crain et al., 2008). Due to the difficulty of controlling and manipulating multiple stressors, multi-stressor experiments are scarce and urgently needed, given their high ecological relevance (Wernberg et al., 2012).

From the experiments reported in this thesis, multiple stressors were accounted for in three studies (Chapter 3: pH and temperature; Chapter 6: hydrostatic pressure, temperature and copper exposure; and Chapter 7: copper exposure and sediment burial). In Chapter 3 and 6 interactions between stressors could be identified while results of Chapter 7 were less clear to investigate as this experiment was not performed under controlled laboratory conditions. On an organism level, increased hydrostatic pressure was shown to increase copper sensitivity of *H. disjuncta* but a reduction of temperature at the same time reduced copper sensitivity, thus both factors interacted in an antagonistic way (Chapter 6). On the other hand, temperature and pH had cumulative effects on sediment pore water pH and meiofauna community responses (Chapter 3). Our findings underline that, indeed, multiple co-occurring stressors can have a distinct impact on biota, however, experiments should be carefully designed to also allow for the investigation of responses to each specific stressor in isolation. This would enable us to discern the different mechanisms of each stressor separately and of both stressors in combinations.

8.2. Seawater acidification and warming

8.2.1. Likelihood and scale

The research focussing on impacts of OAW and CCS reported in this thesis was conducted in the framework of the European ECO_2-project (Sub-seabed CO_2 Storage: Impact on Marine Ecosystems) and the UGent BOF GOA project "Assessing the biological capacity for marine ecosystem resilience". The ECO_2 project specifically aimed at investigating potential risks associated with sub-seabed carbon storage using interdisciplinary approaches in an international setting. Within this project, the research described in Chapter 2 contributed to the understanding of extreme pCO_2 conditions on a subtidal community. These conditions with a seawater pH of up to 6.4 are very unlikely to occur within in any of the predicted ocean acidification scenario within the next 100 years (Gattuso et al., 2015; IPCC, 2013), but they are probable in CCS operation scenarios (Blackford et al., 2009).

In Europe, CCS may be located at sites of depleted oil and gas fields or deep saline aquifers located under the continental shelves in relatively shallow water depths <300 m (ECO2, 2015). In an attempt to model seawater acidification resulting

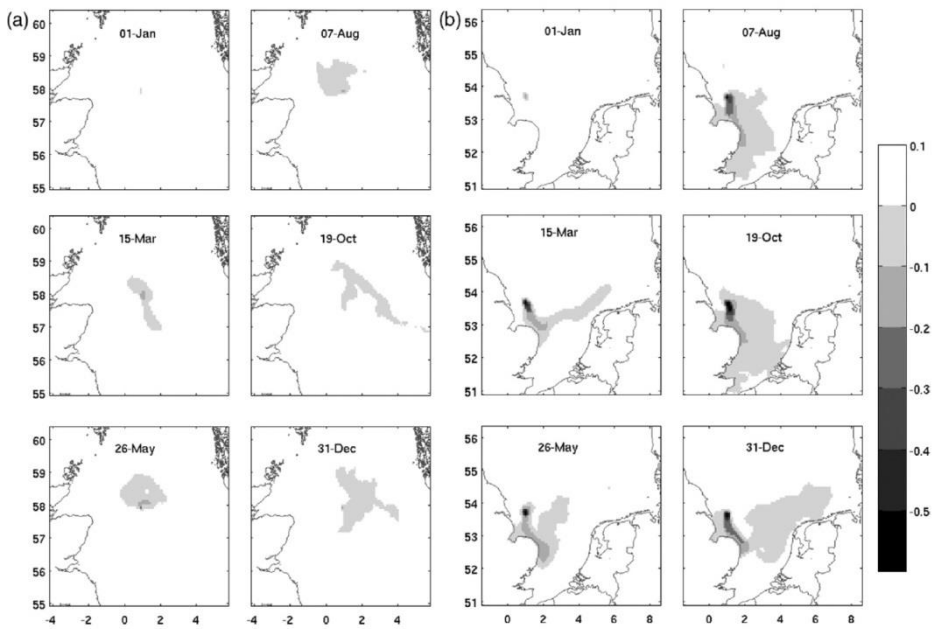

Figure 8.2 Modelled continuous point source CO_2 leakage scenarios over 1 year at two sites in the North Sea: a) North site, 138 m depth and b) South site, 28.5 m water depth. The greyscale indicates pH perturbations. Copyright: Blackford et al. (2009)

from CCS at two sites the North Sea (at 138 m and 28.5 m water depth), Blackford et al. (2009) considered 3 leakage scenarios. These included I) continuous diffuse seepage associated with e.g. reservoir faults, II) temporary point-source leak that may occur during pipe failures and III) continuous point-source leaks that may be associated with leakage through the injection well or failure of abandoned wells. Resulting models indicated that pH perturbations resulting from continuous, diffuse seepage were insignificant with maximum pH perturbations of 0.12 units.

In contrast, pipeline failure (temporary point-source leak) may reduce the seawater pH by 0.5 for 1-5 days in a worst-case scenario (50x pipeline capacity)(Blackford et al., 2009). Finally, based on the models, strongest pH perturbations can be expected in a continuous point-source leakage scenario where perturbation maxima exceeding 1 unit may be recorded at localized areas around the leak (Blackford et al., 2009, Fig. 8.2). However, depending on the water depth and duration of the leak, lower perturbations may be recorded even several kilometres from the source impacting coastal ecosystems (Blackford et al., 2009, Fig. 8.2). As indicated by these models, CO_2 leaks from CCS may be temporarily and spatially confined, nevertheless, it is important to assess the environmental impact and potential recovery of benthic communities from these disturbances and develop efficient methods for the monitoring of CCS sites.

It has been shown that strong decreases in seawater pH, occurring in the vicinity of a CCS leak, can affect marine organisms. Reported effects of elevated pCO_2 (ΔpH=-1-2) observed in laboratory experiments include, amongst others, growth inhibition (Klok et al., 2014), physiological impairment (Hammer et al., 2011), changes in immunological responses (Ellis et al., 2015), changes in behaviour (Murray et al., 2002; Rodríguez-Romero et al., 2014a) and mortality (Rodríguez-Romero et al., 2014a, Chapter 2). Furthermore, severe drops in seawater pH have the potential to increase metal bioavailability in polluted sediments, potentially affecting survival, growth and larval development (Rodríguez-Romero et al., 2014b; Szalaj et al., 2017). Although impacts from pipe failure or other CO_2 leaks associated with CCS are expected to be regional, the impact on local populations may be severe, affecting the structure of the metapopulation network and consequently the resilience of populations to additional stressors (Cowen et al., 2007). Source populations may be geographically distinct and depletion of these populations through reduced survival, reproduction or larval development may affect many other populations in the population network (Lipcius et al., 2008). Effects of strong pH disturbances from CCS on these source populations therefore have the potential to indirectly affect species population dynamics and increase the scale of secondary impact from CCS.

In contrast to CCS, ocean acidification and warming is a global phenomenon happening at this very moment and will continue to worsen in the future. Even if carbon emissions would be considerably reduced (RCP 2.6 scenario), ocean acidification would continue, though at a slower pace, and still cause reductions of -0.14 units until 2100 (Collins et al., 2013; Gattuso et al., 2015). At that rate of change, however, mitigation and restoration options to keep environments and animal stocks on healthy levels would be more efficient than in more severe scenarios (Gattuso et al., 2015). Thus, it is almost certain that ocean acidification and warming will continue to increase in the next 100 years and will occur on a global scale (Collins et al., 2013). Due to regional differences in water temperature and carbonate chemistry, some parts of the ocean, such as the polar seas are more sensitive to OA and reduced carbonate and aragonite saturation (Egleston et al., 2010). Furthermore, hypoxic conditions resulting from upwelling of oxygen-depleted waters or eutrophication may amplify ocean acidification in coastal zones (Melzner et al., 2013). This will lead to more severe pH reductions in particular regions of the ocean than the predicted global average.

The rate of OA and warming plays a major role in determining long-term impacts on marine species. Low rates of change may allow animals to physiologically adapt to the new conditions, which may especially be true for organisms with high turnover rates, such as most phyto- and zooplankton (Lohbeck et al., 2012). Similarly, most meiofauna organisms exhibit short generation times, therefore, adaptation to changing abiotic conditions may also be relevant for this group (Giere, 2009).

8.2.2. Monitoring and risk assessment

The evidence of potential environmental impacts following leakage from CCS and associated seawater acidification urges scientists, policy makers and environmental management agencies to research and develop effective measures to improve detection of such leaks so that immediate and appropriate mitigation measures can be taken. The behavioural responses of cockles and other bivalves offer a cheap and effective solution to this problem. In response to strong seawater acidification, the reduced burrowing activity and surfacing of moribund bivalves (Rodríguez-Romero et al., 2014b, Chapter 2) should alert managers of CCS sites if large accumulations of bivalves on the seafloor are detected in the vicinity of storage sites as it may be a first indication of a CO_2.leak which should then be further investigated.

Next to the detection of environmental impacts, another focus should lie in long-term baseline studies on the biological and biogeochemical properties of the ecosystem over multiple seasons and years before and during the implementation

of CCS. This would allow to distinguish natural variability from effects directly or indirectly caused by external stressors. As the results of our study pointed out, these baseline studies should include the study of all benthic size classes to account for changes in trophic links or biotic interactions (Chapter 2). Furthermore, attention should be paid to the extent of metal pollution in sediments lying above CO_2 storage sites and potential release of heavy metals during acidification events (Rodríguez-Romero et al., 2014b).

Due to the global, gradual process and passive character of ocean acidification and warming, they have to be distinguished from other, more directly caused anthropogenic disturbances (e.g. CCS, dredging, mining, pollution) with regard to environmental risk assessment. An environmental monitoring in the sense of a BACI design (Before-After-Control-Impact) is not feasible in an OAW context. Simply stated, a Control situation does not exist. Furthermore, the rate of pH and temperature change is expected to increase, therefore, rendering attempts to predict the future by modelling the past is difficult (Wernberg et al., 2012). Therefore, we are in need of more predictive ecology and ecosystem based research to integrate the vast body of current knowledge on the topic (Montoya and Raffaelli, 2010). Experimental research can significantly contribute to understanding the responses of natural communities to pH perturbations and results may feed into more complex models that aim to predict future changes (Artioli et al., 2014). Additionally, the investigation of marine communities inhabiting natural CO_2 vents or their surroundings can provide useful information to forecast adaptation potentials of species to long-term pH reductions (Guilini et al., 2017).

The study of individual organism responses to hypercapnia has already revealed some general patterns, such as the elevated sensitivity of calcifying organisms to high pCO_2.(Orr et al., 2005) and increased energy needed to maintain basal metabolism (Fabry et al., 2008; Pörtner et al., 2004). But multiple-species experiments reveal a more complex picture of OA impacts on marine communities by changing species behaviour and biotic interactions (Briffa et al., 2012; Manríquez et al., 2014; Watson et al., 2017), Chapter 2 and 3). However, and perhaps more importantly, cumulative effects of other stressors can considerably modify the impact of OA on marine organisms (Crain et al., 2008; Harvey et al., 2013). Both stressors have shown significant additive or antagonistic responses but depended strongly on the life-stage and general physiology (e.g. calcifying vs. non calcifying)(Harvey et al., 2013).

Therefore, an effective adaptive management of ecosystems under ocean acidification is only feasible through experimental studies incorporating multiple

species and trophic levels under realistic conditions including multiple stressors. These experimental studies should, however, be accompanied by long-term monitoring of the specifically studied environments. Seasonal and annual variation in pH can be very high in particular environments, such as coastal zones and estuaries, whereas in other areas, e.g. open ocean or coral reefs, pH is more stable (Hofmann et al., 2011). By separating annual variability and correlations with other abiotic variables, pH time series have revealed that hypoxic conditions intensify acidification of the seawater in coastal areas (Melzner et al., 2013).

Due to their direct connection between land and open sea, coastal ecosystems are particularly affected by multiple anthropogenic pressures such as habitat restructure, eutrophication, toxicants and waste pollution in addition to OAW (Lotze et al., 2006). The cumulative effects of hypoxia on seawater acidification (Melzner et al., 2013) but also the increase in metal bioavailability resulting from reduced pH (Rodríguez-Romero et al., 2014a; Szalaj et al., 2017) are just some examples of possible stressor interactions that may affect responses of biological communities. This underlines the importance to move OAW research away from single species / single stressor experiments and towards an integrated, ecosystem based approach.

8.3. Deep-sea mineral extraction

Chapter 4 to 7 focus on the environmental risks of potential deep-sea mineral extraction and submarine tailings disposal where two main impacts were studied: burial with sediments and coarse material, and the toxicity of metals. In Chapters 5 and 7 cumulative effects of both stressors are reported. In the next sections we give an overview of the likelihood and scale of both impacts and the wider implications of our finding with suggestions for environmental management.

8.3.1. Likelihood and scale

The nodule-rich sediments are generally composed of a very fine, semi-liquid top layer of approximately 10 cm thickness (Oebius et al., 2001). Already the shear movement of the heavy equipment at speeds of 0.3 – 0.6 m s^{-1} to ensure an economically viable mining operation will create strong disturbances of the very loose abyssal sediment. In addition to that, the upper 10-15 cm of the sediment will be collected together with the nodules, separated from the nodules inside the collector and ~90% of the sediment will be released back to the seafloor through an exhaust at the rear end of the collector (Murphy et al., 2016). Therefore, sediment plume creation, dispersion via currents and re-deposition at sites further from the mined site is very likely.

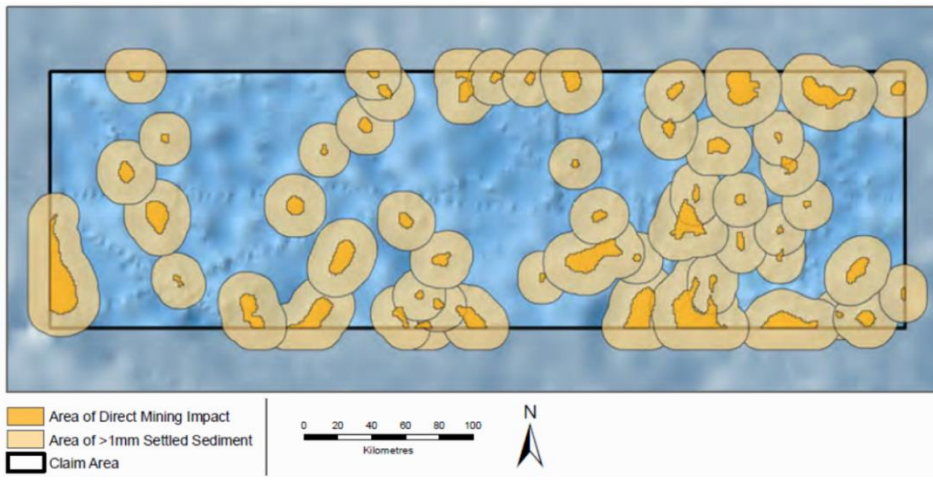

Figure 8.3 Areas affected by direct mining activity (dark yellow) and >1 mm annual sediment deposition (light yellow) in a 75,000 km² hypothetical licence area after 30 years of mining in an intermediate mining scenario with a production rate of 2.5 mio t y^{-1} and medium nodule abundance of 15 kg m^{-2}. Modified from (Murphy et al., 2016), copyright Environmental Resources.

At a speed of 0.6 m s^{-1} a total of 8 million tonnes of sediment will be released annually through the collectors' exhaust (Murphy et al., 2016). Furthermore, about 10 % of the sediment will be carried to the support vessel together with the nodules and will be released in the dewatering discharge contributing to an additional sediment plume (Murphy et al., 2016). However, due to uncertainties of the release depth (probably around 1000 m water depth) and thus, uncertain relevance for benthic fauna, sediment plumes from dewatering discharges are ignored in this discussion.

In an average mining scenario with medium nodule abundance (15 kg m^{-2}) and intermediate production rate (2.5 mio t y^{-1}) the area that is directly affected by the nodule collector amounts to 167 km² per year whereas the area indirectly affected by sediment deposition of >1 mm is 3.6 times larger (Murphy et al., 2016). Fig. 8.3 gives an indication of the expected area affected by the sediment plume after 30 years of intermediate mining intensity. However, it is important to note that this model uses average plume dispersal measures and that real sedimentation rates will vary depending on the location and topography of the mined area (Murphy et al., 2016). Furthermore, flocculation effects of the sediment plume were not included in the model that may limit plume dispersal but at the same time may increase sediment deposition depth at closer sites (Oebius et al., 2001).

The thickness of the deposited sediment will mostly depend on the proximity of the directly mined site (see Fig. 8.4). It is expected that different sediment fractions will settle in different distances from the disturbance site depending on their sinking rate and height in the water column (Oebius et al., 2001). Very coarse

fractions and nodule particles are expected to settle in close proximity of the mined tracks, while light fractions may be carried away several km from the mined site. This will also result in a differential thickness of the deposited sediment depending on the distance from the mined site. After one year of mining, the area in a radius of ~1 km around the mined site will be affected by >1 cm of sediment deposition (blue contour in Fig. 8.4).

In association with the sediment plume, our research in chapters 4, 5 and 7 has pointed out that meiofaunal organisms will respond to this by changing their vertical position in the sediment through upward migration. An analysis of the proportion of nematodes remaining in the natural sediment in chapters 4, 5 and 7 and the proportion of stained nematodes in the whole core as an indication of mortality presented in chapter 4 revealed opposite, linear trends (Fig. 8.5). In this analysis, we pooled all results from our experiments, regardless the nature of the applied substrate (crushed nodules, tailings, natural sediment and artificial sediment) and it is interesting, that these patterns are seen throughout different experiments with nematodes from different habitats.

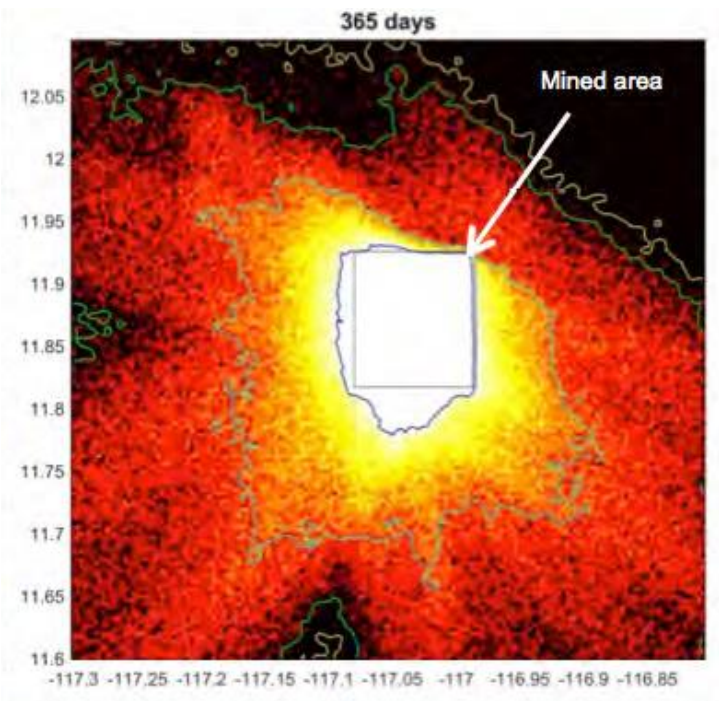

Figure 8.4 Area affected by sedimentation after simulated mining of a 12x12 km plot (white box) for 365 days. 1-10 cm = Blue contour (white area), 1mm-1cm= Cyan contour (yellow and orange area), 0.1 – 1 mm = Green contour (red area). Modified from (Murphy et al., 2016), copyright Environmental Resources.

This means that proportionally more nematodes will leave the original sediment with increasing burial depth (Fige 8.5). Based on the results of Chapter 4, increased burial depth also entails higher mortality of nematodes (Fig 8.5). However, it remains to be investigated if the increase in mortality is a more widespread phenomenon or if it was associated with the specific conditions of the experiment in Chapter 4. If burial- induced mortality is a widespread and universal phenomenon, this would have severe consequences for the environmental management of mining operations.

The very consistent vertical escape response of meiofauna between experiments was striking and has also been reported before by Schratzberger et al. (2000). The authors could show a much more complete migration out of the sediment with only 4.4 – 33.9 % of nematodes remaining in their original sediment when buried with 3 or 6 cm of sandy and muddy sediment for 2 months. In this experiment, the authors also found that instantaneous burial caused more severe changes in nematode assemblage structure than burial in smaller, but more frequent doses which may represent a more realistic scenario in the context of mineral extraction compared to instantaneous burial.

Maurer et al. (1986) reported vertical migration of different macrobenthic species which the authors could associate with rapidly decreasing pore water oxygen concentrations and an increase in ammonia as a result of burial with 30 - 40 cm of sediment with different sand and clay contents (Maurer et al., 1986, 1985). The change in biochemical conditions also led to severe mortality of macrofauna subsequent to burial, already visible 1 day after sediment addition (Maurer et al., 1986, 1985). Similarly, changing abiotic conditions as a result of substrate burial were observed in our experiment after 11 and 15 days of burial with mine tailings and natural sediment, respectively, and were directly or indirectly associated with the mortality of nematodes (Chapter 4).

Unfortunately, biochemistry of the pore water in the other experiments reported in Chapter 5 and 7 was not assessed and we can only hypothesize on causal mechanisms of the vertical meiofauna migration. However, the high sensitivity of copepods to low oxygen conditions (De Troch et al., 2013; Grego et al., 2014) combined with the vertical migratory response of these animals suggest that sediment oxygen penetration depth may have declined as a result of substrate burial, which requires further investigation.

A potential decline in oxygen penetration depth could also have consequences on metal bioavailability inside the sediments. In nodule fields, heavy metals are strongly associated with MnO_2 but could be released under reducing conditions

that may occur under anoxic conditions or when pore water pH drops considerably (Koschinsky et al., 2001a).

Not only sediment burial may affect pore-water chemistry, but also the direct physical disturbance during mining through e.g. sediment mixing, sediment removal and sediment compaction. The assessment of changing sediment properties, particularly with regard to oxygen and pH, resulting from sediment deposition should be a topic of interest in future research on the impacts of mining. Ideally this would then be accompanied by an assessment of metal contents and, more importantly, metal speciation and bioavailability.

Deep-sea ecotoxicology research, however, is not only of particular interest for polymetallic nodule mining scenarios, but also for the mining of SMS or polymetallic crusts. Especially, polymetallic crusts are often found on seamounts in intermediate water depths (Petersen et al., 2016). At these depth, we may also find so called oxygen minimum zones (OMZ), bodies of oxygen depleted seawater at depths between 300 - 700 m, that are expected to expand significantly over the coming century as a consequence of global change (Stramma et al., 2008). If the appearance of an OMZ coincides with a mining activity for polymetallic crusts, the hypoxic seawater may potentially cause metal releases from the disturbed substrates, similar to nodule field sediments. The mining of SMS deposits will

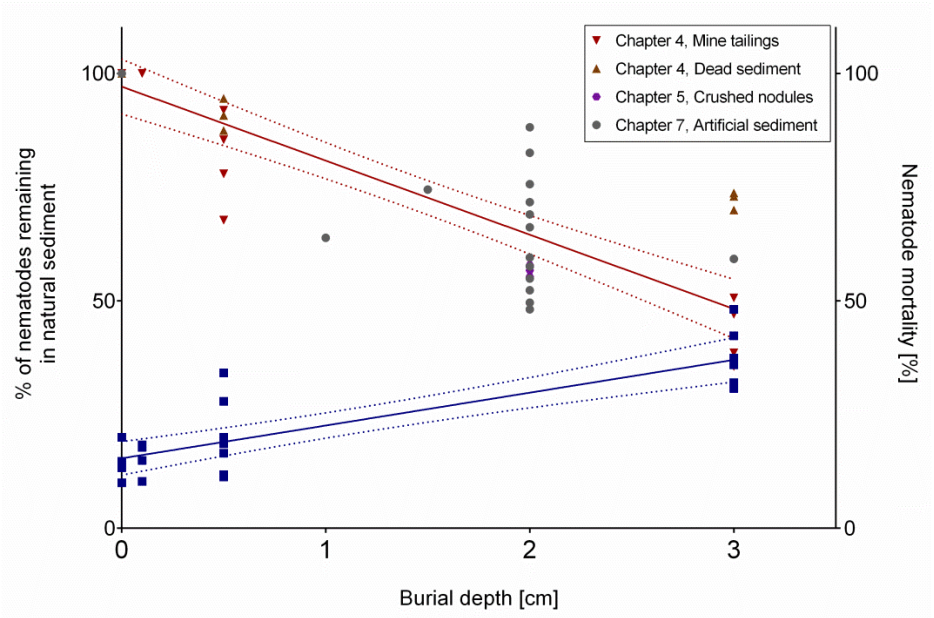

Figure 8.5 Relationship between burial depth and percentage of nematodes remaining in the natural sediment (left y-axis, red), derived from results of Chapter 4, 5 and 7, and percentage of stained nematodes indicating mortality (right y-axis, blue). Lines are linear regressions with 95 % confidence intervals (CI). Error bars depict 95 % CI.

expose large fresh mineral reserves that lie under the mined layer to the corrosive surrounding seawater which may result in the release of metals to the water column (Fallon et al., 2017). Similarly, upon return of the extraction water from the support vessel, significant amounts of dissolved solids would be exposed to oxygen rich water initiating the oxidative dissolution of sulphides with a potential release of metal fractions (Fallon et al., 2017).

8.3.2. Monitoring and risk assessment

Our research on the impacts of deep-sea mining have considerably contributed to the gathering of baseline information on the responses of meiobenthos to substrate burial and toxic effects. The relatively high abundance of meiofauna in abyssal sediments, offers a clear advantage over other benthic soft-sediment organisms for monitoring. The vertical migration pattern observed in Chapter 4, 5 and 7 might serve as a first indication of potential harm to the environment and may be used to monitor the impacts of sediment plume dispersal. The negative effects on the functioning of bathyal fjord communities found in Chapter 4 urge the further investigation of similar patterns in abyssal ecosystem. Nevertheless, awaiting further investigations, the fine scale vertical distribution of meiofauna could be considered when monitoring mining impacts based on a precautionary approach. This could entail for example that the location and intensity of mining activities could be reconsidered when significant shifts in meiofaunal vertical distribution are found in the area of secondary impact (a few km from the actively mined site).

To define significant changes it would be advisable to include long-term monitoring of the meiofaunal communities and abiotic factors in different control areas to study natural variability and heterogeneity on different spatial scales. "Significant shifts" could then be defined as changes of the meiobenthic distribution in impacted areas that significantly deviate from these naturally observed patterns.

For the assessment of impact from metal toxicity more research is needed on the potential release of metal fractions when abiotic variables change and how these would change upon mining disturbance (burial, sediment mixing, compaction). Additionally, measuring of the metal uptake in particular organisms could give an indication of the bioaccumulation and uptake of heavy metals in animal tissues and associated toxic effects (Rainbow, 2007). This is already common practice in shallow-water habitats using mussels as bioindicators (Mestre et al., 2017; Rodríguez-Romero et al., 2014b). The measurements of nematodes using μ-XRF provided some interesting insights in the differential copper uptake upon burial with different types of substrates but require further investigation (Chapter 5 and

7). Nevertheless, our results indicate that bioaccumulation of heavy metals in nematodes is variable. Therefore, nematode copper burden may be an important parameter to assess during the monitoring of mining activities which could provide an indication of changing toxicity inside the sediments.

8.4. Future outlook and recommendations

This thesis covered many different types of environmental impact on the marine meiofauna. Once again it was proven that these tiny organisms are a valuable asset in the study of anthropogenic impacts (Zeppilli et al., 2015). Based on the insights generated by the research performed in this thesis, we recommend to incorporate two different approaches in future research on impacts of anthropogenic stressors on benthic ecosystems.

On the one hand, future research should be aiming at a more holistic, ecosystem-based approach that focusses on different components of the benthic food web with emphasis on biotic interactions and changes over time. As previously discussed, this also includes the study of multiple stressors in combination in realistic, but logistically feasible scenarios. Approaches integrating structural responses of the benthic fauna with functional aspects such as nutrient cycling and food-web dynamics are preferred. Ideally, experimental studies should be accompanied by time-series measurements to discern the stressor-related impact from natural variability in the field.

On the other hand, increased attention should be paid to the physiological mechanisms underlying the various responses and how these may differ between species. This can be accomplishes through single-species experiments including multiple stressors relevant for the ecosystem that is being investigated (e.g. hydrostatic pressure in the deep sea or pollution effects in estuarine ecosystems, see Chapter 6). The analysis of the physiological responses to one or multiple stressors should be aided by established, state-of-the-art methodologies such as genomic approaches to identify sublethal consequences on gene expression and to identify impacted metabolic pathways. Furthermore, the potential of species to adapt to new biotic conditions could be investigated through selection experiment in short-lived species as described in Lohbeck et al. (2012). The authors exposed multiple generations of the coccolithophore *Emiliana huxleyi* to increasing seawater pH conditions and revealed that after 500 generations, individuals from increased CO_2 conditions significantly increased their tolerance to high sweater pH compared to control organisms. In this regard, meiofauna research may also offer the potential for further investigation of the adaptive evolution of species.

The gathered information from experimental research (both, ecosystem based and species-specific) and field monitoring could then feed into ecosystem-specific, dynamic models that may allow predictions of future changes in different scenarios.

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List of Publications

A1 Publications

Brown, A., Wright, R., Mevenkamp, L., Hauton, C., 2017. A comparative experimental approach to ecotoxicology in shallow-water and deep-sea holothurians suggests similar behavioural responses. *Aquat. Toxicol.* **191**, 10–16. doi:10.1016/j.aquatox.2017.06.028

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C2 Publications (Dissertations, conference abstracts and internal reports)

Dissertations

Mevenkamp, L. (2013). Selective settlement of deep-sea and intertidal meiofauna - an experimental approach. *MSc Thesis*. Ghent University, Marine Biology Research Group: Gent. 31 pp.

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Hauton, C., Martins, I., Mestre, N.C., Auguste, M., Bebianno, J., Brown, A., Carreiro-Silva, M., Carvalho, A., Godinho, A., De Grave, J., Lins, L., Mevenkamp, L., Vandenberghe, D., Vanreusel, A., Wright, R., 2016. Report on the biological response of bathyal and abyssal meio - to megafauna to

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