CO₂ leakage can cause loss of benthic biodiversity in submarine sands

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ABSTRACT

One of the options to mitigate atmospheric CO₂ increase is CO₂ Capture and Storage in sub-seabed geological formations. Since predicting long-term storage security is difficult, different CO₂ leakage scenarios and impacts on marine ecosystems require evaluation. Submarine CO₂ vents may serve as natural analogues and allow studying the effects of CO₂ leakage in a holistic approach. At the study site east of Basiluzzo Islet off Panarea Island (Italy), gas emissions (90–99% CO₂) occur at moderate flows (80–120 L m⁻² h⁻¹). We investigated the effects of acidified porewater conditions (pH range: 5.5–7.7) on the diversity of benthic bacteria and invertebrates by sampling natural sediments in three subsequent years and by performing a transplantation experiment with a duration of one year, respectively. Both multiple years and one year of exposure to acidified porewater conditions reduced the number of benthic bacterial operational taxonomic units and invertebrate species diversity by 30–80%. Reduced biodiversity at the vent sites increased the temporal variability in bacterial and nematode community biomass, abundance and composition. While the release from CO₂ exposure resulted in a full recovery of nematode species diversity within one year, bacterial diversity remained affected. Overall, our findings showed that seawater acidification, induced by seafloor CO₂ emissions, was responsible for loss of diversity across different size-classes of benthic organisms, which reduced community stability with potential relapses on ecosystem resilience.

1. Introduction

The combustion of fossil fuels and industrialization are the main causes of the current exceptionally high rates of increase in atmospheric CO₂, which in turn contributes to climate change (Blanco et al., 2014). In the short term, CO₂ Capture and Storage (CCS) in sub-seabed geological formations is considered as the most significant means of reducing net carbon emissions into the atmosphere (Turkenburg, 1997; Bachu and Adams, 2003), while the search for other profitable and renewable energy sources is ongoing. Since it is, however, difficult to predict long-term underground storage security, the inevitable risk of a CO₂ leak from storage reservoirs is one of the arguments raised to dispose CO₂ in aquifers located off-shore instead of land inwards (Turkenburg, 1997; House et al., 2006). Nevertheless, in the advent of a leak, the escaping CO₂ will rapidly dissolve into sediment porewater and into bottom-seawater, reducing the pH and carbonate saturation state and increasing acidity of either (Chen et al., 2005; Millero, 2007). Modeling and experimental studies have shown that the majority of biological impacts from CCS leakage are likely to occur in benthic or epibenthic communities of less mobile organisms, with the magnitude of the impact depending on the duration, the scale and the intensity of the leak, and on the local hydrodynamic regime (Blackford et al., 2009, 2013; Zeppilli et al., 2015).

The majority of CCS leakage impact studies that measured the response of benthic, infaunal or microbial communities applied acidified seawater (pH ≥ 5.6) above sediments in laboratory-based, circulation system experiments (e.g. Dashfield et al., 2008; Ingels et al., 2018; Widdicombe et al., 2009; Schade et al., 2016; Thistle et al., 2006), injected liquefied CO₂ into corrals deposited on the deep-sea floor (porewater pH: 5.4; Carman et al., 2004; Barry et al., 2005) or released CO₂ gas via a borehole at 11 m below the seafloor (porewater pH: 7.5; Widdicombe et al., 2015). Those experiments are very relevant in determining the effects of more or less severe, acute (max. 140 days; Rastelli et al., 2015) CO₂ leakage from CCS sites or injection pipelines. However, they also recognize the inability to predict the impact of more chronic CO₂ leakage or to generalise the results to a more complex...
natural environment, where other environmental and ecological processes can affect the observed responses (Widdicombe et al., 2013). Studies at natural submarine CO2 vents, where escaping gas is mainly composed of CO2 and lacks toxic compounds (e.g. sulphide) or temperature increases, may serve to gain this complementary information as they allow to study ecological consequences of relatively long-term (e.g. multi-decadal; Gambi et al., 2003; Jones et al., 2014) exposure to high pCO2 and low pH porewater conditions in seafloor surface sediments, where porewater pH exhibits a steep decline within the first few millimeters (e.g. pH2 range: 7.7–5.5; Moli et al., 2018). So far, only few studies have used natural gas seeps to investigate the effect of seabed CO2 leakage on benthic biodiversity (Dias et al., 2010; Pettit et al., 2013; Johnson et al., 2015; Rauf et al., 2015; Hassenznck et al., 2016), focusing only on specific size-class of organisms. Studies at such natural analogues may provide answers to whether species from originally undisturbed natural environments are able to persist and tolerate, and eventually adapt to the highly acidified conditions, or whether they are being replaced by colonizing tolerant species and what consequences this might have for the biodiversity and the role they fulfill within an ecosystem under high biochemical pressure (Moli et al., 2018; Zeppill, 2015).

Moli et al. (2018) found that long-term, high CO2 conditions at the vents East of Basiluzzo Islet (N of Sicily, SE Tyrrhenian Sea, 14–21 m water depth) led to substantial structural and functional shifts in the bacterial and invertebrate communities, accompanied by a decrease in biomass and abundance, which led to a reduced efficiency in carbon transfer along the food web. Based on a sediment transplantation experiment, Moli et al. (2018) observed that CO2 leakage impacts on the composition of the benthic communities were already apparent after one year. The recovery of the system after one year of release of exposure to acidic conditions was, however, far from complete. In this study, we focus on the effect of CO2 leakage on the biodiversity of the vent system at Basiluzzo Islet and explore the relation between biodiversity and temporal stability in structural aspects of the benthos (i.e. community composition, abundance, biomass; data presented in Moli et al., 2018). While we focus on bacteria at the level of operational taxonomic units (OTUs) (i.e. internally transcribed spacer or ITS phylotypes corresponding to binned ARISA peaks), we specifically addressed the most dominant meio- and macrofauna taxa (i.e. nematodes and polychaetes) at species level and tested the following hypotheses:

1) Both one year and at least 3 years of exposure to acidic porewater conditions reduces OTU or species diversity of benthic organisms belonging to different size classes (i.e. microbio, meio- and macrofauna).
2) Benthic bacterial OTU and nematode species diversity do not recover after one year of release of exposure to acidic conditions.

2. Material and methods

2.1. Study site and sampling

The study site is located east of the Basiluzzo Islet, ca. 4 km NE of the Aeolian Island Panarea (Italy), in the SE Tyrrhenian Sea (Fig. 1A). The mainly submarine Aeolian volcanic structure that lies along the NW-oriented faults (Beccaluva et al., 1982; Gabbianelli et al., 1993) is responsible for the visible release of gas from the seabed at several sites around Panarea. Gas emissions at Panarea submarine fumarolic fields have been reported to occur since two thousand years (Italiano and Nuccio, 1991, and references therein; Lucchi et al., 2013, and references therein). Specifically, at the south side of Basiluzzo Islet, shallow (8–13 m water depth) gas emissions have been observed since more than 25 years (Calanchi et al., 1995). This vent area east of Basiluzzo Islet was selected as natural analogue as it fulfills the following criteria: (i) continuous, dispersed degassing of CO2 through sand causing low pH, (ii) similar oxygen availability and negligible co-emission of toxic substances or microbial energy sources such as sulfide and methane, (iii) no significant temperature anomalies from hydrothermalism (Moli et al., 2018). The sampled locations include two CO2 vent sites (“CO2-R”, N 38°39.749′ E 15°07.123′, 15–17 m water depth; “CO2-G”, N 38°39.820′ E 15°07.137′, 21 m water depth) with rather evenly distributed gas leakage (density of 2–3 gas bubble strings per m2, 120 L m−2 h−1 and 97–99% CO2 at CO2-R, 80 L m−2 h−1 and 90–97% CO2 at CO2-G) and one reference site (“REF”, N 38°39.827′ E 15°07.118′, 14–17 m water depth), with no gas emissions and similar hydrological and sedimentological characteristics as found at the vent sites. CO2 venting through the sandy sediments resulted in a loss of solid phase carbonate and a decrease in porewater pH by 1–2 units relative to the reference site. A detailed description of the biogeochemistry of the bottom seawater, porewater and, sediments of the selected sampling sites is provided by Moli et al. (2018).

The scuba-diving team collected natural sediment samples in the month June of 2011, 2012 and 2013 for the investigation of the effects of at least 3 years of continuous CO2-emissions and porewater acidification on the benthos. Macrofauna samples were collected with a push core with an inner diameter of 6.4 cm (only in 2012, n = 5 per site). The upper 5 cm of the sediment was retained and stored unsealed in seawater-buffered formalin (final concentration of 4%). meiofauna samples were gathered with pre-cut and taped push cores with an inner diameter of 4.7 cm (2011, n = 3 per site) or 5 cm (2012, 2013, n = 3 per site). The sediments were vertically sectioned in 2 cm slices, down to 8 cm depth. All sample sections were preserved in a 4% seawater-buffered formalin solution. Sediment samples for investigating bacterial community composition and diversity, via Automated Ribosomal Intergenic Spacer Analysis (ARISA; Ramette, 2009), were obtained with push cores with an inner diameter of 5.4 cm (2011, n = 2 per site; 2012–2013, n = 3 per site), vertically sectioned in 2 cm slices down to 10 cm depth (n layers = 5), and surficial sediment (0–2 cm) collected with 50 ml plastic tubes (2011, n = 15 per site; 2012–2013, n = 20 per sample). Samples were stored frozen at −20 °C. Additionally, 454-Massively Parallel Tag Sequencing (454-MPTs; Sogin et al., 2006) was used for better characterization of the low abundant and rare bacterial taxa in the 0–2 and 4–6 cm sediment layers collected in 2012 (n = 3 per site), as representative for oxic/suboxic and anoxic sediment environments, respectively (Moli et al., 2018).

Additionally, the response of benthic organisms to one year of CO2-venting or reference conditions was assessed with an in situ sediment transplantation experiment. In 2012, sediment samples were transplanted in situ within and between REF and CO2-R sites: (i) reimplanted at the same site (within habitat: REF in REF and CO2-R in CO2-R) to control for transplantation effects, and (ii) transplanted to the other habitat type (across habitat: REF in CO2-R and CO2-R in REF) to assess the effect of the different environmental setting (Moli et al., 2018, Fig. 1B). Each transplant was collected after pushing a cylindrical TUBO device into the sediment for 15 cm. Thirty liters of sand were scooped out and transferred into a mesh bag (vinyl-coated fiberglass mosquito net: fiber diameter 280 μm and 1.8 mm × 1.6 mm mesh) covered by an additional plastic bag to protect the sediment from the surrounding seawater during transportation of the bags. At the site of interest, the bags were inserted into an empty TUBO hole (n = 5 per treatment). The TUBO and the plastic bag were removed, and the mesh bags were closed (Moli et al., 2018). Samples for microbial and meiofaunal
Sequences were clustered into operational taxonomic units at a 3% nucleotide difference (hereafter referred to as OTU0.03). The whole dataset comprised a total of 169,490 operational taxonomic units at a 3% nucleotide difference (hereafter referred to as OTU0.03). The whole dataset comprised a total of 169,490 high-quality bacterial sequences, which were clustered into 9674 OTUs0.03. From the whole 454-MPTS subset only OTUs0.03 > 0.1% of high-quality bacterial sequences, which were clustered into 9674 OTUs0.03. From the whole 454-MPTS subset only OTUs0.03 > 0.1% of high-quality bacterial sequences, which were clustered into 9674 OTUs0.03. From the whole 454-MPTS subset only OTUs0.03 > 0.1% of high-quality bacterial sequences, which were clustered into 9674 OTUs0.03. From the whole 454-MPTS subset only OTUs0.03 > 0.1% of total bacterial sequences per sample were used for analysis of dominant classes.

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The metazoan organisms were quantified and classified at higher taxon level under a stereoscopic microscope. Fifty to sixty nematodes per sediment layer (down to 8 cm for the natural sediments, down to 4 cm for the transplant experiment) were randomly handpicked with a fine needle, transferred to glycerine (De Grise, I, II and III, Seinhorst, 1959), mounted on glass slides and identified to species (natural sediment) and genus (transplant experiment) level based on original species descriptions which are available on the Nemys website (Guilini et al., 2017a). Nematode biomass (μg dry weight 10 cm−2) was determined for all identified nematodes based on Andrassy (1956) and assuming a dry-to-wet-weight ratio of 0.25 (Heip et al., 1985).

The macrofauna was extracted from the sediment through sieving and washing on a 1-mm mesh sieve, followed by handpicking all organisms with a forceps or needle under a binocular microscope. All macrofauna organisms were counted and identified to higher taxon level (down to Class), whilst Polychaeta were further identified to species level based on the World Polychaete Database (Read and Fauchald, 2016) and Gil (2011).

2.3. Data analysis

Alpha diversity was assessed as species richness, exponential of Shannon index and inverse of Simpson index, corresponding to Hill's numbers of order q = 0 (H0), q = 1 (H1) and q = 2 (H2), respectively (Hill, 1973; Chao et al., 2014). The diversity measures spread along Hill's continuum provide us with a more complete understanding of shifts in rare and abundant species and a simplified interpretation of results because units are always in effective number of species (Jost, 2006). These effective species numbers behave as one would intuitively expect when diversity is doubled or halved, while other standard indices of diversity do not (Jost, 2006). For the natural sediments, the overall alpha diversity was visualized in interpolation and extrapolation species diversity curves based on replicates' average of the sum of sequences/individuals per layers. Additionally, for Bacteria, richness estimates (Chao1) were calculated with 100 random re-sampling runs to the smallest number of sequences per sample in the dataset (n = 4346), to account for differences in sequencing depth between samples. Diversity indices were also calculated for bacterial dominant classes, representing together more than 80% of total OTUs0.03 at each sites (Molari et al., 2018). For testing the effect of space ("Site" and sediment "Layer") and time ("Year") on variations observed in bacterial, nematode and polychaete alpha diversity from natural sediments, multifactorial ANOVA analyses were performed based on a three factor design with Layer nested in Site. The nested design was chosen to take into account the site-specific environmental gradients due to presence/absences of acidified porewater effluxes at CO2-venting sites and reference, respectively (for more details see Molari et al., 2018). In order to account the effect due to transition between aerobic and anaerobic environments, the diversity indices of single layer were grouped in oxic/suboxic (i.e. 0–2 cm) and anoxic (below 2 cm) layers. Moreover, for bacteria we had more observations for 0–2 cm layer (n = 15–20) than for other layers (n = 3), thus grouping together layers below 2 cm evenlyed sampling effort. In order to account for the dependency of observation from the same sediment core, a mixed effects model with "Sediment Core" as random factor was carried out for Nematode data. Three-way ANOVA was applied to Bacteria and one-way ANOVA to Polychaeta data due to high contribution of independent observations for layer 0–2 cm and one observation per core, respectively. To test the effect of short-term exposure to acidified porewater conditions on alpha diversity of bacteria and nematodes, a two-way split-spot ANOVA was performed with the factors "Treatment" (within habitat transplants and across habitats transplants) and sediment "Layer" (nested in
“Treatment”), and “Sediment Core” as random factor. Transplants pairwise comparison was carried out using non-parametric Kruskal-Wallis tests with Benjamini–Hochberg (BH) adjusted P-values.

Temporal stability (S), which is an index of the stability of a community or population over time (Tilman, 1999; Lehman and Tilman, 2000; Tilman et al., 2006), was considered for bacterial and nematode density, biomass and community composition (data which are presented in Molari et al., 2018). S was estimated as the ratio between the temporal mean (μ) of community-level density, biomass and Bray-Curtis similarity values for species composition and their standard deviation (σ), per sampling site. Larger S values indicate higher temporal stability. The relationship between temporal stability and biodiversity could only be visually stated since three sample sites did not allow performing simple linear regressions.

Beta diversity (i.e. OTUs/species turnover) was calculated as the Jaccard dissimilarity distance on presence/absence data for each of the bacteria, nematode and polychaete datasets. Turnover of OTUs or species were quantified between the layers and between the years at each site for Bacteria (ARISA) and nematodes, and between sites for bacteria (ARISA and 454-MPTS), nematodes and polychaetes. The Jaccard dissimilarity distance was used to calculate beta similarity (i.e. the number of shared OTUs/species), and similarity percentage breakdown procedure (SIMPER; Clarke, 1993) was carried out to assess the average percentage of shared OTUs/species within and between the groups (sites and layers per each year, and years per each site) for Bacteria (ARISA and 454-MPTS) and Nematoda datasets. One- or two-way ANOVA, after verifying that the assumptions of normality and homoscedasticity were met, was applied to test significant differences between average of shared OTUs/species. Following significant ANOVA between the investigated sites and/or years, Tukey post-hoc comparison tests were applied. In case the assumptions for ANOVA were not met, Kruskal-Wallis tests and post-hoc Dunn-tests were performed instead, with BH-adjusted P-values.

Multivariate Redundancy Analyses (RDA) were performed to investigate which environmental variable or set of variables (Table A0.1) could best explain the patterns in bacterial and nematode diversity (Hill’s numbers) observed with depth in the sediment (0–10 cm and 0–8 cm, respectively) and in the oxic/suboxic layer (0–2 cm) for the years 2012 and 2013. Prior to the analyses, the explanatory environmental variables were standardized (i.e. Z-scored) and assessed for collinearity based on the variance inflation factor (VIF). The variables pH, chlorophyll a (Chl-a), total organic carbon (TOC), median grain size (MGS), silicate, and dissolved inorganic carbon (DIC) were retained based on a VIF < 3, indicating no or little collinearity among these variables. Additionally, variation partitioning (VP) analysis was used with multiple partial RDAs to assess the explanatory power of each significant explanatory variable and proportion of variance explained by variables multicollinearity. All statistical analyses were performed in R (v. 3.3.1) (R Development Core Team, 2014; https://www.R-project.org) using packages iNEXT (Hsieh et al., 2016), vegan (Oksanen et al., 2015), usdm (Naimi et al., 2014), lme4 (Bates et al., 2015), and ggplot2 (Wickham, 2009).

3. Results

The rarefaction curves indicated that bacterial (OTUs), nematode (species) and polychaete (species) richness in the natural sediments was well captured for the abundant taxa in all three study sites, while diversity of the less abundant taxa and rare biosphere remained largely underestimated (Fig. 2, Fig. A1 and A2). REF contributed the largest fraction of absolute singletons (OTUs with single-sequence; SSOabs) and unique OTUs0.05, followed by CO2-G and then by CO2-R (Table A.2). Hill numbers (Fig. 3 and A.3) showed a general reduction in diversity at the CO2-venting sites compared to the reference site (ANOVA, p < 0.001, redundancy ≥ 63.1%; Table A.3), with no more than 1.5% and 21% of the variance explained by “Year” (2011–2013) and sediment “Layer” (0–2 cm and 2–10 cm for bacteria, 0–2 cm and 2–8 cm for nematodes), respectively, for both bacteria and nematodes. Bacterial diversity (OTUs) was generally the lowest at CO2-R with almost all
dominant classes showing a decrease of richness and evenness at the seep sites (Fig. A.4). Diversity of polychaete species was equally low at both vent sites for H0 and H2 (ANOVA pairwise test, \( p \leq 0.03 \)). Redundancy analysis (ANOVA pairwise test, \( p = 0.35 \)) and lower at CO2-R compared to CO2-G. Nematode species diversity was equally low at both vent sites for H0 and H2 for all indices (ANOVA pairwise test, \( p \geq 0.72 \)) while seep sites (Fig. A.4). Diversity of polychaete species was equally low at both vent sites for H0 and H2. The number of shared OTUs/species in the natural sediments was generally lower between the reference and CO2-venting sites than between the two vent sites (Table 2; Bacteria (ARISA): ANOVA, \( F \) value = 8.19, adjusted \( P < 0.05 \); Bacteria (454-MPTS): Kruskal-Wallis, \( \chi^2 = 12.79 \), adjusted \( P < 0.001 \); Nematoda: Kruskal-Wallis, \( \chi^2 = 9.78 \), adjusted \( P < 0.001 \); Polychaeta: Kruskal-Wallis, \( \chi^2 = 22.6 \), adjusted \( P < 0.001 \)). Beta similarity was different among size-class organisms here investigated, as REF shared 9–10% of nematode species and 0–3% of polychaete species, while 82–85% (ARISA) and 16–20% (454-MPTS) of bacteria OTUs with the vents. The number OTUs and species shared between the top sediment layer (0–2 cm) and underlying layers decreased with sediment depth at REF and CO2 vents in all years (Table 3). Even, the beta similarity between top and deepest layers of the sediment were higher at REF than at vent sites both for Bacteria and nematodes (Bacteria (ARISA): ANOVA, \( F \) value = 25.91, adjusted \( P < 0.001 \); nematodes: Kruskal-Wallis, \( \chi^2 = 25.36 \), adjusted \( P < 0.001 \)). Temporal variation in bacterial and nematode community composition was lower at REF than at CO2-venting sites (Table 2; Bacteria (ARISA): ANOVA, \( F \) value = 18.02, adjusted \( P < 0.01 \); Nematoda: ANOVA, \( F \) value = 128.09, adjusted \( P < 0.0001 \)).

4. Discussion

4.1. CO2 leakage affects bacterial diversity

The results from few previous studies investigating effects of acidified porewater on bacterial alpha diversity in soft sediments at CO2-venting sites showed contradictory responses (Yanagawa et al., 2013; Kerfahi et al., 2014; Rauf et al., 2015; Hassenrück et al., 2016), likely as a result of the inherent high heterogeneity of environmental gradients at natural vents (German and Seyfried, 2014). Where no pure CO2 release occurs, fluid and gas emissions of reduced elements provide fixed pH values are the averages in the 0–2 cm sediment layer.

Fig. 3. Sediment integrated alpha diversity expressed in Hill's numbers (q = 0, 2) for A) Bacterial OTUs (0–10 cm, ARISA, undisturbed sediments 2011–2013, n = 9, transplanted sediments 2013, n = 3) and B) Nematoda genera in the natural sediments at REF, CO2-G and CO2-R (0–4 cm, n = 9), and in the transplanted sediments within and across REF and CO2-R (0–4 cm, n = 3).
reported for other ocean acidification natural analogues (Raulf et al., 2015; Hassenrück et al., 2016) and typically described for seeps and hydrothermal vents (Ristova et al., 2015; Raulf et al., 2015). Besides, the temporal variation in community composition was comparable with that observed in other coastal sandy sediments (Böer et al., 2009; Gobet et al., 2012). Direct comparison of beta diversity values between studies could be biased by application of different sequencing techniques (e.g. different sequencing depth, different primers) and methods for beta diversity estimation. However, here, our results were compared with previous studies applying the same methods to investigate microbial diversity (i.e. ARISA and/or 454-MPTS) and to assess beta diversity (i.e. Jaccard dissimilarity distance). Both long- (at least three years) and short-term (one year) exposure to CO2 emissions resulted in a decrease

Table 1
Results of variation partitioning and partial redundancy analyses illustrating which predictor variables explain the variance in Bacteria (ARISA) and Nematoda diversity (Hills numbers) for 2012 and 2013, considering the entire sediment profile (all layers) and restricted to the upper 2 cm of the sediment. Proportion of variance explained (%) and p values (ANOVA) are reported for significant variables, as identified by RDA (single source). Proportion of variance explained by variables multicollinearity is also reported, as identified by variation portioning analysis (multiple sources). RDA and partial RDA details are reported in Table A0.5.

| Habitat | Assemblage | Layer | Bacteria | Nematoda |
|---------|------------|-------|----------|----------|
|         | Undisturbed sediments | 0–10 cm | 0–2 cm | 0–8 cm | 0–2 cm |
|         | Undisturbed sediments | observations | n = 90 | n = 18 | n = 72 | n = 18 |

| Variance (%) | P-value | Variance (%) | P-value | Variance (%) | P-value | Variance (%) | P-value |
|--------------|---------|--------------|---------|--------------|---------|--------------|---------|
| Single source |         |              |         |              |         |              |         |
| pH           | 40      | 0.001        | 55      | 0.001        | 56      | 0.001        | 38      | 0.001 |
| MGS          | < 1     | ns           | < 1     | nt           | 0       | nt           | 11      | 0.005 |
| Silicate     | 2       | 0.003        | 6       | 0.007        | 0       | nt           | 5       | 0.047 |
| Chl-a        | 0       | nt           | 0       | nt           | 1       | 0.022        | 0       | nt    |
| Multiple sources |     |              |         |              |         |              |         |
| pH + MGS     | 6       | 1            | 0       | 12           |          |              |         |
| pH + Silicate| 10      | 18           | 0       | 0            |          |              |         |
| pH + MGS + Silicate | 20 | 12 | 0 | 16 |          |              |         |
| pH + Chl-a   | 0       | 1            | 0       | 17           |          |              | 0       |       |
| Unexplained  | 20      | 8            | 25      | 18           |          |              |         |

MGS = median grain size; Chl-a = chlorophyll a; ns = not significant; nt = not tested.

Fig. 4. Relationships between sediment integrated OTUs and species richness ($H_0$) and temporal stability ($S$) over 3 years across all study sites (n = 9). A) Bacterial temporal stability based on community composition (left Y-axis) and abundance (right Y-axis), and B) nematode biomass, density and community composition based on species level, across all study sites (circle: REF, square: CO2-G, triangle: CO2-R).

Table 2
Beta similarity between sites for three years and between years within study sites, presented as the average percentage (± standard deviation) of shared OTUs/species derived from Jaccard dissimilarity index based on presence/absence data for bacterial OTUs (ARISA, n = 3; 454-MPTS, n = 9), nematode (n = 3) and polychaete (n = 5) species. 454-MPTS was only applied on samples collected in 2012, therefore replicates’ pairwise comparisons were used to test differences in beta similarity between site, and beta similarity between years is not available (na).

|                   | Shared bacterial OTUs (%) | Shared nematode species (%) | Shared polychaete species (%) |
|-------------------|---------------------------|------------------------------|-------------------------------|
| Between sites     |                           |                              |                               |
| REF vs CO2-R      | 68 ± 7                    | 16 ± 1                       | 9 ± 7                         |
| REF vs CO2-G      | 73 ± 3                    | 20 ± 2                       | 10 ± 7                        |
| CO2-R vs CO2-G    | 82 ± 2                    | 32 ± 2                       | 20 ± 8                        |
| Between years     |                           |                              |                               |
| REF vs CO2-R      | 88 ± 1                    | na                           | 47 ± 6                        |
| CO2-R vs CO2-G    | 76 ± 3                    | na                           | 29 ± 7                        |

the temporal variation in community composition was comparable with that observed in other coastal sandy sediments (Böer et al., 2009; Gobet et al., 2012). Direct comparison of beta diversity values between studies could be biased by application of different sequencing techniques (e.g. different sequencing depth, different primers) and methods for beta diversity estimation. However, here, our results were compared with previous studies applying the same methods to investigate microbial diversity (i.e. ARISA and/or 454-MPTS) and to assess beta diversity (i.e. Jaccard dissimilarity distance). Both long- (at least three years) and short-term (one year) exposure to CO2 emissions resulted in a decrease
of microbial diversity in benthic microbial assemblages at Basiluzzo. Remarkably, the decrease in richness and evenness was observed within almost all dominant bacterial classes, even those favored at CO2 vent sites (Molari et al., 2018). Together, these findings suggest a stable and univocal response of dominant bacterial functional groups to CO2 emissions here investigated. Molari et al. (2018) showed that alongside the lowering of pH, the CO2 emissions have an indirect effect on productivity (i.e. stimulating microphytobenthos) and sand mineralogy (i.e. dissolution of carbonate fraction). The trophic status and sediment composition are known to be factors shaping microbial communities (e.g. Schöttner et al., 2011; Bienhold et al., 2012). However, our results showed that bacterial diversity was highly related with pH rather than with others environmental factors, suggesting that at venting sites CO2 emissions led to the selection for those bacteria physiologically tolerant to acid stress.

Neutralophilic bacteria, growing at pH range of 5–9, can use different mechanisms for pH homeostasis (Booth, 1985; Slonczewski et al., 2009). Specifically for respiratory bacteria, which dominated microbial communities at our study site, acid stress is met by direct active efflux of protons by redox potential-driven pumps, light-driven pumps or bond energy-driven pumps (Kruhlisch et al., 2011). Recently, a 9-days mesocosm experiment showed that a pH reduction of 0.2 units stimulated expression of different pH homeostasis genes in marine heterotrophic bacterioplankton (Bunse et al., 2016). These stimulated mechanisms to export protons across the cell membrane are energy demanding, which implies that the physiological acclimation of bacteria to seawater acidification may increase energy demand for cell maintenance, potentially resulting in a reduction of growth efficiency. Thus, under exposure to constant and moderate flows of acidified seawater, and depending on the extent of acidification, we can expect that an increase of metabolic cost could select for those bacteria with more versatile metabolism (e.g. resource exploitation, regulation of growth efficiency), with consequences for diversity and carbon cycling. This is exactly what we have observed at acidified Basiluzzo sites, where the unchanged bacterial abundance was accompanied with a loss of diversity and an increase of organic carbon remineralization (Molari et al., 2018). Future studies need to be carried out to look closely at the effects of seawater acidification on microbial physiology and energy allocation for elucidating the consequence on carbon and energy fluxes in the microbial food web. Nevertheless, for the first time transplantation experiments and three years of field observations provide robust evidence that seawater acidification, induced by seafloor CO2 emissions, can be responsible for the loss of bacterial diversity.

### 4.2. CO2 leakage affects invertebrate diversity

The results of this study demonstrate that at least 2 years of exposure to diffuse CO2 leakage reduced polychaete species diversity. These findings complement the results from laboratory and field experiments investigating more or less severe, acute CCS leakage impacts on benthic macrofaunal diversity (Christen et al., 2012; Hale et al., 2011; Ingels et al., 2012; Widdicombe et al., 2009, 2015). The threshold at which impacts were observed on infaunal macro-invertebrate diversity in laboratory experiments that mimic a CO2 rich plume, was identified at seawater pH levels below 7, but only after five weeks of continuous exposure (Widdicombe et al., 2015). An experimental sub-seabed release of CO2 caused a reduction in macrobenthic diversity within a few days, although porewater pH did not drop below 7.5 (Widdicombe et al., 2015). In both scenarios, impact thresholds are likely determined by natural processes, such as carbonate buffering and permeability, which influence the carbon chemistry of the sediment (Widdicombe et al., 2015). Moreover, the vulnerabilities to high pCO2/low pH conditions differ between and within phyla (Christen et al., 2012 and references therein). The reduction in biodiversity in these relatively short-term CO2 leakage simulations result from the loss of species that hamper the ability to regulate the acid-base balance of internal fluids to maintain a number of key pH sensitive physiological processes (Kroeker et al., 2013) or in case of calcifying organisms, fail to maintain important physiological processes (e.g. growth, reproduction, immune function) as a result of an energetically more demanding calcification process under reduced calcite or aragonite saturation states (Pörtner, 2008; Wood et al., 2008). In general, annelids were classified among the taxa most resilient to highly elevated pCO2/low pH (Christen et al., 2012; Hale et al., 2011). The relatively long-term
perspective and natural environment in our study allows to consider whether the vacant niches of species that disappear on the short term are being occupied by colonizing tolerant species and what consequences this might have for the biodiversity. The low number of shared polychaete species between the reference and vent sites and the severely reduced species diversity at the vent site leaves no doubt that polychaete communities cannot cope with diffuse CO₂ leakage over a period of at least several years. This reduction in species diversity coincides with a reduction in functional diversity (i.e. feeding modes), density and biomass (Molari et al., 2018) and therefore warrants for a reduction in the optimal functioning of the benthic ecosystem.

Nematodes, generally the numerically dominant and species-rich members of the meiofauna, have most often been the focus of meiofaunal response measures to acute seawater acidification (Ingels et al., 2012, 2018; Kurihara et al., 2007). This is the first study, however, that indicates that one to at least three years of exposure to acidified porewater conditions (pH₂ minimum: 5.5) in a natural environment severely reduces nematode species diversity. Earlier acute acidification exposure experiments testing the effect of seawater pH ≥ 6 over a maximum of 12 weeks on the meiofauna found no negative effects on nematode abundance, community composition and diversity (Takeuchi et al., 1997; Dashfield et al., 2008; Ingels et al., 2018; Schade et al., 2016). Merely once an increase in nematode abundances occurred, which was attributed to the reduction of ecological constraints (predation, competition) resulting from a decrease in macrofaunal abundance (Hale et al., 2011). Only when seawater acidity was decreased below what appears to be a threshold of 6 pH units for a maximum of 20 weeks, nematode community structure changed and species diversity decreased (Widdicombe et al., 2009), abundance decreased (Takeuchi et al., 1997; Barry et al., 2004; Ishida et al., 2005), or high mortality was suggested based on changed morphometrics (Fleeger et al., 2010). The nematodes’ impermeable proteinaceous cuticle is suggested to buffer them relatively well to short-term natural fluctuations of porewater pH in the upper few centimeters of sediments (typical pH range of 6.5–8.2; Brusca and Brusca, 1990; Widdicombe et al., 2011). Persistently high CO₂ conditions probably resulted in extracellular acidosis, a condition which might exert lethal physiological stresses on nematodes (Barry et al., 2004; Widdicombe et al., 2009). The highly distinct and species-poor nematode communities found at the vents at Basluzzo Island, however, also demonstrate that there are nematode species which potentially adapted and/or exhibit tolerance to extreme and chronic high pCO₂/low pH conditions, with few opportunistic species demonstrating strong competitive advantages in exploiting these harsh environments (e.g. Microlaimus compridus, Microlaimus honestus, Oncholaimus campylolecoides, and Daptonema Microspiculum; Molari et al., 2018). At the same time, these species-poor vent communities seem prone to a relatively high temporal variability in different structural community characteristics (i.e. community composition, abundance, biomass), which suggests reduced resilience to additional environmental perturbations. These results emphasize the importance of investigating synergistic impacts of environmental factors on multi-species assemblages to enable more informative predictions (Jones et al., 2014; Mevenkamp et al., 2018).

Similar susceptible changes to CO₂ exposure were also observed for other meiofaunal groups (Thistle et al., 2005). For example, for many species of harpacticoid copepods, alterations in CO₂ concentrations resulted in general mortality rates of around 80% of the total community (Thistle et al., 2006), but it also indicated that some species are less susceptible than others to changes in the environment (Thistle et al., 2006, 2007).

In general, the meiofauna is less affected by environmental changes compared to the macrobenthos (Ingels et al., 2012; Zeppilli et al., 2015). Some species can even be favored by extreme environmental conditions. Within the meiofauna, copepods are considered particularly susceptible to stress in comparison with nematodes, suggesting the high versatility and different responses of this size class in predicting major global changes by identifying sensitive and tolerant species (Zeppilli et al., 2015).

4.3. Recovery of benthic diversity after CO₂ leakage has ceased

Transplanting sediments from the CO₂ vent site to the reference site allowed evaluating the recovery potential of high pCO₂/low pH-impacted nematode communities. Our results show that nematode species diversity could re-establish within one year, while Molari et al. (2018) additionally demonstrates a recovery of the nematode community composition, though with reduced overall population densities. These findings are in agreement with the results presented by Widdicombe et al. (2015), where the macrofauna community composition had recovered from the impact of a subseabed CO₂ injection 18 days after the leakage was stopped. Neither the bacterial community composition (Molari et al., 2018), nor the diversity of bacteria recovered within one year, suggesting that colonization time exceeded one year. However, as mentioned above, the CO₂ leakage promoted dissolution of carbonate sands, an effect that was still recorded in the sediments transplanted to the reference site (Molari et al., 2018). Without carbonate sands the sediment lost its buffering capacity, resulting in porewater with a pH value 0.4–0.7 units lower than what is typically found in undisturbed sediments. Thus, we cannot exclude that bacterial diversity may be affected by variations of pH values even smaller than those observed at CO₂-venting sites.

Although both experiments have shown that the chemical effects of CO₂ leakage on benthic invertebrate diversity are not long lasting, we need to consider that both study areas were spatially relatively small (35–200 m²). The time needed for recovery from CO₂ leakage events will eventually depend on the area of the impacted site, the flux of CO₂ and the potential physical disruption of the sediments, the species dispersal capabilities, their rate of recruitment, and the distance between the disturbed site and the unaffected source populations.

5. Conclusions

In summary, this study proved that porewater acidification, induced by seafloor CO₂ emissions, was responsible for reducing 30–80% species diversity of dominant benthic organisms across size-classes (Bacteria, Nematoda and Polychaeta). With the transplantation experiment we provided evidence that the impact on microbe and nematode diversity was already apparent after one year of exposure to CO₂ emission. Additionally, the transplantation experiment showed that recovery of an impacted area relatively small in size was fairly quick (i.e. within one year), at least for nematodes. The lack of recovery of bacterial communities pointed out that effects of CO₂ leakage on sediment properties may last for a long time after the disturbance is ceased, with consequences on benthic biodiversity that may vary according to the size and ecology of organisms (e.g. dispersion, sessile/motile) and to the scale of the impacted area.

High microbial and nematode species richness at the reference site revealed more stable communities over time, while loss of species at the vent sites resulted in higher temporal variability in community characteristics, suggesting reduced resilience to additional environmental perturbations at impacted locations. These results contribute to the growing body of evidence that highlights the importance of biodiversity for ecosystem stability.
Data accessibility

Data can be accessed through the PANGAEA database (https://www.pangaea.de). Biodiversity is calculated based on data that are deposited in the following datasets: #830368, #846151, #846154, #846155, #846597, #873500. During the review process, the reviewers can access these data and also the supporting data from Molari et al. (2018) via https://doi.pangaea.de/10.1594/PANGAEA.871453.

Authors’ contributions

K.G. processed the macro- and meiofauna samples, identified the nematodes to species level, analysed the faunal data, and wrote the manuscript; M.M. processed the bacterial samples, analysed the bacterial data, and wrote the manuscript; L.L. processed the meiofauna samples and wrote the manuscript; A.V., A.R. and K.G. designed the study. All authors revised the article critically and gave final approval of the version to be published.

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Appendices

Fig. A.1. Rarefaction curves based on Hill numbers (q = 0, 1 and 2) for A) abundance of Nematoda for samples collected in 2011 (n = 3) and B) abundance of Nematoda for samples collected in 2013 (n = 3). Interpolation (full), extrapolation (dashed) lines and their 95% confidence intervals (shaded area) are indicated.
Fig. A.2. Rarefaction curves calculated from A) numbers of bacterial sequences based on the ARISA (2011–2013) and B) numbers of genera based on 454-MPTS (2012; n = 3).

Fig. A.3. Alpha diversity of Polychaeta species (n = 5) expressed in Hill's numbers (H₀, H₁, H₂) for each study site (REF, CO2-G, CO2-R).
Fig. A.4. Alpha Diversity as described by estimated richness (Chao1) and Hills numbers (H0 and H2) for dominant bacterial classes, based on 454-MPTS data (OTUs > 0.1%; n = 6; 0–2 cm 4–6 cm layer were combined). Significant difference between the vent sites and the REF are indicated with an asterisk according to pairwise comparison adjusted P values (* < 0.05, ** < 0.01, *** < 0.001). In the plot statistic F-ratio (F), with subscript numbers reporting the degrees of freedom between groups and within groups, respectively, and probability level (P) for total ANOVA model are also reported. Chao1 was calculated based on repeated random subsampling of the 454-MPTS data sets for each class: Actinobacteria = 175 sequences; Alphaproteobacteria = 132 sequences; Bacteroidia = 11 sequences; Caldilineae = 32 sequences; Candidatus Thiobios = 19 sequences; Chlorobia = 15 sequences; Cyanobacteria unclassified (uncl.) = 122 sequences; Clostridia = 32 sequences; Deltaproteobacteria = 470 sequences; Flavobacteria = 185 sequences; Gammaproteobacteria = 860 sequences; Sphingobacteria = 187 sequences.
Verrucomicrobia = 39 sequences.

Fig. A.5. Redundancy analyses plots illustrating the contribution of environmental variables to explain the variance in OTUs / species diversity (Hills numbers) of Bacteria (ARISA; A: 0–10 cm, B: 0–2 cm) and Nematoda (C: 0–8 cm, D: 0–2 cm) at the three study sites (REF, CO2-G, CO2-R) based on abundance data of 2012 and 2013.

Table A.1

Sedimentary environmental data for the study locations sampled in 2012 and 2013, as presented in Molari et al. (2018). Data are presented as the average ± standard deviation (n = 3).

| Year | Site | Layer | Silicate (μM) | Phosphate (μM) | Ammonium (μM) | Nitrite-Nitrate (μM) | pH (mM) | DIC (mM) | TA (mM) |
|------|------|-------|--------------|---------------|---------------|---------------------|---------|----------|---------|
| 2012 | REF  | 0–2   | 7.6 ± 2.7    | 0.5 ± 0.4     | 1.2 ± 0.4     | 1.6 ± 1.1           | 7.5 ± 0.1| 2.5 ± 0.1| 2.5     |
|      |      | 2–4   | 16.7 ± 1.2   | 0.5 ± 0.1     | 1.3 ± 0.2     | 1.6 ± 0.6           | 7.5 ± 0.0| 2.5 ± 0.0| 2.5     |
|      |      | 4–6   | 21.8 ± 3.4   | 0.5 ± 0.1     | 2 ± 2.2       | 0.6 ± 0.5           | 7.4 ± 0.1| 2.4 ± 0.0| 2.5     |
|      |      | 6–8   | 23.6 ± 6.3   | 0.5 ± 0.2     | 3.7 ± 2.0     | 0.4 ± 0.5           | 7.4 ± 0.1| 2.5 ± 0.2| 2.5     |
|      |      | 8–10  | 28.9 ± 21.5  | 0.8 ± 0.7     | 4.9 ± 3.1     | 0.4 ± 0.3           | 7.4 ± 0.0| 2.4 ± 0.0| 2.5     |
|      | CO2-G| 0–2   | 131 ± 163.9  | 3.7 ± 5.7     | 2.3 ± 3.9     | 0.4 ± 0.3           | 5.8 ± 0.3| 19.5 ± 18.5| 8.8     |
|      |      | 2–4   | 158.9 ± 156.1| 3.8 ± 5.6     | 4 ± 4.2       | 0.4 ± 0.5           | 5.6 ± 0.2| 19.5 ± 19.5| 9.3     |
|      |      | 4–6   | 163.3 ± 149.5| 5.6 ± 8.5     | 4.2 ± 6.9     | 0.4 ± 0.5           | 5.6 ± 0.2| 26 ± 16.2  | 9.7     |
|      |      | 6–8   | 235.8 ± 123.3| 7.7 ± 8.2     | 10.4 ± 9.4    | 0.5 ± 0.3           | 5.7 ± 0.1| 29.3 ± 15.0| 11.7    |
|      |      | 8–10  | 275.4 ± 108.8| 7.9 ± 8.0     | 11.2 ± 9.9    | 0.3 ± 0.5           | 5.6 ± 0.1| 31.4 ± 13.6| 12.8    |
|      | CO2-R| 0–2   | 161.2 ± 9.5  | 0.6 ± 0.6     | 1.3 ± 0.8     | 1.3 ± 1.8           | 5.7 ± 0.1| 15.2 ± 3.3  | 6.5     |
|      |      | 2–4   | 370.8 ± 148.8| 0.3 ± 0.1     | 3.5 ± 2.0     | 1 ± 1.2            | 5.7 ± 0.1| 24.1 ± 6.7  | 9.4     |
|      |      | 4–6   | 465.3 ± 232.2| 0.4 ± 0.4     | 14 ± 8.9      | 0.8 ± 0.9           | 5.7 ± 0.1| 26 ± 6.5   | 11      |
|      |      | 6–8   | 505.3 ± 224.1| 1.3 ± 1.7     | 14.8 ± 9.6    | 1.1 ± 1.4           | 5.7 ± 0.1| 29.6 ± 7.6  | 10.5    |
|      |      | 8–10  | 285.6 ± 91.4 | 0.6 ± 0.2     | 14.3 ± 13.5   | 1.7 ± 1.3           | 5.7 ± 0.1| 30.4 ± 7.3  | 12.7    |
| 2013 | REF  | 0–2   | 1.4 ± 0.3    | 0 ± 0.0       | 13.2 ± 2.8    | 1.4 ± 1.2           | 7.9 ± 0.0| 2.4 ± 0.1  | 2.4     |
|      |      | 2–4   | 3.6 ± 1.1    | 0 ± 0.0       | 12.2 ± 5.0    | 4.1 ± 2.4           | 7.7 ± 0.1| 2.5 ± 0.1  | 2.4     |
|      |      | 4–6   | 4.9 ± 1.0    | 0 ± 0.0       | 12.8 ± 4.2    | 5.7 ± 1.5           | 7.7 ± 0.1| 2.5 ± 0.1  | 2.4     |
|      |      | 6–8   | 7.2 ± 2.7    | 0 ± 0.0       | 12 ± 3.7      | 7.7 ± 3.4           | 7.7 ± 0.2| 2.5 ± 0.1  | 2.3     |
|      |      | 8–10  | 10.3 ± 3.6   | 0 ± 0.0       | 11.8 ± 2.7    | 7.5 ± 3.3           | 7.6 ± 0.2| 2.5 ± 0.1  | 2.4     |
### Table A.2

Alpha diversity for Bacteria based on 454-MPTS data considering the sediment layers 0–2 cm and 4–6 cm of sediment cores collected in 2012 (n = 3). Data are presented as the average ± standard deviation (n = 3).

| Site | Layer | Total number of sequences | Chao1 | OTUs Alpha diversity | SS OBS | Unique OTUs | Orders diversity |
|------|-------|---------------------------|-------|----------------------|--------|-------------|-------------------|
|      |       |                           |       | H0                   | H1     | H2          | H3                |
|      |       |                           |       | %                    | %      | %           | n. orders          |

**DIC:** dissolved inorganic carbon; **TA:** total alkalinity; **Chl-a:** chlorophyll a; **MGS:** median grain size.

1 Calculated on subsampled 454-dataset (number sequences = 4346).
2 Absolute singletons (SSOabs) are OTUs consisting of sequences occurring only once in the full dataset (percentage relative to total OTUs of whole dataset).
3 Unique OTUs are the dominant OTUs (OTUs0.03 > 0.1%) present exclusively in one of three site investigated (percentage relative to total OTUs of whole dataset).
4 Total number of bacterial orders.
Table A.3
Details of the statistical analyses on the Hill's numbers (H_0, H_1, H_2) from the natural sediments. Bacterial OTUs (ARISA) was analysed with three-factor ANOVA (model: Hill number ~ Site + Year*Site + Layer-nested-Site, data), Nematode species diversity with three-factor split-plot ANOVA with "sediment core" as random factor (model: Hill number ~ Site + Year*Site + Layer-nested-Site, random = ~1|Core, data), Polychaete species diversity with one-way ANOVA (model: Hill number ~ Site, data).

| Size-class organisms | Hill numbers | Factors | Df | Sum Sq | Mean Sq | F value | P  | Redundancy (%) |
|----------------------|--------------|---------|----|--------|---------|---------|----|----------------|
| Bacteria             | H_0          | site    | 2  | 397590 | 198795  | 241.259 | < 0.0001 | 63  |
|                      |              | year    | 1  | 134    | 134     | 0.162   | 0.687 | 0              |
|                      |              | site:year | 2  | 393    | 196     | 0.238   | 0.788 | 0              |
|                      |              | site:layer | 3  | 411    | 137     | 0.166   | 0.919 | 0              |
|                      | H_1          | site    | 2  | 386367 | 193183  | 407.817 | < 0.0001 | 73  |
|                      |              | year    | 1  | 1475   | 1475    | 3.115   | 0.0787 | 0              |
|                      |              | site:year | 2  | 1490   | 745     | 1.573   | 0.2092 | 0              |
|                      |              | site:layer | 3  | 2397   | 799     | 1.667   | 0.17  | 0              |
|                      | H_2          | site    | 2  | 154090 | 77045   | 281.747 | < 0.0001 | 64  |
|                      |              | year    | 1  | 2319   | 2319    | 8.482   | 0.00387 | 1   |
|                      |              | site:year | 2  | 1676   | 838     | 3.064   | 0.04823 | 1   |
|                      |              | site:layer | 3  | 3712   | 1237    | 4.525   | 0.00406 | 0   |
| Nematode             | H_0          | site    | 2  | 1984.6 | 992.3   | 139.972 | < 0.0001 | 72  |
|                      |              | year    | 1  | 5.63   | 5.63    | 0.7946  | 0.3828 | 0              |
|                      |              | site:year | 2  | 15.35  | 7.68    | 1.083   | 0.3568 | 1              |
|                      |              | site:layer | 3  | 102.54 | 34.18   | 4.8212  | 0.0039 | 4              |
|                      | H_1          | site    | 2  | 395.55 | 197.774 | 66.2195 | < 0.0001 | 60  |
|                      |              | year    | 1  | 1.52   | 1.519   | 0.5086  | 0.4863 | 0              |
|                      |              | site:year | 2  | 3.8    | 1.899   | 0.6359  | 0.5394 | 1              |
|                      |              | site:layer | 3  | 5      | 1.667   | 0.5581  | 0.6443 | 1              |
|                      | H_2          | site    | 2  | 175.782 | 87.891 | 39.2373 | < 0.0001 | 47  |
|                      |              | year    | 1  | 1.25   | 1.25    | 0.558   | 0.4633 | 0              |
|                      |              | site:year | 2  | 2.774  | 1.387   | 0.6193  | 0.5479 | 0              |
|                      |              | site:layer | 3  | 0.658  | 0.219   | 0.098   | 0.9609 | 0              |
| Polychaete           | H_0          | site    | 2  | 43.364 | 21.6821 | 19.312  | < 0.0001 | 78  |
|                      |              | year    | 1  | 25.092 | 12.5466 | 18.603  | < 0.0001 | 77  |
|                      |              | site:layer | 3  | 15.6196 | 7.8098 | 18.691  | < 0.0001 | 77  |

Table A.4
Details of the statistical analyses on the Hill's numbers (H_0, H_1, H_2) from the transplant experiment. Bacteria (ARISA) and Nematode species diversity was analysed with three-factor split-plot ANOVA with "sediment core" as random factor (model: Hill number ~ Treatment + Layer-nested-Treatment, random = ~1|Core, data).

| Size-class organisms | Hill numbers | Factors | Df | Sum Sq | Mean Sq | F value | P  | Redundancy (%) |
|----------------------|--------------|---------|----|--------|---------|---------|----|----------------|
| Bacteria             | H_0          | Treatment | 3  | 7092.3 | 2364.1  | 41.077  | < 0.0001 | 57  |
|                      |              | Treatment:Layer | 4  | 2816.1 | 794.02  | 12.233  | < 0.0001 | 23  |
|                      | H_1          | Treatment | 3  | 3059.6 | 1019.86 | 22.2783 | < 0.0001 | 49  |
|                      |              | Treatment:Layer | 4  | 1201.1 | 300.28  | 6.5595  | 0.0003 | 19  |
|                      | H_2          | Treatment | 3  | 959.46 | 319.82  | 5.136   | 0.0239 | 24  |
|                      |              | Treatment:Layer | 4  | 533.51 | 133.38  | 2.2994  | 0.0738 | 13  |
| Nematode             | H_0          | Treatment | 3  | 302.648 | 100.883 | 15.9288 | < 0.0001 | 79  |
|                      |              | Treatment:Layer | 4  | 16.333 | 4.083   | 0.6447  | 0.6459 | 4   |
|                      | H_1          | Treatment | 3  | 171.503 | 57.168  | 21.396  | < 0.0004 | 78  |
|                      |              | Treatment:Layer | 4  | 21.398 | 5.35    | 2.0022  | 0.1872 | 10  |
|                      | H_2          | Treatment | 3  | 90.895 | 30.2985 | 14.6966 | 0.0013 | 71  |
|                      |              | Treatment:Layer | 4  | 15.775 | 3.9437  | 1.9129  | 0.2017 | 12  |

Table A.5
Output of standard and partial redundancy analyses (RDAs). Significant variables used for variation portioning analysis and partial RDA are in bold.

| 0–10 cm Bacteria (n = 90) |
|---------------------------|
| RDA: Hill Numbers ~ pH + Chl.a + TOC + MGS + Silicate | adjR^2 | 0.804 |
|                           | ANOVA  | Df   | SS      | F      | P      |           |          |
|                           | pH     | 1    | 3582.2  | 346.7  | 0.001  |
|                           | Chl.a  | 1    | 22.5    | 2.2    | 0.133  |
|                           | TOC    | 1    | 22.6    | 2.2    | 0.148  |
### 0–2 cm Bacteria (n = 18)
#### RDA: Hill Numbers ∼ pH + Chl.a+TOC + MGS + Silicate

| ANOVA | Df | SS | F | P  |
|-------|----|----|---|----|
| pH    | 1  | 236.5 | 72.4 | 0.001 |
| Chl.a | 1  | 4.8  | 0.026 |
| TOC   | 1  | 0.6  | 0.439 |
| MGS   | 1  | 1.2  | 0.285 |
| Silicate | 1  | 13.6 | 0.007 |
| Residual | 66 | 11.9 |

### 0–10 cm Bacteria (n = 90)
#### RDA: Hill Numbers ∼ pH + Chl.a+TOC + MGS + Silicate

| ANOVA | Df | SS | F | P  |
|-------|----|----|---|----|
| pH    | 1  | 47.6 | 26.6 | 0.001 |
| Chl.a | 1  | 1.6  | 0.233 |
| TOC   | 1  | 1.1  | 0.315 |
| MGS   | 1  | 4.1  | 0.099 |
| Silicate | 1  | 6.8  |
| Residual | 69 | 12.6 |

### 0–8 cm Nematoda (n = 72)
#### RDA: Hill Numbers ∼ pH + Chl.a+TOC + MGS + Silicate

| ANOVA | Df | SS | F | P  |
|-------|----|----|---|----|
| pH    | 1  | 156.3 | 12.6 | 0.001 |
| Chl.a | 1  | 4.7  | 0.022 |
| TOC   | 1  | 4.7  | 0.022 |
| MGS   | 1  | 1.2  | 0.285 |
| Residual | 69 | 12.6 |

### 0–2 cm Nematoda (n = 18)
#### RDA: Hill Numbers ∼ pH + Chl.a+TOC + MGS + Silicate

| ANOVA | Df | SS | F | P  |
|-------|----|----|---|----|
| pH    | 1  | 47.6 | 26.6 | 0.001 |
| Chl.a | 1  | 1.6  | 0.233 |
| TOC   | 1  | 1.1  | 0.315 |
| MGS   | 1  | 4.1  | 0.099 |
| Silicate | 1  | 6.8  |
| Residual | 14 | 7.2  |

### RDA: Hill Numbers ∼ pH + Condition (MGS,Silicate)

| adjR² | 0.401 |
|-------|-------|

### ANOVA

| Df | SS  | F   | P  |
|----|-----|-----|----|
| pH | 1   | 236.5 | 72.4 | 0.001 |
| Chl.a | 1  | 4.8  | 0.026 |
| TOC | 1   | 0.6  | 0.439 |
| MGS | 1   | 1.2  | 0.285 |
| Silicate | 1  | 13.6 |
| Residual | 66 | 11.9 |

### RDA: Hill Numbers ∼ G.size + Condition (pH + Silicate)

| adjR² | 0.006 |
|-------|-------|

### ANOVA

| Df | SS  | F   | P  |
|----|-----|-----|----|
| pH | 1   | 2969.5 | 72.4 | 0.001 |
| Chl.a | 1  | 4.7  | 0.022 |
| TOC | 1   | 1.2  | 0.285 |
| MGS | 1   | 1.2  | 0.285 |
| Silicate | 1  | 13.5 |
| Residual | 15 | 358.5 |

### RDA: Hill Numbers ∼ pH + Condition (MGS,Silicate)

| adjR² | 0.561 |
|-------|-------|

### ANOVA

| Df | SS  | F   | P  |
|----|-----|-----|----|
| pH | 1   | 28.6 | 12.6 |
| Chl.a | 1  | 4.7  | 0.022 |
| TOC | 1   | 1.2  | 0.285 |
| MGS | 1   | 1.2  | 0.285 |
| Silicate | 1  | 13.5 |
| Residual | 69 | 12.6 |

### RDA: Hill Numbers ∼ pH + Condition (Chl.a)

| adjR² | 0.561 |
|-------|-------|

### ANOVA

| Df | SS  | F   | P  |
|----|-----|-----|----|
| pH | 1   | 28.6 | 12.6 |
| Chl.a | 1  | 4.7  | 0.022 |
| TOC | 1   | 1.2  | 0.285 |
| MGS | 1   | 1.2  | 0.285 |
| Silicate | 1  | 13.5 |
| Residual | 69 | 12.6 |

### RDA: Hill Numbers ∼ pH + Condition (MGS,Silicate)

| adjR² | 0.376 |
|-------|-------|

### ANOVA

| Df | SS  | F   | P  |
|----|-----|-----|----|
| pH | 1   | 13.6 | 26.4 | 0.001 |
| Chl.a | 1  | 7.2  | 0.018 |
| TOC | 1   | 1.6  | 0.233 |
| MGS | 1   | 3.1  | 0.099 |
| Silicate | 1  | 4.1  |
| Residual | 14 | 7.2  | 0.018 |
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n: number of observations; Hill number: H0, H1, H2; Chl.a: chlorophyll a; TOC: total organic carbon; MGS: mean grain size; adjR2: adjusted R2; Df: degrees of freedom; SS: sum of the squares; F: statistic; P: probability level; RDA: standard redundancy analysis; pRDA: partial redundancy analysis.

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