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Microbial biodiversity and viral impact in benthic deep-sea ecosystems

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State of art

Microbiome of deep-sea sediments: abundance, distribution and diversity of prokaryotes and viruses

Deep-sea environments (the waters and sediments of the ocean interior beneath 200m depth) are the largest biome of the world, representing more than 65% of the Earth's surface and more than 95% of the global biosphere (Herring, 2001; Corinaldesi, 2015). In general, abyssal (3,000–6,000 m) and hadal (>6,000 m) environments are characterized by low temperature, high hydrostatic pressure and the absence of solar radiation (Bartlett, 1992; Lauro & Bartlett, 2008), and their exploration is very challenging because of their extreme conditions. In fact, despite the technological advancement, current estimates indicate that only 5% of the deep oceans have been explored in detail, and less than 0.001% has been studied in terms of microbiology and biodiversity (Rex et al., 2006; Wei et al., 2010; Danovaro et al., 2015). However, there is evidence that microbes in the deep-sea represent the 'hidden majority' of all life forms, representing 50-80% of the Earth's total microbial biomass and 10-30% of the Earth's total living biomass (Whitman et al., 1998, Teske, 2005).

Prokaryotic abundance ranges from ca. 10^8 to 10^9 cells cm^{-3} in deep-sea surface sediments (Orcutt et al., 2011). Typically the dominant group of Bacteria inhabiting deep-sea sediments are *Gammaproteobacteria*, *Deltaproteobacteria*, *Planctomycetes*, *Actinobacteria*, and *Acidobacteria* (Zinger et al., 2011; Bienhold et al., 2016). Recent investigations highlighted the presence of a core microbiome consisting of 18 OTUs, with two predominantly cosmopolitan OTUs affiliated with the JTB255 clade of the order *Xanthomonadales*, and one with the OM1 clade of the order *Acidomicrobiales*. The exact environmental functions of these two clades are actually unknown, even though it has been hypothesized a sulphur-oxidizing activity related with the JTB255 clade

(Bowman & McCuaig, 2003; Dyksma, 2016). A number of studies provide evidence of the important role of Archaea in benthic deep-sea systems (Karner et al., 2001; Schippers et al., 2005; Biddle et al., 2006; Schippers & Neretin, 2006; Lipp et al., 2008; Lloyd et al., 2013; Xie et al., 2013; Danovaro et al., 2015). Different lineages of Euryarchaeota affiliated with *Methanosarcinales* (e.g., ANME-1, ANME-2, and ANME-3) and phylotypes of Crenarchaeota (e.g., Marine Benthic Group B and Miscellaneous Group) have been detected in benthic sediments of different marine systems (Boetius et al., 2000; Orphan et al., 2001; Orphan et al., 2002; Schleper et al., 2005; DeLong & Pace, 2001; Jørgensen & Boetius, 2007). Especially, ANME lineages perform the sulphate-dependent anaerobic methane oxidation process syntrophically with sulphate reducing bacteria (Dong et al., 2015), being also able to fix N_2 thus presenting an important role in regulating biogeochemical cycles in deep-sea sediments (Dekas et al., 2009). As regard Crenarchaeota, the Marine Benthic Group B occurs widely in methanogenic and anaerobic sediments, while the Miscellaneous Crenarchaeotic Group are hypothesized to be heterotrophic anaerobes able to use and assimilate complex organic substrates (Teske & Sørensen, 2008).

Viruses are the most abundant life forms in the ocean (Weinbauer et al., 2011) showing abundances in the order of 10^{30}–10^{31} and exceeding those of Bacteria and Archaea by approximately 15-fold (Suttle, 2007). Previous studies highlighted high virus abundances, in the order of 10^{8} per gram of sediment in different benthic marine ecosystems (Danovaro et al., 2002; Danovaro et al., 2008). Such high viral abundances in deep-sea sediments could be due to the supply of virioplankton adsorbed onto sinking particles (Mari et al., 2007; Zhang et al., 2014) and/or to the in situ viral production rates (Danovaro et al., 2008; Siem-Jørgensen et al., 2008), even though there is evidence that they are mostly produced in *situ* (Danovaro & Serresi, 2000; Danovaro et al., 2008). Information on viral diversity in benthic deep-sea ecosystems is very limited (Yoshida et al., 2013; Anderson et al., 2013; Tangherlini et al. 2016). However, available studies allowed hypothesizing that deep-sea viral metagenomes could be highly diversified and characterized by a large portion of new genes and potential functions, even involved in microbial adaptation (Tangherlini et al., 2016; Yoshida et al., 2015; Danovaro et al., 2016, Rohwer & Thurber, 2009; Rohwer & Youle, 2011). Viral

diversity in benthic deep-sea ecosystems is characterized by the presence of either double-stranded DNA phages, mainly belonging to the order Caudovirales (i.e., Myoviridae, Podoviridae, Siphoviridae) that are believed to infect prokaryotes (Angly et al., 2006; Rosario and Breitbart, 2011; Hurwitz and Sullivan, 2013), or by single-stranded DNA viruses belonging to Circoviridae and infecting eukaryotes (Labonté and Suttle, 2013; Yoshida et al., 2013).

Recognizing the fundamental role of microbes (prokaryotes and viruses) in influencing all of the biogeochemical cycles in each benthic marine habitat, appears to be of fundamental importance to expand and deepen the actual knowledge on their distribution, functions and diversity for a better understanding of the deep-sea ecosystem functioning.

Virus-prokaryote interactions in deep-sea sediments

Every second, approximately 10^{23} viral infections occur in the ocean influencing the composition of marine communities (Suttle, 2007). Growing evidence suggests that viruses are important players in microbial food webs, not only as agents of mortality but also as mediators of carbon and nutrient cycling (Bonilla-Findji et al., 2008) thus contributing importantly to functioning of the deep-sea trophic webs. Viral infections in deep-sea surface sediments are responsible for the abatement from 40 to up to >80% of the overall heterotrophic carbon production by bacteria and archaea (below 1000-m depth), causing the release of ~0.37–0.63 Gt C $year^{-1}$ on a global scale (Danovaro et al., 2008). By killing their hosts through lytic infections, viruses can transform the living biomass into organic detritus (mostly dissolved organic matter; DOM), which can then be used again by other microbes stimulating their growth (Corinaldesi et al., 2007; Corinaldesi et al., 2012). Viral infections may play also key roles in modifying prokaryotes abundance and in shaping their diversity by laterally transferring DNA between microbes in different biomes, altering their phenotypic characteristics and modifying their fitness (Hewson et al., 2003; Sano et al., 2004; Paul, 2008; Weitz & Wilhelm, 2012). Previous studies, indeed, found that in different investigated benthic systems, >80% of the total variance of the prokaryotic assemblage composition was

explained not only by the availability of trophic resources (in term of phytopigments, biopolymeric C and C released by the viral shunt) but also by the 'viral predatory pressure' (Corinaldesi et al., 2012).

Different models have been developed aiming at describing the interactions between prokaryotes and viruses. One of the main models describing the possible influence of viral infection on prokaryotic diversity is the concept 'Killing the Winner' (KtW; Thingstad and Lignell, 1997), which indicates that lytic viruses can keep in check competitive dominants allowing the co-existence of less competitive populations (Weinbauer & Rassoulzadegan, 2004). Recently, a new model (Piggyback-the-Winner, PtW) has been proposed. It predicts that lytic dynamics can be suppressed at high host density and the lytic to temperate switching of viral communities is promoted (Knowles et al., 2016).

Recent studies reported that viral infection in deep-seabed can affect selectively Bacteria and Archaea, having, on average, a significantly higher impact on the Archaea component, especially on specific taxa belonging to MG-I Thaumarchaeota, the most represented archaeal group in surface deep-sea sediments, being one of the main drivers of chemoautotrophic production (Danovaro et al, 2016).

Virus-prokaryote interactions and their understanding are fundamental keys to comprehend the ecological processes that establish the basis for the sustainment of the ocean, even in its remote and unknown deep-sea systems.

Hadal trenches and microbial assemblages

The deepest areas of the ocean, commonly defined as ultra-abyssal or hadal zones (> 6500 m depth), represent ~1.5% of the global deep-sea floor (Belyaev, 1989). They are almost exclusively represented by trenches, which are one of the most remote and least explored ecosystems on Earth (Jamieson et al., 2010). To date 37 deep-sea trenches, 28 of which are localized in the Pacific Ocean

including the 9 deepest one (9-11 Km depth), have been discovered (Belyaev, 1989; Watling et al., 2013). Trenches origin at convergent margins, where one plate sub-ducts beneath another one (Orcutt et al. 2011). They are characterised by absence of light, high hydrostatic pressures (up to 1100 bar or approximately 1.1 tonnes per cm^2) and, regarding the Pacific trenches, temperatures ranging from ca. 1 to 2.5 °C (Jamieson, 2010). There are bottom currents that flow trough the trenches (e.g., up to 9.9 cm s^{-1} within the Izu-Ogasawara Trench and 8.1 cm s^{-1} within the Mariana Trench; Taira, 1987; Taira et al., 2004) exhibiting tidal cycles with semi-lunar and lunar periodicity, comparable to those observed on abyssal plains (Gould & McKee, 1973). Dissolved oxygen concentrations can change widely, not only among the different trenches (with the lowest reported from the Banda trench 2.03-2.38 ml l^{-1}), but also within the same trench as in the case of the Cayman and Puerto Rico trenches (from 4.9 to 6.9 ml l^{-1}, Belyaev, 1989). In general, trench habitats are thought to be food-rich but physically unstable compared to the most of the abyssal seafloor (Smith & Demopoulos, 2003). A number of investigations carried out in the Mariana, Atacama and the northern Japan trenches, provided evidence that these deep-sea habitats represent active bioreactors due to the elevated rates of deposition of organic matter of photoautotrophic origin resulting in high microbial remineralization rates (Danovaro et al., 2003; Oguri et al., 2013; Glud et al., 2013; Turnewitsch et al., 2014). However, the organic matter inputs can be extremely different between trenches located close to the continental shelves (e.g., Atacama Trench; Danovaro et al., 2003) and open-ocean trenches. Indeed, recent studies conducted in the Kermadec Trench (New Zealand), based on a mathematical model of gravitational lateral sediment transport, showed that the topographic characteristics of these hadal ecosystems may play a fundamental role in driving regional and local scale patterns in food availability and, as a consequence, in benthic biomass distribution in trench environment.

Past studies indicate prokaryotic abundances in the order of 10^6-10^8 cells cm^{-3} from superficial sediments of hadal trenches (Danovaro et al. 2003, Glud et al. 2013), abundance that falls in the range of values found in other deep-sea ecosystems (Wei et al., 2010; Middelboe et al 2006; Danovaro et al. 2005). Higher microbial biomass and more elevated diagenetic activity in hadal

trenches compared those of the abyssal plain were detected within the Japan, the Tonga and the Mariana trenches probably sustained by the higher food supply that characterizes these systems and also thanks to the presence of tectonic activity thought to be able to favour biological hot spots based on chemosynthetic communities (Wenzhöfer et al., 2016). In fact, trenches are regions of high tectonic activity and therefore possibly places able to support chemosynthesis where sulfide or methane escapes are present (Blankenship-Williams & Levin, 2009). Due to their isolation, distribution and geomorphology, trenches represent an important realm of speciation and may contribute significantly to deep-sea biodiversity (Levin and Dayton, 2009; Blankenship-Williams and Levin, 2009). These hadal systems potentially host endemism and species highly specialized in living in the extreme environmental conditions characterizing these habitats. Past studies of microbial isolates origin from the Mariana Trench observed microbial genetic adaptations to high-pressure conditions (Takami et al., 1997; Kato et al., 1997; Tamegai et al., 1997) as well as diverse and indigenous marine actinobacteria (Pathom-aree et al., 2006). León-zayas et al. (2015) revealed novel metabolic capabilities, including those associated with nitrogen, sulphur, carbon, and energy acquisition mechanisms, related with isolates from water samples and amphipods collected in the Puerto Rico Trench. Especially, they identified the potential for the isolated *Thaumarchaeota* to synthesize fatty acids and utilize alternate ways to recover ammonia, CO_2, and methyl groups. Despite their isolation, several studies suggest that part of the microbial assemblages inhabiting hadal trenches origin from other ocean habitats. Past analysis of psychrophilic bacteria isolated from the deep-sea water of the Japan Trench identified the same phylogenetic and phenotypic group of bacteria dominated in extremely cold and remote environments such as Antarctic surface waters, hypothesizing that such microorganisms originate in polar regions and are transported by cooled seawater and/or fecal pellets driven by deep-current directed toward this trench (Maruyama et al., 2000).

The hypothesis that hadal trenches may host microorganisms supplied from other habitats was also sustained by Takai et al. (1999) that isolated a novel extremely thermophilic bacterium from the Mariana Trench sediments at 10897 m depth, named *Thermaerobacter marianensis,* thought to

have originated from shallow marine hydrothermal environments. A recent study on bacterial diversity inhabiting the first 10 cm of sediment of the Mariana Trench bottom revealed a predominance of Archaea Marine Group I, Planctomycetes, Chloroflexi and Firmicutes (Yoshida et al., 2013). However, studies on prokaryotic diversity in hadal trenches are mainly limited to the water column, leading to a constant lack of knowledge on benthic microbial communities. The predominance of Gammaproteobacteria, Bacteroidetes, SAR406, Planctomycetes and Thaumarchaeota in water within the Mariana and the Japan trenches was recently described (Nunoura et al., 2015; Nunoura et al., 2016). Bottom water samples within the Challenger Deep, 1 m above the seafloor, were also collected by Tarn et al. (2016) and sequence-based analysis were carried out observing that the most abundant microbial groups belonged to Gammaproteobacteria. They found also Alpha-, Beta- and Deltaproteobacteria, Chlorobi, Atribacteria, Chloroflexi, Marinimicrobia, Cyanobacteria, Gemmatimonadetes and Nitrospirae, as well as previously cultured piezophiles such as *Colwellia piezophila* and *Moritella abyssi*. Archaea were dominated by Nitrosopumilus, methane-oxidizing archaea (ANME-1 and 2D), Marine Group II and III (Euryarchaeota) and Marine Benthic Group A.

Conversely, data on trench virome are very scant. Benthic viruses were detected to be relatively low in the Izu-Ogasawara and Mariana trenches since they ranged from 10^6 to 10^7 viruses cm^{-3} (Yoshida et al., 2013). It was detected the predominance of ssDNA viruses belonged to the Circo-Nano and Microviridae groups and it was observed the presence of novel viromes, distinct from the viral genotypes previously identified in ocean environments.

If data on distribution, activity and diversity of hadal microbial assemblages are highly limited, information on virus-prokaryote interactions are completely absent. Because of the fundamental importance of such interactions in deep-sea benthic ecosystems, and the potential novel prokaryotic diversity that may be hidden in hadal trenches seabed, it is necessary to deepen the knowledge on microbial assemblages living the ocean's deepest environments.

Deep-sea mining impact on the seafloor assemblages

Benthic deep-sea ecosystems can be highly vulnerable to anthropogenic impacts including trawling activities, exploitation of resources as methane hydrates and polymetallic nodules, and spill of contaminants and wastes of different nature (Pusceddu et al. 2014, Thiel, 2003 Ramirez-Llodra, 2011). Increasing evidence suggests that the exploitation of mineral resources will be in the near future one of the main threats of deep-sea habitats. In any given year, deep-sea mining could disrupt seafloor communities over areas of 600 to 8,000 km^2, and 15 years of such activity could conceivably affect 120,000 km^2 of seafloor (Smith et al., 2009). High coverage of nodules was recorded in five main ocean regions: between the Clarion and Clipperton Fracture Zones (CCFZ) in the equatorial North Pacific and extending westwards into the northern sector of the Central Pacific Basin, in the abyssal plain area around the Musicians Seamounts in the Northeast Pacific Basin, in the central sector of the Southwestern Pacific Basin, in an E-W trending belt in the Southern Ocean coincident with the Antarctic Convergence, and in the northern sector of the Peru Basin (Glasby, 2006).

Manganese nodules are polymetallic concretions consisting mainly of manganese (26–30%) and iron (6–7%), but also including economically significant amounts (3% combined) of nickel, copper, cobalt, lithium, molybdenum, zirconium, and rare earth elements (Cronan, 1980; Halbach et al., 1988; Rühlemann et al., 2011; Hein et al., 2010, 2013; Mewes et al., 2014). They are end products of chemical precipitation of dissolved Fe and Mn within the marine bottom sediments under oxic or suboxic conditions, which is followed by the incorporation of other metals thanks to the involvement of microbial processes (Wang et al., 2009; Somayajulu, 2000).

Different environmental programs, such as DOMES (Deep Ocean Mining Environmental Study) and MESEDA (Metalliferous Sediments Atlantis II Deep), have been developed since the 1970s in order to study the possible impacts of mining activity on the seabed and the inhabiting communities trying to establish the risk assessments of this exploitation. In fact, manganese nodules can be used as substrate from benthic organisms for their growth, protection and grazing (Mullineaux, 1987,

Veillet et al., 2007, Veillet et al., 2007), therefore their removal represents an un-repairable threat for deep-sea habitats (Oebius et al., 2001). Not only the removal of the nodules but also the destruction of the seabed can lead to biodiversity loss. Past experiments of simulated mining activity revealed an intense decrease of benthic assemblages inhabiting the surface bottom sediments after the mechanical impact of mining devices (Thiel, 2001; Ingole et al., 2001), as also data obtained from a long-term *Dis*turbance and Re*col*onisation experiment (DISCOL) in the deep Peru Basin demonstrated that meio-, macro- and megafauna community structure were still disturbed also several years after the impact with irregular distribution of species and different diversity patterns compared to the pre-impact condition (Borowski & Thiel, 1998; Borowski, 2001; Bluhm, 2001). Deep-sea mining causes intense sediment resuspension and resedimentation processes other than seabed destruction and dredging. Past experiments to evaluate the impact of such processes on benthic assemblages observed a stronger effect of the forming sediment plume on deposit feeders than on suspension feeders and omnivores (Shirayama, 1999), results that confirm the inevitable shift in species diversity of the impacted communities. Other investigations indicated that phenomena of re-colonisation of species and re-establishment of balanced communities, even though over long time periods, are reasonably foreseen, but the perspective is that the new stable community will be differently structured from the one existing before the collection of the nodules (Thiel & Tiefsee-Umweltschutz, 2001). In addition, there is proof that the impact of mining activity implies also severe modifications of the chemical gradient and of the geochemical structure of seabed sediments that seem not to be restored by any diffusive or bioturbation processes even for long and unknown period of time (Khripounoff et al. 2006). Similar impacts can be caused by a more studied human activity, the deep-sea trawling, which can be considered for comparison to foresee the possible consequences of mining disturbances on benthic habitats. Pusceddu et al. (2014) revealed lower organic C turnover and significant depletion of organic matter in deep-sea trawled sediments with important effects on food availability for benthos and severe implications for the key ecosystem function of C cycling. Conversely, Raghukumar et al. (2001) observed an increase in total organic carbon content after a simulated mining activity through the use of a hydraulic device, probably due to the strong bioturbation

involving the subsuperficial sediment layers, and hypothesized the benefit of such increase for meio- and macrobenthos biomass production.

On the overall, the impact of anthropogenic activities on marine ecosystems has been investigated by assessing changes in different physical-chemical variables and biological components including mega, macro, and meiofauna (Kaneko & Maejima, 1997; Thiel & Tiefsee-Umweltschutz, 2001; Lodge et al., 2014), while the microbial component has often been neglected despite potentially affected by such activities (Nogales et al., 2011). Few studies on the impact of mining activity on microbial assemblages observed a drastic reduction of bacterial number after the nodule removal (Raghukumar et al., 2001; Raghukumar et al., 2006).

Data on prokaryotic diversity in the sediments rich in polymetallic nodules (e.g., containing Mn and Fe) were carried out especially for commercial and technological purposes in order to highlight the diversity of bacteria associated with metals and involved in the sulphur and nitrogen cycling (Wang et al., 2010). Unique microbial assemblages living within the nodules were indeed identified, distinct from those in the surrounding sediments and possibly involved in the formation and/or growth of nodules (Wu et al., 2013).

Despite the increased knowledge acquired during the last fifty years and addressed to assess the impact of mining activity on deep-seas, information on the effects of such human exploitation on virus-prokaryote interactions and on microbial assemblages diversity and structure changes are completely lacking. Their investigation is crucial for a better comprehension of the consequences of such anthropogenic impact on the benthic food webs and biogeochemical cycles of deep-sea ecosystems.

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Chapter 1.

Impact of viral infection on prokaryotic assemblages in hadal benthic ecosystems of the Pacific Ocean

1.1 Introduction

The deepest areas of the ocean, commonly defined as ultra-abyssal or hadal zones (> 6500 m depth), represent ~1.5% of the global deep-sea floor (Belyaev, 1989). They are almost exclusively represented by trenches, which being habitats characterized by extreme pressures, low temperature, and geographic isolation (Lauro & Bartlett, 2008; Nunoura et al., 2015), are one of the most remote and least explored ecosystems on Earth (Jamieson et al., 2010). To date 37 deep-sea trenches have been discovered, 28 of which are localized in the Pacific Ocean including the 9 deepest ones (9-11 Km depth), (Belyaev, 1989; Watling et al., 2013).

Previous studies suggested that deep-sea trenches represent traps of organic matter supporting high benthic biomass and highly active microbial communities (Danovaro et al. 2003; Glud et al. 2013; Ichino et al. 2015).

Benthic prokaryotic abundances from Pacific hadal trenches have been reported to be in the order of 10^6-10^8 cells cm^{-3} of surface sediment (Danovaro et al. 2003, Glud et al. 2013, Wenzhöfer et al., 2016) falling in the range of values found in other deep-sea ecosystems (Wei et al., 2010; Middelboe et al 2006; Danovaro et al. 2005).

Despite there is evidence that deep-sea microbes (prokaryotes and viruses) represent 50-80% of the total microbial biomass and 10-30% of the total living biomass of the Earth (Whitman et al., 1998; Corinaldesi, 2015), their dynamics and diversity in these extreme systems are still largely

unknown. Even less information are available dealing with the role of viruses in such ecosystems and their interactions with prokaryotic hosts.

Previous studies have revealed that beneath a depth of 1000 m viral infections are responsible for the abatement of 80% of prokaryotic heterotrophic production, increasing with water depth and releasing on a global scale ≈0.37-0.63 Gtonnes of carbon per year, essentially contributing to the functioning of the largest ecosystem on Earth (Danovaro et al., 2008). However, benthic viral abundances reported so far from the Izu-Ogasawara and Mariana Trenches are relatively low since these ranged from 10^6 to 10^7 viruses cm^{-3} (Yoshida et al., 2013), leading to hypothesize a low control on their hosts.

This study aimed at analysing the distribution of prokaryotes and viruses in surface sediments of three different North-Pacific trenches: Japan Trench, Izu-Ogasawara Trench, and Mariana Trench, and their interactions in relation with the environmental and trophic characteristics and the implications of viral infection on the functioning of the benthic food webs and biogeochemical processes of these extreme ultra-abyssal systems.

1.2 Materials and Methods

1.2.1 Site description and sampling

Our study was focused on three hadal trenches located in the Northwest-Pacific Ocean: Japan, Izu-Ogasawara, and Mariana Trenches. The Japan Trench has a maximum depth of approximately 8000 m (Nunoura et al., 2016) and it is 800 km in length. This is the northernmost trench among the three considered in this study and is subjected to high accumulation of sedimentary material also of terrestrial origin being located under relatively high productive surface waters and near the land (Lutz et al, 2007; Watling et al., 2013; Turnewitsch et al, 2014). The Izu-Ogasawara Trench presents

a bottom depth that exceeds the 9700 m and extends from a mesotrophic area to the northernmost section of the Mariana Trench, which instead is located under oligotrophic surface waters (Wenzhöfer et al., 2016). The Mariana Trench is long 2550 km and reaches its known maximum depth of ca. 11.03 km at the Challenger Deep, a small slot-shaped valley in its southernmost end and the deepest point on Earth (Pathom-aree et al., 2006). Despite the different productivity that characterized the areas under which these three trenches lie, food availability in hadal systems is thought to be influenced by the topographically driven concentration of organic matter (Ichino et al., 2015; Leduc et al., 2015). In fact, a lateral downslope transport of sedimentary material in addition to the vertical primary flux from the overlying water column has been reported in all of these trenches (Turnewitsch et al., 2014), which are also characterized by cyclonic currents of deep waters flowing northward (Mantyla & Reid, 1983; Johnson, 1998; Fujio et al., 2000). As usually hadal systems are known to be, these trenches are subjected to frequent earthquakes (Hashimoto et al., 2009; Oguri et al., 2013) leading to occasional gravity-driven sediment slides.

Surface sediment samples (n=3) were collected by using a gravity corer with the Lander system of the ROV *ABISMO* within the Izu-Ogasawara Trench at 9776 m depth (29°09.00' N, 142°48.12' E) and in the adjacent abyssal plain at 5747 m depth (29°16.79' N, 143°46.04' E) during the KR11-11 cruise on board of the research vessel Kairei in collaboration of the Japan Agency for Marine-Earth Science & Technology (JAMSTEC) in December 2011 (Figure 1). Sediment samples (n=3) were also collected in the Japan Trench at 8000 m depth (36°04.00' N, 142°44.00' E) during the KR12-19 cruise on board of the R/V Kairei (December 2012) and in the abyssal site used as a control (5253 m depth, 39°00.0' N, 146°00.1' E). Finally, sediment samples (n=3) were collected within the Challenger Deep of the Mariana Trench at 10901 m depth (11°22.0533' N, 142°25.4486' E) and in the surrounding abyssal plain (4700 m depth, 10°17.9803' N, 142°36.0157' E), during the KR14-01 cruise on board of the R/V Kairei (January 2014). Sub-aliquots of the sediment surface were used to carry out the analysis of the biochemical composition of the organic matter and microbiological parameters. Immediately after the sediments collection, the samples for analyses of the trophic parameters (in terms of proteins, carbohydrates, lipids and total phytopigments) were frozen and

stored at -20°C. For the analyses of prokaryotic abundances, samples were fixed with buffered 2% formalin and stored at 4°C until processed. Viral production experiment was set up and processed immediately after the collection of the sediments, and samples for analysing viral abundances were stored at -20° without the addition of any preservative. All analyses were carried out on the top 0-2 cm of sediment from each sediment core in 3 sub-replicates.

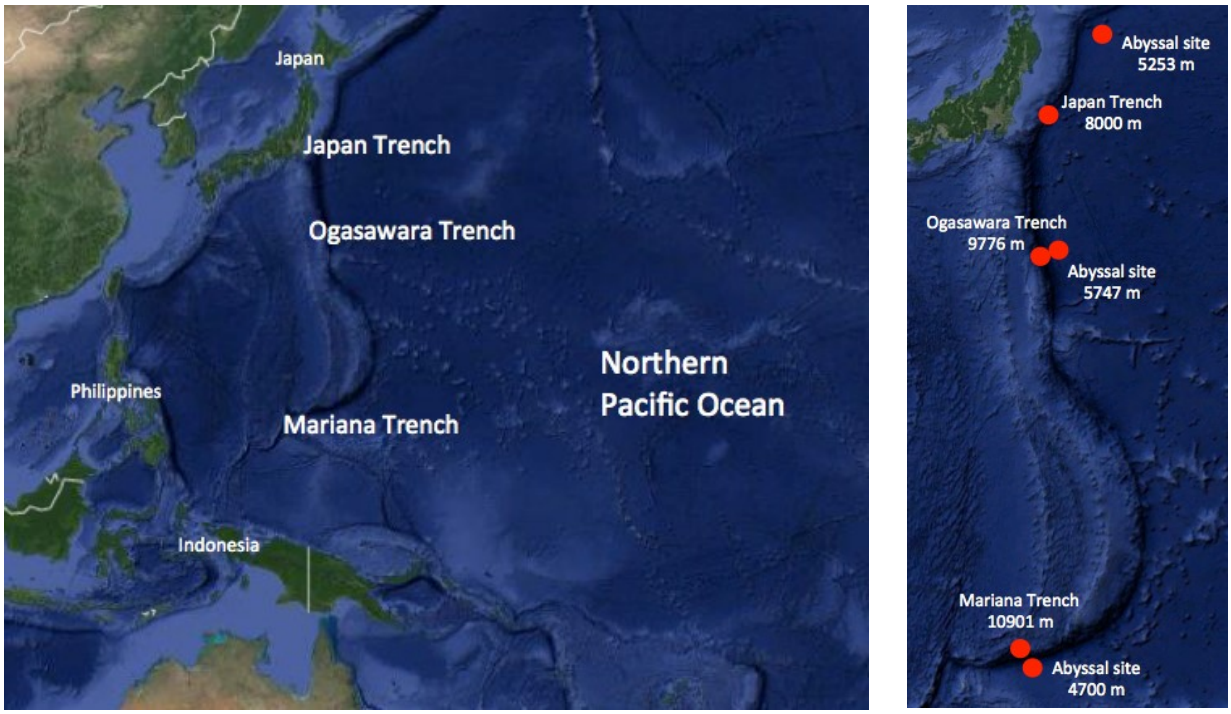

Fig. 1 Sampling sites

1.2.2 Sedimentary organic matter

Protein content (PRT) was determined according to Hartree (1972) modified by Rice (1982) to compensate for phenol interference. Concentrations were calculated from calibration curves of standard solutions of BSA, normalized to sediment dry weight and expressed as milligram of albumin equivalents per gram of dry sediment. Carbohydrates (CHOs) were analysed according to Gerchacov and Hachter (1972) and expressed as glucose equivalents normalized to sediment dry weight. The method is based on the same principle as the widely used method of Dubois et al. (1956), but it is specifically adapted for carbohydrate determination in sediments. Lipids (LIPs) were extracted by direct elution with chloroform and methanol and analysed according to Marsh and Wenstein (1966). Lipid concentrations were reported as tripalmitine equivalents normalized

to sediment dry weight. For each biochemical analysis, blanks were made with the same sediment samples as previously treated in a muffle furnace (450°C, 2 h). The sum of the carbohydrate, protein and lipid concentrations converted into carbon equivalents (using the conversion factors of 0.40, 0.49 and 0.75 mg C mg^{-1}, respectively) was defined as the biopolymeric organic carbon (Pusceddu et al, 2009). With the exception of Ogasawara Trench and it related abyssal site samples, due to the limited amount of available sediment, chloroplastic pigments (chlorophyll-a and phaeopigments) were analysed fluorometrically according to Lorenzen and Jeffrey (1980). Pigments were extracted with 90% acetone (24 h in the dark at 4°C). After centrifugation (800 X g), the supernatant was used to determine the functional chlorophyll-a and acidified with 0.1 N HCl to estimate the amount of phaeopigments. Total phytopigment concentrations (CPEs) were defined as the sum of chlorophyll-a and phaeopigment concentrations (Danovaro et al., 1999a,b).

1.2.3 Prokaryotic abundance and biomass

For prokaryotic counting, *ca.* 1 cm^3 of sediment from the top 0-2 cm of each sediment core were analysed as previously described by Danovaro et *al.* (2002). Benthic bacteria were detached from sediment by using pyrophosphate at a final concentration of 5 mM, and were sonicated by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W) to increase the extraction efficiency. For bacterial counting, subsamples were diluted 50-fold with sterilized 0.2 µm-filtered seawater and stained with Acridine Orange (final concentration, 0.01%). After the incubation at the dark, the samples were filtered onto 0.2-µm-pore-size black Nuclepore filters with vacuum pressure of 150 mm of Hg by using sterile glass filter holders for 25 mm diameter filters with 15 ml glass funnel (Millipore). Acridine Orange was used instead of SYBR Green I for bacterial analysis because it provided similar counts but did not result in the overestimation of biovolume. All filters were analysed by epifluorescence microscopy using a Nikon ECLIPSE Ni-U microscope equipped with a micrometric scale to examine the cell dimensions and estimate the biovolume. Ten to 50 fields were viewed at a magnification of X1000, and a minimum of 400 bacterial cells was counted. The prokaryotic abundance was calculated

considering the optical field coefficient (filtration area/counting area), the extraction coefficient (1.44), and the dilution factor and it was expressed as viruses per g dry sediment after desiccation at 60°C for 24 h. Bacterial biovolume, expressed as μm^3 $cell^{-1}$, was measured on the base of three different sizes, small (<0.5 μm), medium (0.5 < x < 1.0 μm), and large (≥ 1 μm), and considering each cell like a sphere [(4/3) $r^3 \times \pi$]. The biovolume was converted to carbon content (bacterial biomass) by using a conversion factor of 310 fg of C μm^{-3} (J. C. Fry, 1990).

1.2.4 Extracellular enzymatic activities

Analyses of l-aminopeptidase, β-d-glucosidase, and alkaline-phosphatase activity were carried out on sediment sub-samples (n = 3). Sediment slurries (prepared with pre-filtered sterilized seawater, 1:1 v/v) were incubated for 2 h (enzymatic activity increased linearly with time) in the dark and at in situ temperature with l-leucine-4-methylcoumarinyl-7-amide (MCA), 4- methylumbelliferone β-d-glucopyranoside (β-Glu), and 4-methylumbelliferone phosphate (MUF-P) respectively as substrates (final concentrations of MCA 200 μM, MUF 200 μM, and MUF-P 50 μM determined after kinetic experiments). Sediment sub-samples incubated without fluorogenic substrates were used as blanks and processed according to the procedure followed for fresh sediment samples. After incubation, samples were centrifuged (800X g) and the supernatants were analysed fluorometrically according to Hoppe (1993). Enzymatic activities were expressed as nmol of substrates hydrolysed in one hour and normalized to sediment dry weight. The ratio between enzymatic activities and prokaryotic abundances leaded to estimate the specific degradation activity per cell that were expressed as nmol of substrate hydrolysed in one hour normalized to sediment dry weight per cell.

1.2.5 Viral abundance

Viral counting was carried out on *ca.* 1 cm^3 of sediment from the top 0-2 cm of each sediment core as described by Danovaro et *al.* (2001). Sediment sub-samples were diluted in a final volume of 5 ml of virus-free seawater filtered at 0.02 μm pore size using disposable syringe filters (Anotop 25, Whatman), and treated with pyrophosphate at a final concentration of 5mM. Samples were sonicated by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W) to increase the extraction yield. The samples were then diluted 25-fold in virus-free seawater and incubated with the addition of DNase I from bovine pancreas (2-5 Uml^{-1}) in order to eliminate uncertainties in virus counting due to extracellular DNA interference. After the incubation, samples were filtered onto 0.02 μm pore size filters (Anodisc Al^2 O^3; 25mm diameter, Whatman) with vacuum pressure of 150 mm of Hg by using sterile glass filter holders for 25 mm diameter filters with 15 ml glass funnel (Millipore). Then the filters were stained using SYBR Gold (10000X concentrated in anhydrous dimethyl sulphoxide; Molecular Probes-Invitrogen, Grand Island, NY, USA) at a final concentration of 2X. SYBR Gold was used instead of SYBR Green I to detect both double- and single-stranded DNA and RNA viruses, and because its strong brightness and stability as fluorescence stain. Viral counting was performed under epifluorescence microscopy, using a Nikon ECLIPSE Ni-U microscope, by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. The viral abundance was calculated considering the optical field coefficient (filtration area/counting area), the extraction coefficient (1.66), and the dilution factor, and it was expressed as viruses per g dry sediment after desiccation at 60°C for 24 h.

1.2.6 Viral production, virus-induced prokaryotic mortality (VIPM) and turnover

Viral production was analysed as previously described by Dell'Anno et *al.* (2009). Sub-replicate sediment samples were transferred into sterile tubes and mixed with an equal volume of 0.02 μm pre-filtered virus-free seawater. Aliquots of 0.5 ml were collected at 0, 3, 6 and 12 hours. After the

extraction of viruses from each sediment samples as described above, viral counting was performed under epifluorescence microscopy by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. The increment in virus number was determined calculating the difference between the maximum viral abundance value, considering the abundances of the samples of all the incubation times, and the viral abundance at the t 0h (VA t_n - VA t_0) normalized to the number of hours of incubation.

The number of prokaryotes killed by viruses was estimated from the ratio between viral production and burst size, which were calculated on the base the equation: BS = 30.2 + (261.4 × cell biovolume μm^3; Weinbauer & Hofle, 1998). VIPM was estimated as the ratio between the number of prokaryotes killed by viruses and the total number of prokaryotes present in the samples and expressed as percentage. Viral turnover was calculated as the ratio between viral production and abundance and expressed as turnover time (h).

1.2.7 Carbon contribution to microbial C cycle due to viral shunt

The concentration of C released from the infected cells due to viral lysis in each sediment sample (expressed as mg C m^{-2} d^{-1}) was calculated by multiplying the cell biomass for the number of cells killed per hour, which was deducted by the ratio between viral production and burst size. The potential contribution of the C released by host cells due to viral lysis to the prokaryotic C degraded was obtained by values of extracellular aminopeptidase and β-glucosidase activities converted in C equivalents degraded per m^{-2} per day (Hoppe et al., 1988).

1.2.8 Statistics

A one-way univariate analysis of permutational variance (ANOVA) was applied to assess differences between sampling stations (6 levels) and each analysed variable by using the PRIMER 6+ software.

1.3 Results

1.3.1 Biochemical composition of organic matter content and photosynthetic pigments

The results of the biochemical composition of organic matter in the surface sediments of the investigated sites are shown in Figure 2a. Protein concentrations ranged from 0.19±0.08 to 9.51±0.77 mg g^{-1} of dry sediment, respectively in the abyssal site related with the Mariana Trench and in the Japan Trench. Carbohydrate concentrations ranged from 1.02±0.22 to 4.60±0.33 mg g^{-1} (in the abyssal site close to the Mariana Trench and the Japan Trench respectively), while lipid concentrations from 0.11±0.03 to 2.48±0.55 mg g^{-1} (in the abyssal site related with Ogasawara Trench and within the Japan Trench, respectively).

The Japan Trench bottom sediments presented significantly higher protein and lipid contents ($P<0.05$) compared to the Ogasawara and Mariana trenches, exceeding their concentration 7-19 and 6-3 times respectively. Ogasawara Trench presented the highest carbohydrate concentrations (2.63±0.03 mg g^{-1}) among the three trenches. Both protein and lipid contents were significantly higher within the three trenches if compared with their related abyssal sites, while carbohydrates concentration was double in the abyssal plain respect to the Japan Trench, and between the Mariana Trench and its related abyssal site there was not a significant difference (1.03±0.11 and 1.02±0.22 mg g^{-1} respectively). Ogasawara Trench, instead, showed significantly higher carbohydrates concentrations compared to its relative abyssal site.

Biopolymeric carbon contents (Figure 2b) ranged from 0.56±0.14 to 7.47±0.82 mg g^{-1} with significantly different values among the trenches (the Japan Trench exceeding 3 and 5 times the concentrations that were present in Ogasawara and Mariana trenches, respectively) but higher when compared to their related abyssal sites. In Figure 2c the contributions of the different biogeochemical components of organic matter to biopolimeric carbon are showed. The total phytopigments were significantly higher in the Japan Trench (269.32±13.81 μg g^{-1}) than both the Mariana Trench (6.83±0.25 μg g^{-1}) and the related abyssal site (84.09±11.43 μg g^{-1} Figure 3a). In all

sites the contribution of chlorophyll-a to the CPE was almost negligible (ranged: 1-3%; Figure 3b). CPE concentrations for Ogasawara Trench are not available.

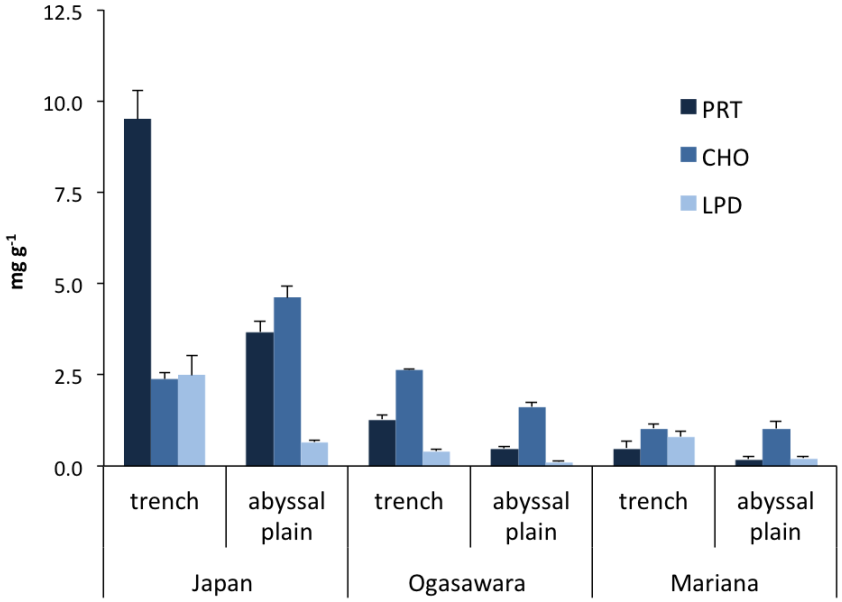

Fig.2a Organic matter content

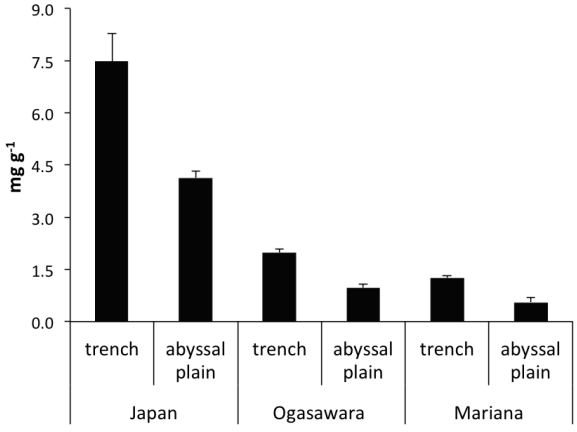

Fig.2b Biopolymeric C content (BPC)

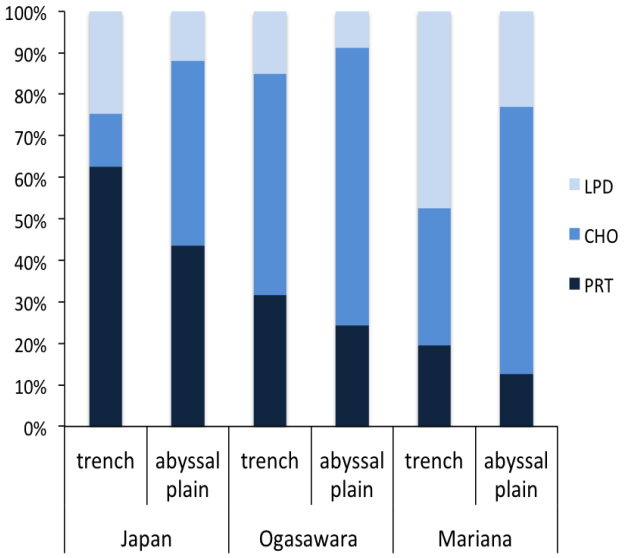

Fig.2c Percentage contribution of each biogeochemical component of organic matter to BPC

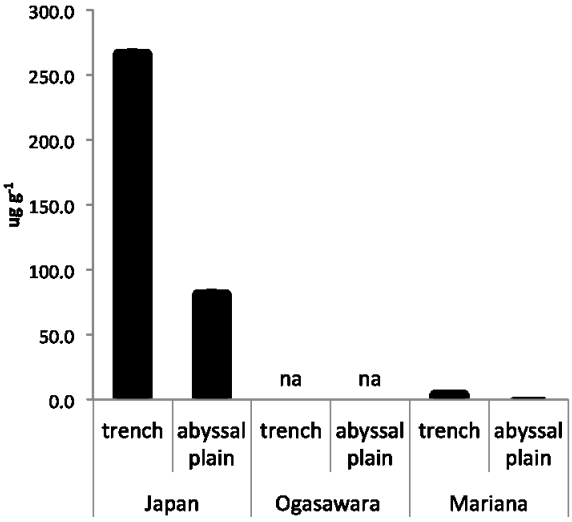

Fig. 3a Total phytopigments content

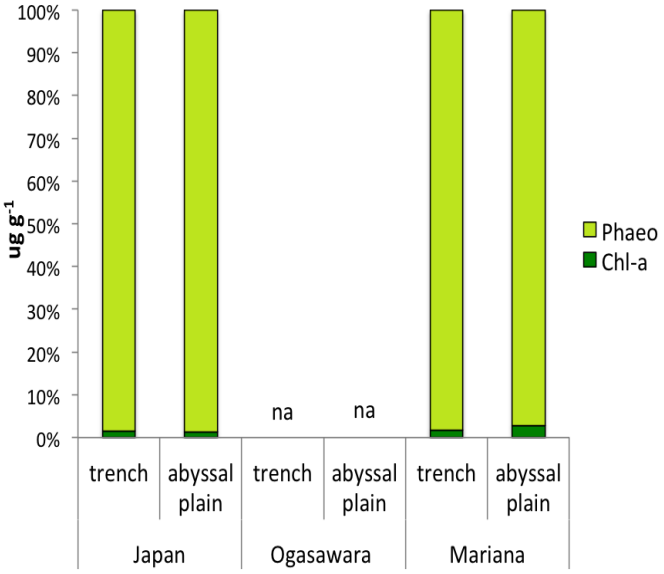

Fig. 3b Chl-a and phaeopigment contribution to total CPE

1.3.2 Prokaryotic abundance and biomass

In general, the prokaryotic abundances were significantly higher ($P<0.05$) within the trenches than in their related abyssal sites, with the highest values in Ogasawara Trench bottom sediments ($2.31\pm0.17 \times 10^8$ cells g^{-1}), followed by those within the Japan and the Mariana trenches ($8.02\pm0.8 \times 10^7$ cells g^{-1} and $4.16\pm0.7 \times 10^7$ respectively; Figure 4a).

Prokaryotic biomasses followed the same pattern, but in this case the highest value was found within the Japan Trench (5.39 ± 0.4 µg C g^{-1}), followed by the Ogasawara and the Mariana Trench (3.3 ± 0.13 and 2.01 ± 0.17 µg C g^{-1}; Figure 4b).

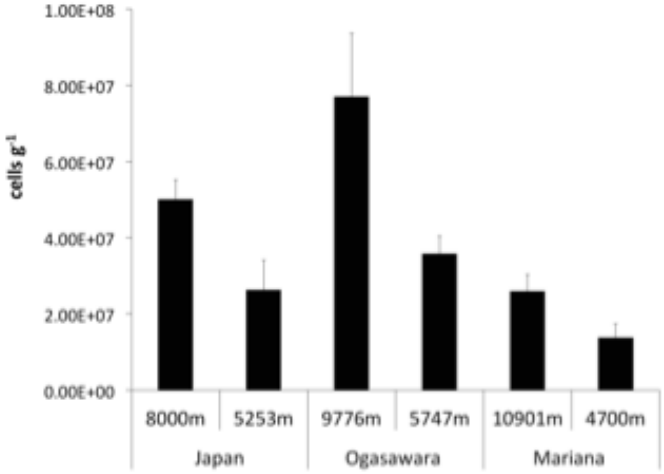

Fig.4a Prokaryotic abundance

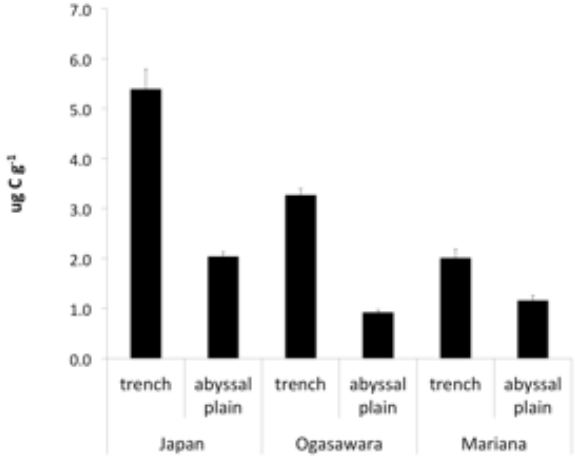

Fig.4b Prokaryotic biomass

1.3.3 Extracellular enzymatic activity and efficiency

Aminopeptidase activity ranged from 2±0.23 to 433.2±54.7 nmol h^{-1} g^{-1} (within the Japan Trench and in its control site, respectively), while alkaline-phosphatase and β-glucosidase activity ranged from 1.50±0.19 to 109.6±10.1 nmol h^{-1} g^{-1} and from 0.02±0.01 to 0.89±0.15 nmol h^{-1} g^{-1} in the abyssal sites surrounding Ogasawara and Japan Trench (Figure 5a-b-c). The Mariana Trench presented significantly ($P<0.05$) higher values for each enzymatic activity compared to the other two trenches. Each trench showed significantly higher enzymatic activities compared to their related abyssal sites with the exception of the Japan Trench. The aminopeptidase activity per cell ranged from 2.49±0.29 × 10^{-8} to 1.02±0.13 × 10^{-5} nmol h^{-1} g^{-1} $cell^{-1}$ in the Japan Trench and its control site, the β-glucosidase activity per cell between 6.1±0.1 × 10^{-10} and 8.39±0.06 × 10^{-8} nmol h^{-1} g^{-1} $cell^{-1}$ in the Ogasawara Trench and the abyssal site related with the Mariana Trench, and the alkaline phosphatase activity per cell ranged between 1.23±0.13 × 10^{-8} and 2.59±0.24 × 10^{-6} nmol h^{-1} g^{-1} $cell^{-1}$ in the Ogasawara Trench and in the abyssal site related with the Japan Trench (Figure 6a-b-c). The specific enzymatic activities were always lower in the hadal trenches than in the abyssal plain sediments with the exception of the aminopeptidase in the Ogasawara Trench.

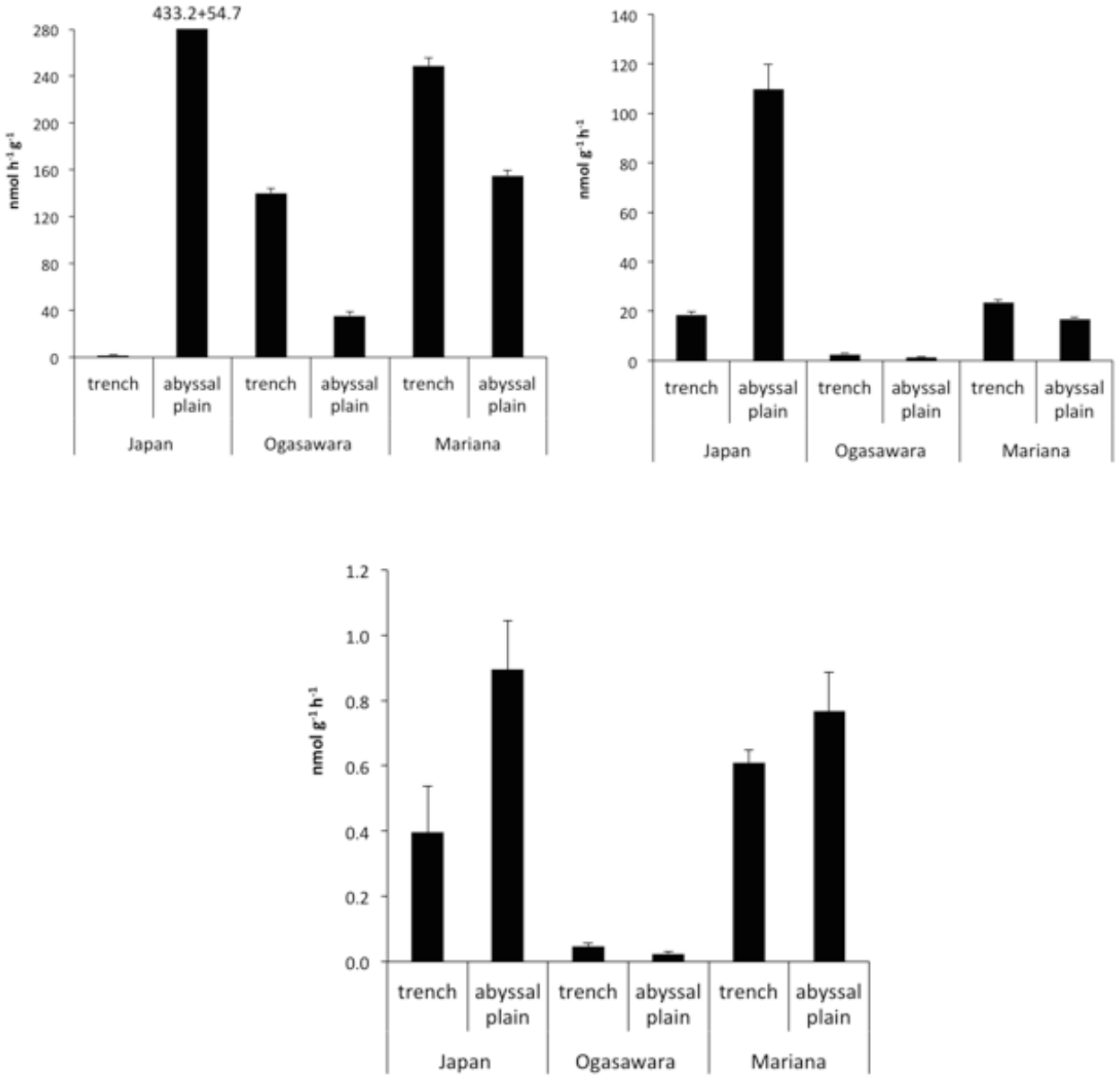

Fig.5 Extracellular enzymatic activity: aminopeptidase; alkaline phosphatase; β-glucosidase activity

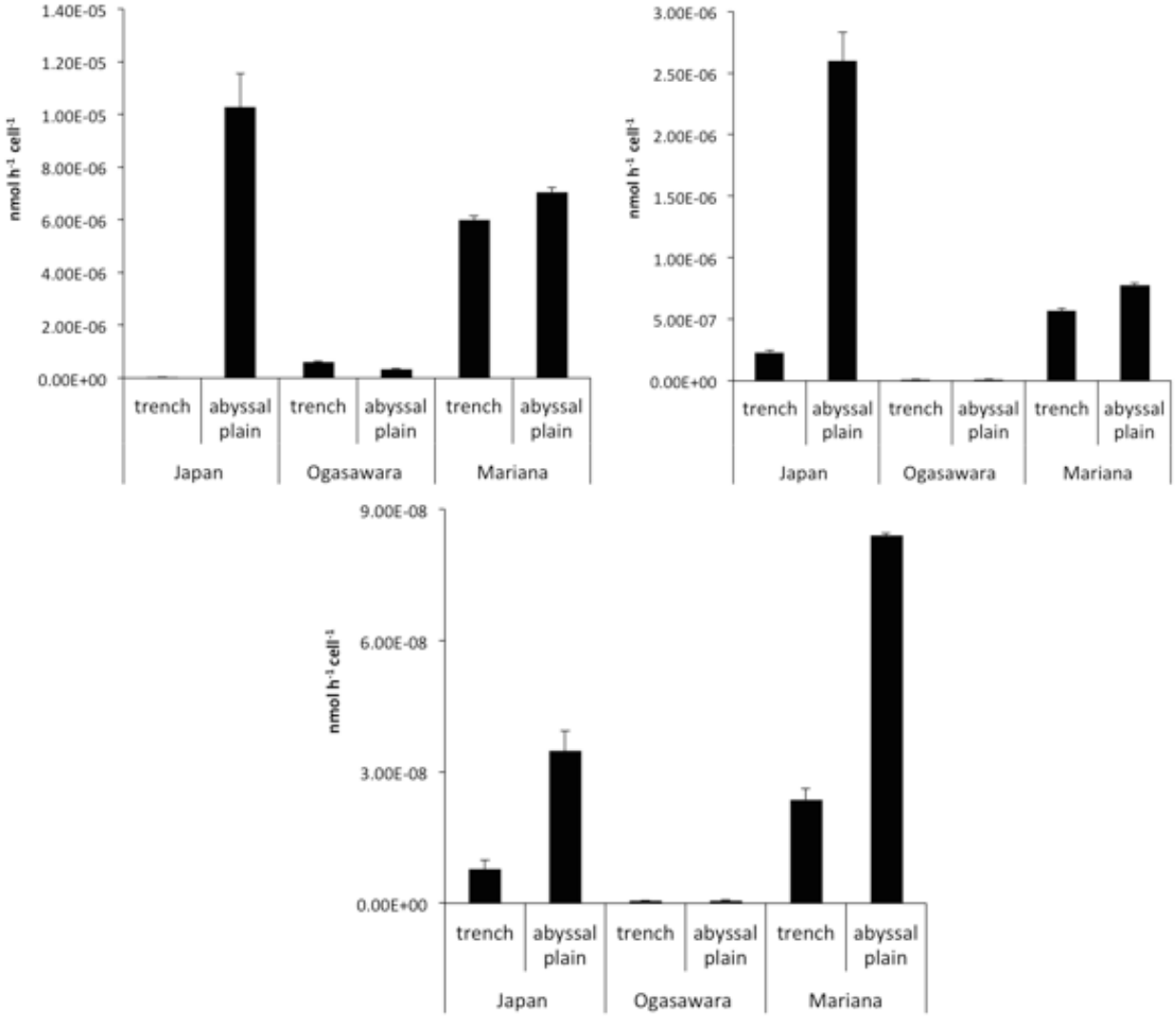

Fig.6 Extracellular enzymatic activity per cell: aminopeptidase; alkaline phosphatase; β-glucosidase

1.3.4 Viral abundance and virus to prokaryote ratio

Viral abundances did not show a clear pattern of distribution across the investigated sites. These ranged from $5.7\pm0.23 \times 10^7$ viruses g^{-1} to $5.8\pm0.11 \times 10^8$ viruses g^{-1} in the Mariana and Ogasawara trenches, respectively (Figure 7a). Among the three trenches, the number of viruses varied significantly. No significant differences were found between viral abundances in the hadal

sediments of the Japan Trench and its related abyssal site, whereas differences were found between the values of the Ogasawara and Mariana trenches and those of the abyssal sediments ($P<0.05$). In particular, within the Ogasawara Trench the number of viruses was significantly higher, while in the Mariana Trench was the opposite. The virus to prokaryote abundance ratio ranged from 0.9 to 9.8 (in the abyssal sites near Ogasawara and Mariana trenches, respectively), with lower values within the trenches than in the respective abyssal sites, with the exception of the Ogasawara Trench (Figure 7b).

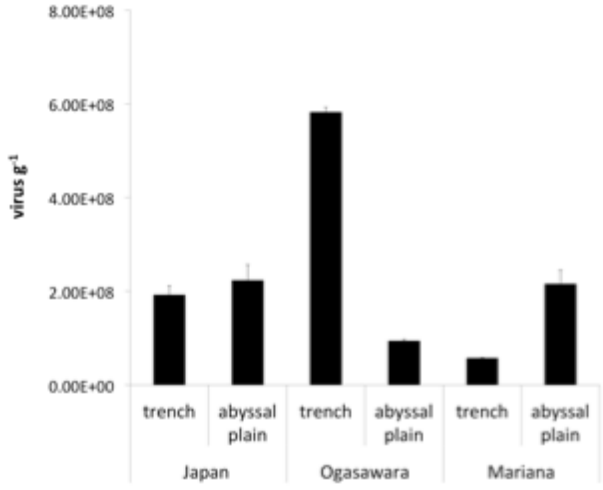

Fig.7a Viral abundance

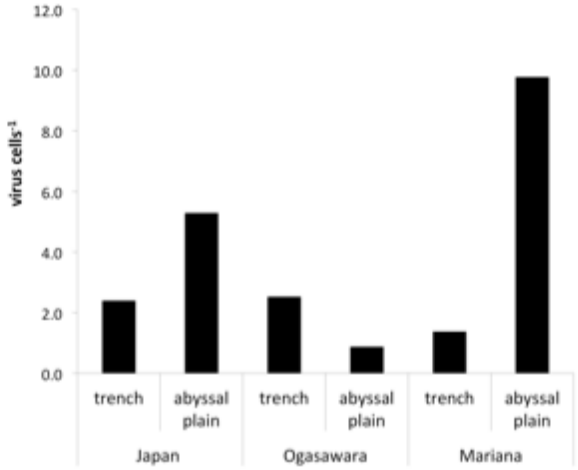

Fig.7b Virus to prokaryote ratio

1.3.5 Viral production and turnover

Benthic viral production ranged from $9.45\pm0.6 \times 10^6$ to $1.94\pm0.76 \times 10^8$ viruses $g^{-1}\,h^{-1}$ in the abyssal site related with Ogasawara Trench and in the Japan Trench, respectively, and showed a significant correlation with prokaryotic biomass (Figure 8c). Viral production among the three trenches was always significantly different with the lower value detected in the Mariana Trench ($3.72\pm0.67 \times 10^7$ viruses $g^{-1}\,h^{-1}$). Overall, viral production was always higher within the trenches than in their relative abyssal sites, but only in Ogasawara Trench differences were significant (Figure 8a). The viral turnover rate varied from 0.1 to 1.01 h^{-1} (in the abyssal site related with Ogasawara Trench and

within the Japan Trench, respectively) with higher replication rates within the trenches particularly in Ogasawara and Mariana Trench (Figure 8b).

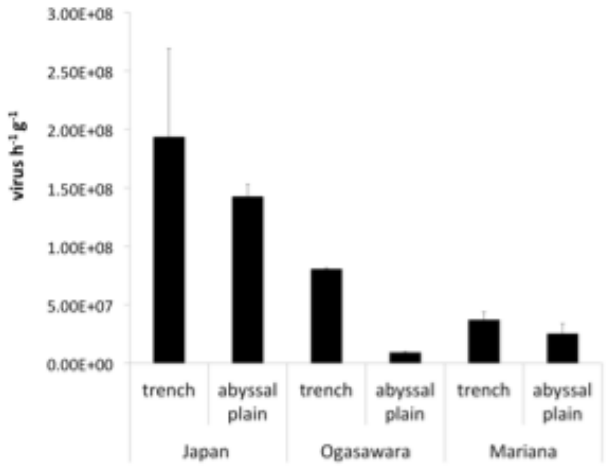

Fig.8a Viral production

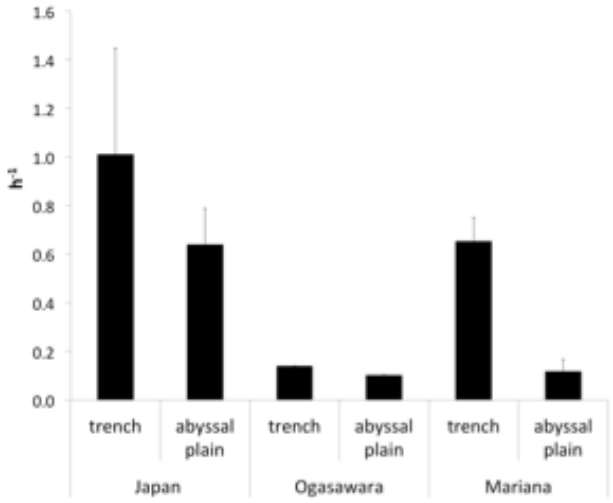

Fig.8b Viral turnover rate

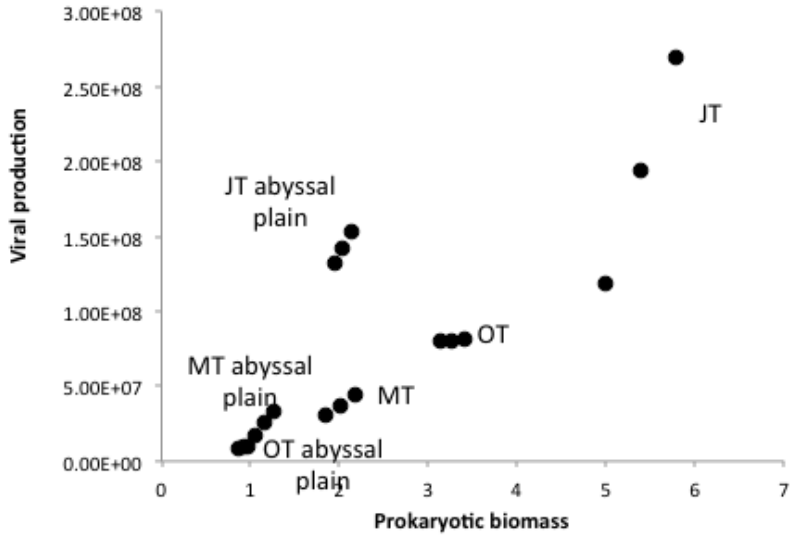

Fig. 8c Relation between prokaryotic biomass and viral production in each sampling site (R = 0.81; P<0.01). JT = Japan Trench; OT = Ogasawara Trench; MT = Mariana Trench.

1.3.6 Virus-induced prokaryotic mortality and C released due to viral shunt

The virus-induced prokaryotic mortality (VIPM) presented the highest value within the Japan Trench among all the trenches (2.78% h^{-1} compared to 0.83% h^{-1} in Ogasawara Trench and 1.26% h^{-1} in Mariana Trench; Figure 9a). Only in Ogasawara Trench VIPM was significantly higher ($P<0.05$) than in the respective abyssal site (0.83 and 0.23% h^{-1} respectively; Figure 8c), the other two trenches did not present significant differences compared their surrounded abyssal plains. Values of Burst Size (BS) ranged from 37 to 87 (in the abyssal site close to Ogasawara Trench and in Japan Trench, respectively; Table 1). The carbon released by killed prokaryotes due to viral lysis was found significantly correlated with prokaryotic biomass (Figure 9b), and corresponded to 32.31 mg C m^{-2} d^{-1} within the Japan Trench (Figure 9c) giving a percentage contribution of 86.8% to the C cycle on the basis of the aminopeptidase and β-glucosidase degradation activities (Figure 9d). In the other trenches, it ranged from 5.49 to 5.87 mg C m^{-2} d^{-1} (in Mariana and Ogasawara trenches, respectively), with a percentage contribution ranged from 0.14 to 0.27. Overall, the C released from killed prokaryotes after viral lysis was higher within the trenches than in their related abyssal sites.

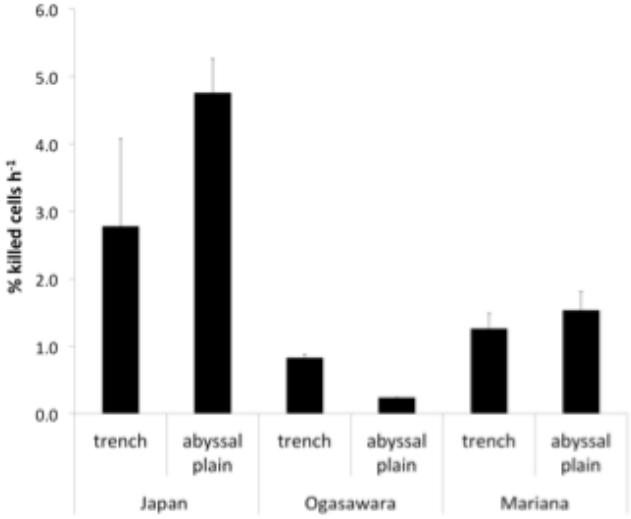

Fig. 9a Percentage of killed prokaryotes due to viral lysis

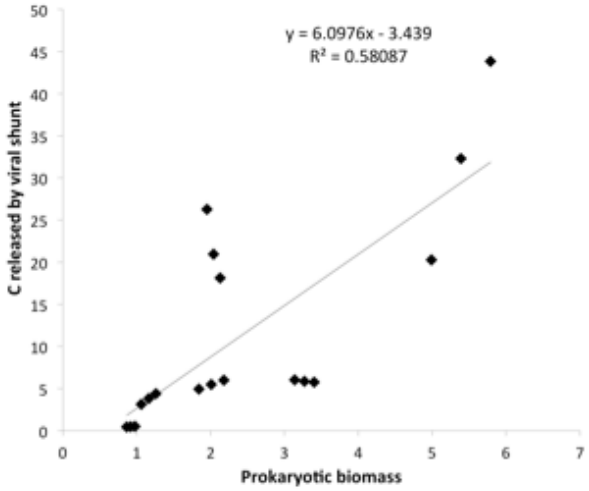

Fig. 9b Relation between prokaryotic biomass and C released by killed prokaryotes ($R=0.76$; $P<0.01$)

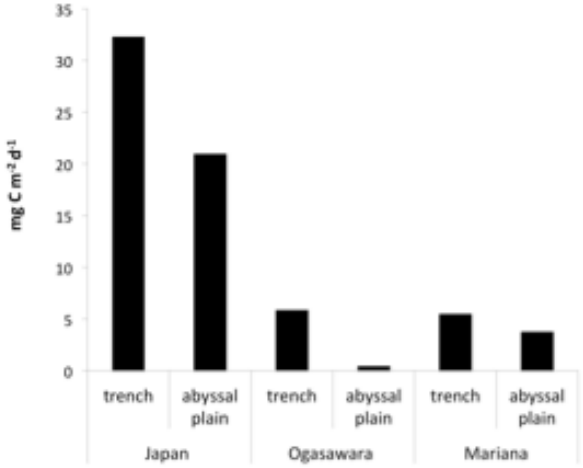

Fig.9c C released from killed prokaryotes after viral lysis

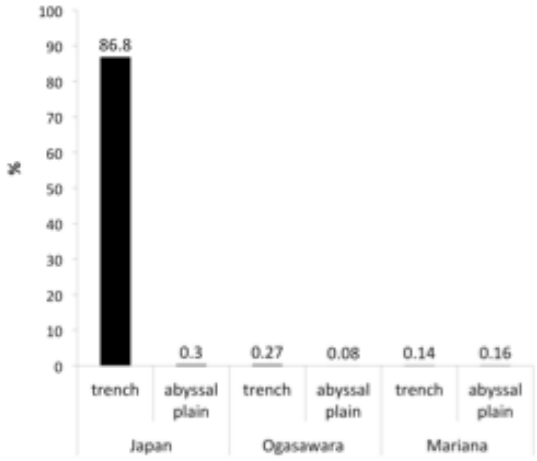

Fig.9d Percentage contribution of C released by killed prokaryotes to the microbial C cycle

		Burst size
Japan	8000m	87
	5253m	71
Ogasawara	9776m	42
	5747m	37
Mariana	10901m	71
	4700m	75

Table 1. Burst size specifically calculated for each sampling site.

1.4 Discussion

Our results showed that prokaryotic abundances within the trenches were comparable to those previously reported for surface deep-sea sediments of the Pacific Ocean in systems characterized by high primary production rates, which ranged from 10^7 to 10^8 cells cm^{-3} (Middelboe, 2006), and higher than their related abyssal sites. Since also biomass values greatly exceeded those abyssal, our findings suggest that hadal trenches can promote significantly the development of prokaryotic assemblages. As expected, enzymatic activities were generally higher in the hadal trenches than in the abyssal sites except for the Japan Trench, which although was characterized by the highest prokaryotic biomass, it showed lower enzymatic activities.

In the sediments of the Japan Trench we found indeed the lowest aminopeptidase activity and enzymatic activity per prokaryotic cell despite the high contribution of proteins and biopolimeric C concentrations. These findings suggest a very low efficiency of the hadal prokaryotic assemblages of the Japan Trench in organic matter degradation despite the high trophic availability. This trench indeed is located in an area characterized by relatively productive surface waters and near the land (Turnewitsch et al, 2014), thus this geographic position can explain the high accumulation of sedimentary material within it.

The discrepancy between low efficiency in organic matter degradation rates and high prokaryotic biomass of the prokaryotes found in the Japan Trench could be explained with an alternative non-heterotrophic pathway of biomass production. Low specific enzymatic activities were also observed in the Mariana and Ogasawara trenches suggesting that all the trenches here investigated could be characterized by an important contribution of mixotrophic and/or chemoautotrophic metabolic pathways as also previously reported (Nunoura et al., 2013).

Virus-prokaryote interactions in hadal sediments

The viral abundances found within the investigated trenches were higher than previously published data from the Mariana Trench (Yoshida et al., 2013) and fell within the range of values already reported for deep-sea viriobenthos abundances (Danovaro et al., 2008b). Comparing data of viral abundance and VPR (virus to prokaryote ratio) between hadal and abyssal sites, we did not observe a clear pattern of distribution. In particular, we found that viral abundance was higher in Ogasawara Trench than in the other trenches, and only in this site VPR exceeded that of the respective abyssal site.

Data of prokaryotic and viral abundances are not sufficient to investigate microbial interactions (Danovaro et al. 2008b), as VPR has to be considered with caution in inferring relationships between viruses and hosts due to the numerous factors that can influence this ratio (Parikka et al., 2016). Therefore, we determined viral production and lysis rates of prokaryotic cells. Viral production was generally high in all trenches, with values similar or even higher to those found in the respective abyssal sites. Viruses within the trenches presented fast turnover rates, especially in the Mariana Trench where they found to be highly dynamic respect viruses of the relative abyssal site, being five times faster. Virus-induced prokaryotic mortality was high in all the trenches suggesting that viruses posses high replication rates and represent a major cause of prokaryotic mortality not only at bathyal and abyssal depths but also at hadal depths.

Among the three investigated trenches, the highest viral production and virus-induced prokaryotic mortality were found in the Japan Trench (five times higher than in the Mariana Trench and double compared to that of the Ogasawara Trench) where very high prokaryotic biomasses were present. Viral replication is known to depend upon abundance and growth rates of host cells (Wommack and Colwell, 2000; Danovaro et al., 2009) and our data suggested that also prokaryotic cell sizes can influence this rate. We found indeed a significantly positive correlation ($P < 0.01$) between viral production and prokaryotic biomasses (Figure 8c) in all trenches. Despite a correlation analysis does not allow us to infer cause-effect relationships it is plausible point out that the high viral lysis rates observed in the Japan Trench are due to the high prokaryotic biomasses.

In the Japan Trench, viral lysis promoted the release of almost 33 mg C m^{-2} d^{-1}. Despite this C amount was lower than in other benthic deep-sea sediments previously investigated (Corinaldesi et al., 2012), it was significantly higher than in the other trenches and respective abyssal sites. This release corresponded to *ca*. 87% of the C degraded by extracellular aminopeptidase and β-glucosidase activities suggesting a relevant role of viruses in funnelling bioavailable organic C within the microbial food-web and in sustaining the abundance and biomass of the microbial assemblage. These data should be taken with caution as only two specific extracellular enzymatic activities (the aminopeptidase and β-glucosidase), among all of the enzymes naturally involved in organic matter remineralization processes in marine sediments (Arndt et al., 2013), were considered.

1.5 Conclusions

Overall, our findings reveal that hadal trenches support high prokaryotic abundances and biomasses compared with the surrounding abyssal sites. Hadal trenches also promote high enzymatic activities, despite the efficiency of degradation of organic matter per cell is low potentially due to non-heterotrophic pathways of biomass production. The high prokaryotic biomasses of hadal trenches favour high rates of viral lysis, which especially in the Japan Trench can stimulate the release of highly bioavailable organic matter for benthic metabolism.

The role of viruses appears to be relevant independently of the hadal trench considered. Indeed viruses, in hadal trenches, can play a fundamental role in funnelling organic C within the microbial food-web and in favouring prokaryotic biomass production.

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Chapter 2.

Virus-prokaryote interactions and prokaryotic diversity in the deepest ecosystem on Earth: the Mariana Trench

2.1 Introduction

The Challenger Deep is the deepest part of the Earth's surface, lying in the Southern Mariana Trench and reaching a maximum depth of ca. 11,000 m (Pathom-aree et al., 2006). This site is characterized by extremely high hydrostatic pressure and by its isolated nature compared to the surrounding ocean. Despite the low productivity of the surface waters under which it is located and the distance from the coast (Turnewisch et al., 2014), recent findings provided evidence that the Challenger Deep is characterised by higher benthic prokaryotic abundance and oxygen consumption rates when compared to adjacent abyssal plain ecosystems (Glud et al., 2013). However, factors leading to such enhanced metabolic activity within Mariana Trench are still to be fully understood.

Viruses are an ubiquitous and numerically dominant component of benthic deep-sea ecosystems worldwide (Danovaro et al., 2008), including abyssal plains, continental margins, seamount, mud volcanoes, and deep-hypersaline anoxic basins (Siem-Jørgensen et al., 2008; Middelboe et al., 2006; Middelboe and Glud, 2006; Danovaro et al., 2009; Corinaldesi et al., 2012; Corinaldesi et al., 2014). Relatively high viral abundances have been also reported in the top 10 cm of the sediment collected at 10.332 m, depth in the Mariana Trench (about 10^7 viruses cm^{-3}; Yoshida et al. 2013). Viruses in benthic deep-sea ecosystems are not only numerically important but also, by killing their prokaryotic hosts play a key role in prokaryotic dynamics, organic matter cycling and nutrient regeneration processes, thus influencing ecosystem functioning in fundamental way (Danovaro et al., 2008; Dell'Anno et al., 2015). However, the extent by which viruses can influence prokaryotic

dynamics and key ecological processes in the deepest ecosystem of the Biosphere is completely unknown.

Viral infections can also influence prokaryotic diversity and assemblage composition (Suttle 2007), which in the Mariana Trench need to be explored yet. Most of the available information on the prokaryotic diversity in the Mariana Trench has been acquired using-culture dependent approaches (Morita, 1976; Yayanos et al., 1981, Takami et al. 1997; Tamegai et al., 1997; Kato et al. 1998; Takai et al. 1999; Takami et al. 2004; Pathom-aree et al., 2006). A recent study, based on sequencing analysis of 16S rRNA genes from clone libraries obtained from DNA extracted from the top 10 cm of the sediment collected at 10.332 m, depth in the Mariana Trench revealed the dominance of sequences belonging to Planctomycetes, Chloroflexi and Firmicutes among Bacteria and to Marine Group I among Archaea (Yoshida et al. 2013).

In this study, we investigated, for the first time, viral abundance and production along with prokaryotic abundance and diversity in surface sediments collected along a transect from the abyssal plains adjacent the Marianna Trench and crossing the whole trench ecosystem. This study aims at providing new insights on the viral impact on prokaryotic assemblages in one of the most extreme ecosystems of the Biosphere and related implications on ecosystem functioning.

2.2 Materials and Methods

2.2.1 Sampling areas

Surface sediments (top 2 cm) were collected along a bathymetric transect of 6 stations including: one station in the abyssal plain at the North of the Mariana Trench at 5385 m depth (11°44.7785' N, 142°06.521' E), one station located along the northern flank of the Marianna trench at 7476 m

depth (11°35.8323' N, 142°25.7723' E), one located at 10901 m depth (11°22.0533' N, 142°25.4486' E), one along the southern flank at 6067 m depth (10°54.9488' N, 142°32.034' E) and two stations located on the abyssal plain at the South of the trench at a depth of 5183 m and 4700 m, respectively (10°38.9897' N, 142°33.0738' E and 10°17.9803' N, 142°36.0157' E; Figure 1). All the sediment samples were collected by using the Lander system of the ROV *ABISMO* during the KR14-01 cruise on board of the research vessel Kairei in collaboration with the JAMSTEC Institute (January 2014). Each sampling site was sampled in three replicates and surface sediments were used for the analysis of the biochemical composition of the organic matter, prokaryotic abundance, biomass and diversity, viral abundance and production and extracellular enzymatic activities. Samples for the determination of extracellular enzymatic activities and viral production were processed immediately after the collection of the sediments. For the prokaryotic counts, sediment sub-samples were fixed with buffered 2% formalin and stored at 4°C until processed. For all other variables investigated samples were stored at -20°C till laboratory analyses.

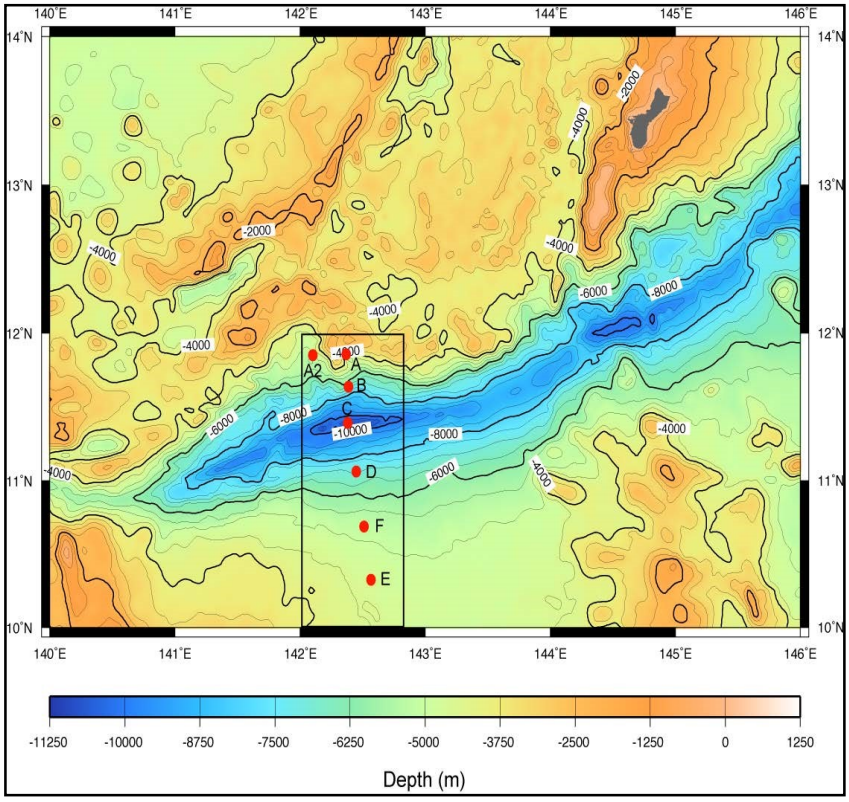

Fig. 1 Sampling area. Site A2 northern abyssal plain (5838 m); site B northern flank (7476 m); site C Challenger Deep (10901 m); site D southern flank of the trench (6067 m); site F (5183 m) and site E (4700 m) southern abyssal plain.

2.2.2 Biochemical composition of organic matter

The biochemical composition of organic matter was analysed in terms of chloroplastic pigment, protein, carbohydrate and lipid concentartions. All analyses were carried out on the first 0-1 cm of sediment from each sediment core.

Chloroplastic pigments (chlorophyll-a and phaeopigments) were analysed fluorometrically according to Lorenzen and Jeffrey (1980). Pigments were extracted with 90% acetone (24 h in the dark at 4°C). After centrifugation (800 X g), the supernatant was used to determine the functional chlorophyll-a and acidified with 0.1 N HCl to estimate the amount of phaeopigments. Total phytopigment concentrations (CPEs) were defined as the sum of chlorophyll-a and phaeopigment concentrations (Danovaro et al., 1999a,b).

Protein content (PRT) was determined according to Hartree (1972) modified by Rice (1982) to compensate for phenol interference. Concentrations are calculated from calibration curves of standard solutions of BSA, normalized to sediment dry weight and expressed as milligram of albumin equivalents per gram of dry sediment. Carbohydrates (CHOs) were analysed according to Gerchacov and Hachter (1972) and expressed as glucose equivalents normalized to sediment dry weight. The method is based on the same principle as the widely used method of Dubois et al. (1956), but it is specifically adapted for carbohydrate determination in sediments. Lipids (LIPs) were extracted by direct elution with chloroform and methanol and analysed according to Marsh and Wenstein (1966). Lipid concentrations are reported as tripalmitine equivalents normalized to sediment dry weight. For each biochemical analysis, blanks were made with the same sediment samples as previously treated in a muffle furnace (450°C, 2 h). The sum of the carbohydrate, protein and lipid concentrations converted into carbon equivalents (using the conversion factors of 0.40, 0.49 and 0.75 mg C mg^{-1}, respectively) was defined as the biopolymeric organic carbon (Pusceddu et al, 2009).

2.2.3 Virus abundance

Viral counting was carried out on *ca.* 1 cm^3 of sediment from the top 0-2 cm of each sediment core as described by Danovaro et *al.* (2001). Sediment sub-samples were diluted in a final volume of 5 ml volume of virus-free seawater filtered at 0.02 μm pore size using disposable syringe filters (Anotop 25, Whatman), and treated with pyrophosphate at a final concentration of 5mM. Samples were sonicated by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W) to increase the extraction yield. The samples were then diluted 25-fold in virus-free seawater and incubated with the addition of DNase I from bovine pancreas (2-5 Uml^{-1}) in order to eliminate uncertainties in virus counting due to extracellular DNA interference. After the incubation, samples were filtered onto 0.02 μm pore size filters (Anodisc Al^2 O^3; 25mm diameter, Whatman) with vacuum pressure of 150 mm of Hg by using sterile glass filter holders for 25 mm diameter filters with 15 ml glass funnel (Millipore). Then the filters were stained using SYBR Gold (10000X concentrated in anhydrous dimethyl sulphoxide; Molecular Probes-Invitrogen, Grand Island, NY, USA) at a final concentration of 2X. SYBR Gold was used instead of SYBR Green I to detect both double- and single-stranded DNA and RNA, and because its strong brightness and stability as fluorescence stain. Viral counting was performed under epifluorescence microscopy, using a Nikon ECLIPSE Ni-U microscope, by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. The viral abundance was normalised to sediment dry weight after desiccation at 60°C for 24 h.

2.2.4 Viral production, turnover and virus-induced prokaryotic mortality (VIPM)

Viral production was analysed in surface sediment samples collected in the investigated area, by the dilution-based procedure specifically developed for sediment samples (Dell'Anno et *al.* 2009). Briefly, sediment samples were transferred into sterile tubes and mixed with an equal volume of 0.02 μm pre-filtered virus-free seawater and incubated at in situ temperature up to 12 hours. Aliquots of samples (0.5 ml) were collected at 0, 3, 6 and 12 hours. After the extraction of viruses

from each sediment samples as described above, viral counting was performed under epifluorescence microscopy by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. After the extraction of viruses from each sediment samples as described above, viral counting was performed under epifluorescence microscopy by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. Viral production was calculated on the basis of the maximum increment of viral abundance occurred over time. Viral turnover was calculated as the ratio between viral production and viral abundance and expressed as turnover time (h).

The number of prokaryotes killed by viral lysis was estimated on the basis of the ratio between viral production and burst size (BS), which was estimated by applying the following equation: BS = 30.2 + (261.4 × cell biovolume μm^3) (Weinbauer & Hofle, 1998). VIPM was estimated as the ratio between the number of prokaryotes killed by viruses and the total number of prokaryotes present in the samples and expressed as percentage.

2.2.5 Prokaryotic abundance and biomass

For prokaryotic counting, *ca.* 1 cm^3 of sediment from the top 2 cm of each sediment core were analysed as previously described by Danovaro et *al.* (2002). Benthic bacteria were detached from sediment by using pyrophosphate at a final concentration of 5 mM, and were sonicated by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W) to increase the extraction efficiency. For bacterial counting, subsamples were diluted 50-fold with sterilized 0.2 μm-filtered seawater and stained with Acridine Orange (final concentration, 0.01%). After the incubation at the dark, the samples were filtered onto 0.2-μm-pore-size black Nuclepore filters with vacuum pressure of 150 mm of Hg by using sterile glass filter holders for 25 mm diameter filters with 15 ml glass funnel (Millipore). Acridine Orange was used instead of SYBR Green I for bacterial analysis because it provided similar counts but did not result in the overestimation of biovolume. All filters were analysed by epifluorescence microscopy using a

Nikon ECLIPSE Ni-U microscope equipped with a micrometric scale to examine the cell dimensions and estimate the biovolume. Ten to 50 fields were viewed at a magnification of X1000, and a minimum of 400 bacterial cells was counted. The prokaryotic abundance was normalised to sediment dry weight after desiccation at 60°C for 24 h. Prokaryotic biomass was estimated on the basis of prokaryotic biovolume using a conversion factor of 310 fg of C μm^{-3} (Fry, 1990; Danovaro et al., 2008).

2.2.6 Extracellular enzymatic activities

Analyses of l-aminopeptidase, β-d-glucosidase, and alkaline-phosphatase activity were carried out. Sediment slurries (prepared with pre-filtered sterilized seawater, 1:1 v/v) were incubated for 2 h (enzymatic activity increased linearly with time) in the dark and at in situ temperature with l-leucine-4-methylcoumarinyl-7-amide (MCA), 4- methylumbelliferone β-d-glucopyranoside (MUF), and 4-methylumbelliferone phosphate (MUF-P) respectively as substrates (final concentrations of MCA 200 μM, MUF 200 μM, and MUF-P 50 μM determined after kinetic experiments). Sediment sub-samples incubated without fluorogenic substrates were used as blanks and processed according to the procedure followed for fresh sediment samples. After incubation, samples were centrifuged (800X g) and the supernatants were analysed fluorometrically according to Hoppe (1993). Enzymatic activities were expressed as nmol of substrates hydrolysed per hour and normalized to sediment dry weight.

2.2.7 DNA extraction

DNA used for the analysis of the prokaryotic diversity was extracted from sediment samples by using the kit PowerSoil DNA isolation kit (MoBio Laboratories Inc., CA, USA). Before DNA extraction, sediment samples were pre-treated with a chemical–physical procedure to remove as much as possible the extracellular DNA (Fortin et al., 2004; Danovaro, 2010).

2.2.8 Sequencing and bioinformatics

Superficial sediment samples from all the investigated sites were used to analyse the genetic diversity of the prokaryotic 16S rDNA sequences associated with the microbial DNA pools. Analyses were conducted by sequencing hypervariable regions V3 and V4 for Bacteria and V4-5-6 for Archaea. Amplicons were generated using the universal primers 314F-805R, (5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3') for Bacteria and 519F-1017R (5'- CAGCMGCCGCGGTAA-3' and 5'- GGCCATGCACCWCCTCTC) for Archaea (Klindworth et al., 2013; Gonzalez-Gil et al., 2015) and were sequenced by applying the Illumina MiSeq, V3 technology, 2x300 paired-end sequencing.

Raw bacterial and archaeal sequences were first subjected to paired-end merging with the FLASH software (Magoč & Salzberg, 2011), and joined pairs were then quality-trimmed by means of the PRINSEQ tool (Schmieder & Edwards, 2011) by removing sequences with an average minim quality of 20 and trimming ends with a quality score <20. The high-quality amplicon reads were subsequently analysed by the QIIME pipeline (Caporaso et al., 2010), aligning them against the GreenGenes database (DeSantis et al., 2006) using the open-reference strategy at 97% of similarity (Rideout et al., 2014). Sequences from each primer set were analysed separately. Each dataset was normalized to the lower number of sequences available and alpha-diversity indices (i.e. Shannon index, Pielou's index and the phylogenetic diversity) were calculated by the same pipeline on this normalized subset.

2.2.9 Statistical analyses

One-way analysis of variance was used for testing differences in the investigated variables among sampling stations. To identify factors influencing prokaryotic abundance a multivariate multiple regression analysis (DistLM) was carried out using virus-induced prokaryotic mortality (VIPM), Chl-a and BPC concentrations, and water column depth as predictor variables. To assess similarity

of the bacterial and archaeal assemblage composition among sampling sites a cluster analysis was also carried out. All statistical analyses were carried out using the PRIMER 6+ software.

2.3 Results

2.3.1 Trophic resources inside and outside the Mariana Trench:

Sediments collected in the northern flank and in the deepest station of the Mariana Trench were characterised by significantly higher chlorophyll-a and BPC concentrations compared to the abyssal sites (Figure 2a,b). Sediments collected in the northern flank and in the deepest station of the Mariana Trench were also characterised by a higher protein to carbohydrate ratio (used as a proxy of the nutritional quality of organic matter for benthic consumers).

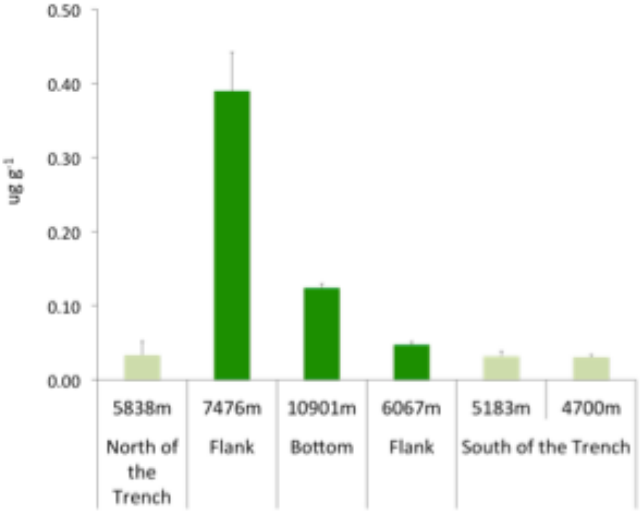

Fig. 2c Chl-a content in the investigated sediments

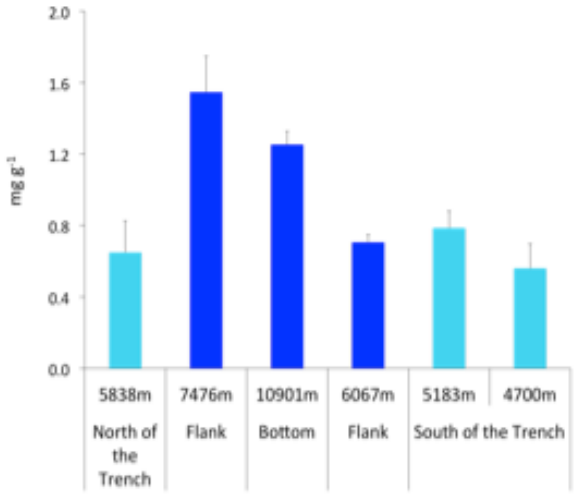

Fig. 2a Biopolymeric C content (BPC) in the investigated sediments

Sites	Depth site	PRT/CHO
North of the Trench	5838m	0.2
Flank	7476m	1.1
Bottom	10901m	0.5
Flank	6067m	0.3
South of the Trench	5183m	0.3
South of the Trench	4700m	0.2

Table 1 Protein to carbohydrate ratio in each sampling site.

2.3.2 Viral abundance, production and turnover rates

The sediments collected at the deepest station of the trench were characterised by the lowest viral abundance ($5.7 \pm 0.23 \times 10^7$; Figure 3a) compared to the other sampling stations.

Conversely viral production values in the deepest station of the trench ($3.72 \pm 0.7 \times 10^7$ viruses g^{-1} h^{-1}) were similar or even significantly higher that those in the adjacent abyssal stations (Figure 3b). Also viral turnover rate (0.7 ± 0.1 h^{-1}) in the sediment of the deepest station of the trench was similar or higher than in abyssal stations (Figure 3c).

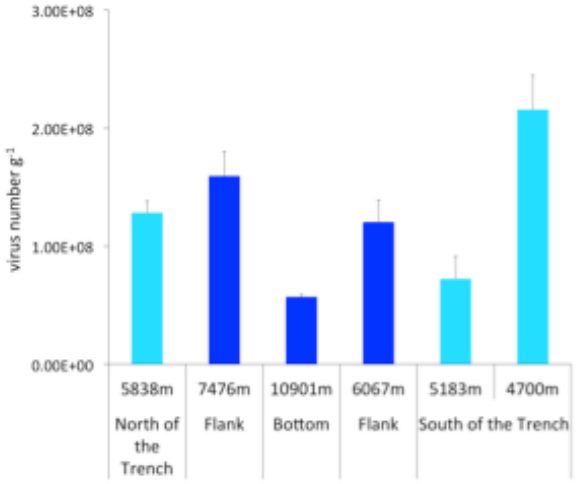

Fig. 3a Viral abundance

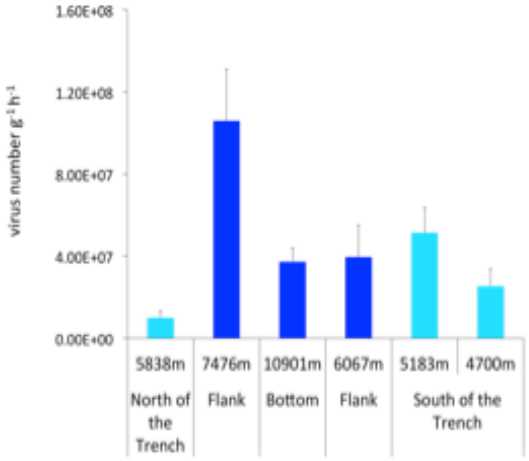

Fig. 3b Viral productions

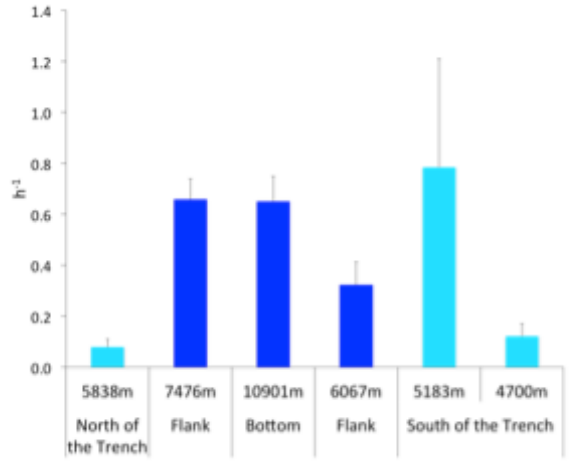

Fig. 3c Viral turnover rate

2.3.3 Prokaryotic abundance and biomass

Prokaryotic abundance and biomass at the bottom of the Mariana Trench ($4.16\pm0.72 \times 10^7$ cells g^{-1} and 2.01 ± 0.17 µg C g^{-1}, respectively) were significantly higher ($p<0.05$) compared to values found in all other investigated benthic sites (Figure 4a,b).

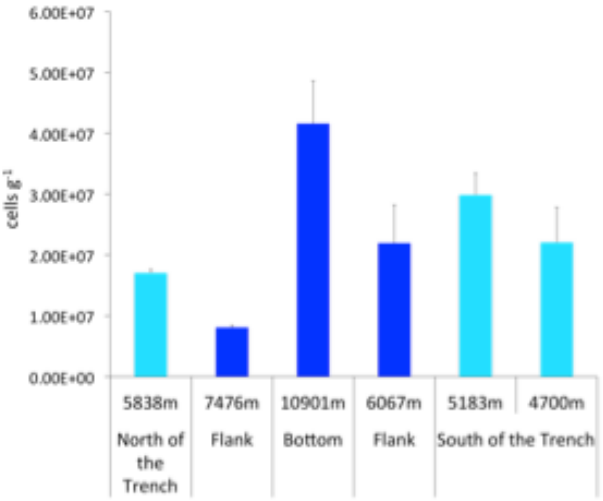

Fig. 4a Prokaryotic abundance

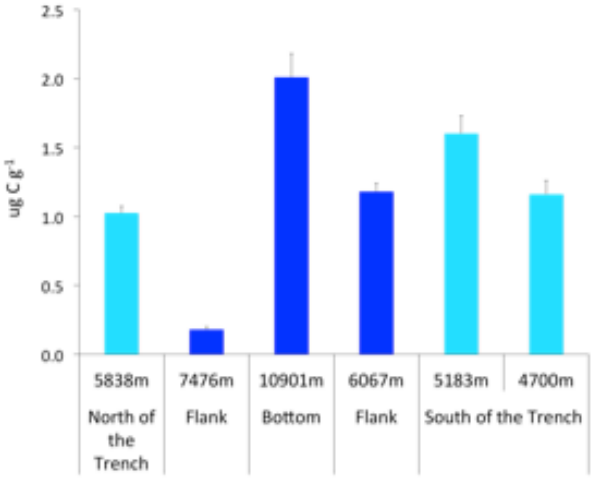

Fig. 4b Prokaryotic biomass

2.3.4 Virus to prokaryote ratio and virus-induced mortality

Values of virus to prokaryote ratio were much lower in the sediments of the deepest stations of the Mariana Trench (1.4) compared to all other sites (range: 2.5-20) (Figure 5a).

The highest values of virus-induced prokaryotic mortality were observed in the sediments along the northern flank of the trench (26 ± 5 % of prokaryotes killed per hour), whereas lowest values were found in the sediments of the northern abyssal plain site (2 ± 0.7 % of prokaryotes killed per hour). Sediments at 10901 m depth within the trench displayed values close to the ones observed in the other abyssal stations (3 ± 0.6 % of prokaryotes killed per hour) (Figure 5b). The Burst Size (BS) values ranged from 49 to 81 (along the northern flank and in the northern abyssal site, respectively; Table 2).

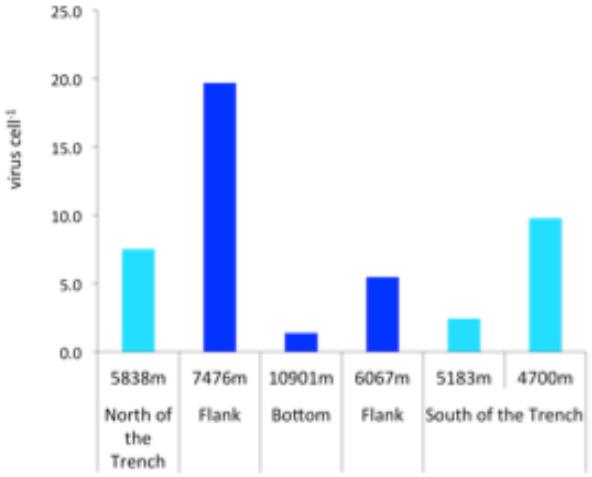

Fig. 5a Virus to prokaryote ratio

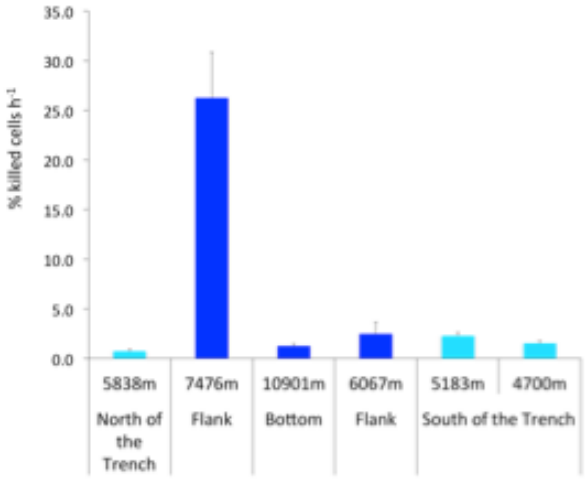

Fig. 5b Virus-induced prokaryotic mortality

Sites	Depth site	BS
North of the Trench	5838m	81
Flank North	7476m	49
Bottom	10901m	71
Flank South	6067m	76
South of the Trench	5183m	75
South of the Trench	4700m	75

Table 2. Burst size values at each sampling site

The DistLM analysis indicated that a major fraction of the variance of prokaryotic abundance was explained by virus-induced prokaryotic mortality (57%, $P<0.01$) and by the availability of trophic resources (i.e. biopolymer carbon 25%, $P<0.01$; Table 3).

Variable	SS	Pseudo-F	P	Prop.	Cumul.
VIPM	1670.7	21.039	0.0015	0.568	0.568
BPC	740.31	20.944	0.0001	0.2517	0.819
Water depth	93.533	2.9988	0.103	0.0318	0.85
Chl-a	173.81	8.596	0.0103	0.0591	0.91

Table 3. Output of the DistLM analysis of prokaryotic abundance variance.

2.3.5 Extracellular enzymatic activity

At all sites, aminopeptidases were the most important extracellular enzymes (Figure 6a-c). Aminopeptidase activities at the bottom of the Mariana Trench (248.9 ± 6.9 nmol h^{-1} g^{-1}; Figure 4a) were significantly higher ($P<0.05$) compared to the other sampling sites with the exception of higher values observed at the site lying on the southern abyssal plain at 4700 m (292.4 ± 29.1 nmol h^{-1} g^{-1}). In the trench bottom the alkaline phosphatase as well as the β-glucosidase activities showed lower values compared to the activities measured in the abyssal sites (23.7 ± 0.8 nmol h^{-1} g^{-1} and 0.6 ± 0.0 nmol h^{-1} g^{-1} respectively), with the exception of the site at 5183 m depth, which was characterised by lower alkaline phosphatase activity. Data on extracellular enzymatic activities related with the site along the flank North are not available.

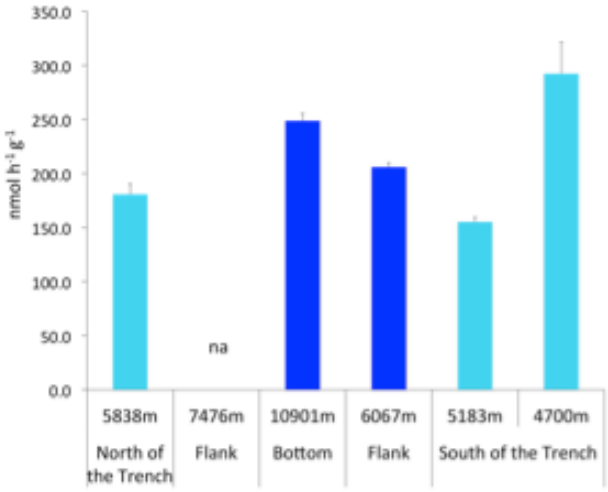

Fig. 6a Aminopeptidase activity

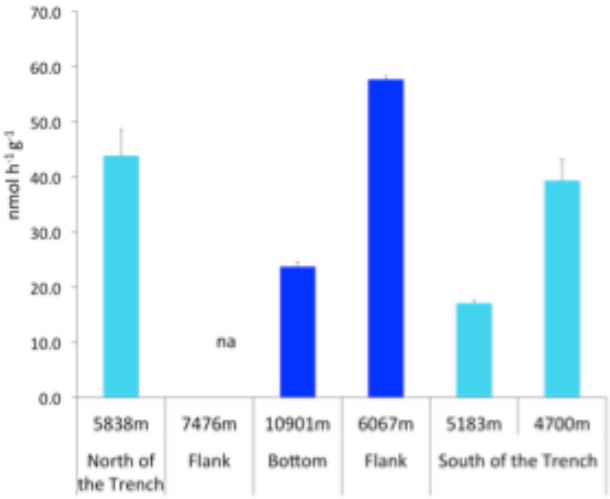

Fig. 6b Alkaline phosphatase activity

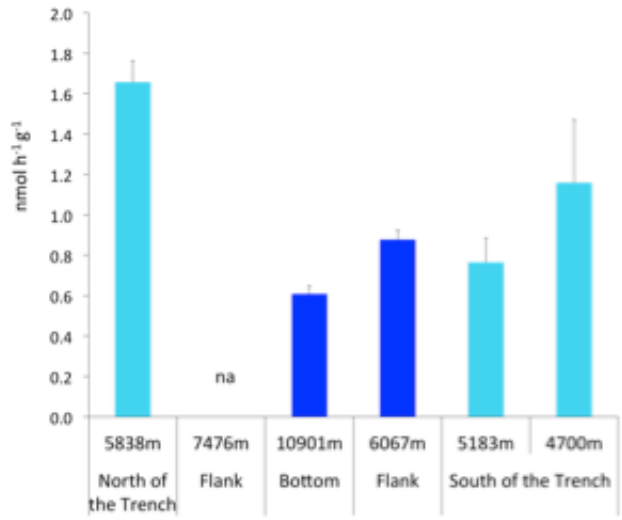

Fig. 6c β-Glucosidase activity

2.3.6 Prokaryotic diversity

Bacterial OTU richness ranged from 7215 to 81688 with the lowest value in the sediments of the bottom of the Mariana Trench and highest value in the northern abyssal site (Figure 7). Archaeal OTU richness ranged from 6034 to 26747 in the sediments of the bottom of the Mariana Trench and in the northern abyssal site, respectively. Estimates of bacterial and archaeal OTU richness based

on Chao1 estimator, which consider also the singletone taxa (very rare taxa represented only by a single read within the prokaryotic community), revealed a similar diversity pattern.

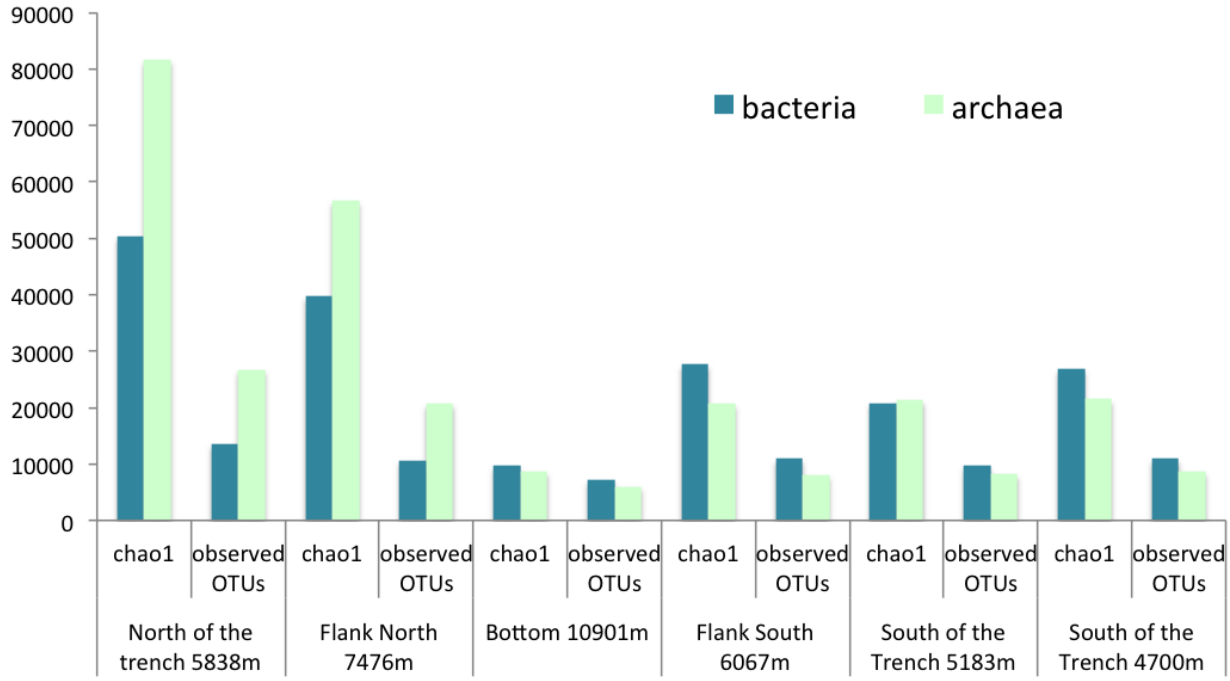

Fig. 7 Observed and estimated (by Chao 1) OTUs number of bacteria and archaea in all investigate sites.

Both bacteria and archaea displayed the lowest values of the Shannon diversity index at the bottom of the trench (10 and 5.8 respectively; Figure 8), while the highest values were observed in the sediment of the northern abyssal site (11 for bacteria and 12 for archaea). Generally, values of Shannon diversity index of both bacteria and archaea were lower in the sediments of the southern than of the northern abyssal sites.

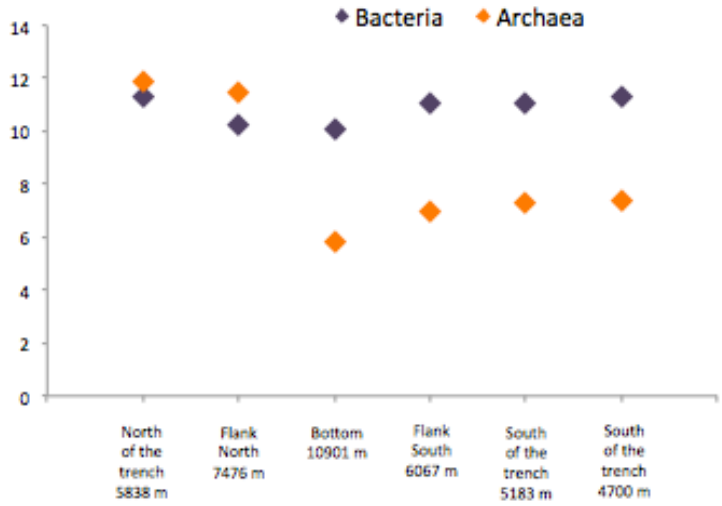

Fig. 8 Bacterial and archaeal Shannon's diversity index.

Equitability values for bacteria assemblages increased moving southward from the trench bottom to the southern sites. The lowest values of equitability for archaea were observed in the sediment of the bottom trench (0.46; Figure 9), while the northern abyssal site showed the highest value (ca. 0.8).

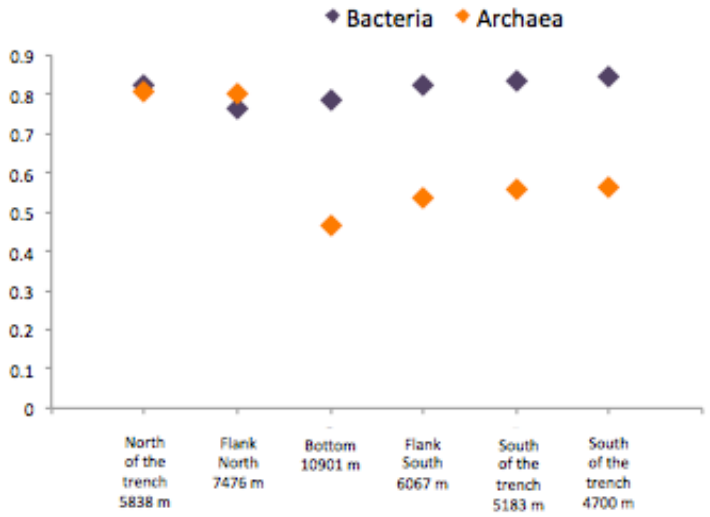

Fig. 9 Bacterial and archaeal Equitability index values.

Rarefaction curves obtained by analysing 40.000 and 80.000 randomly chosen sequences for bacteria and archaea respectively are illustrated in Figures 10 and 11. In some of the sample investigated, such curves did not reach a plateau for both bacteria and archaea.

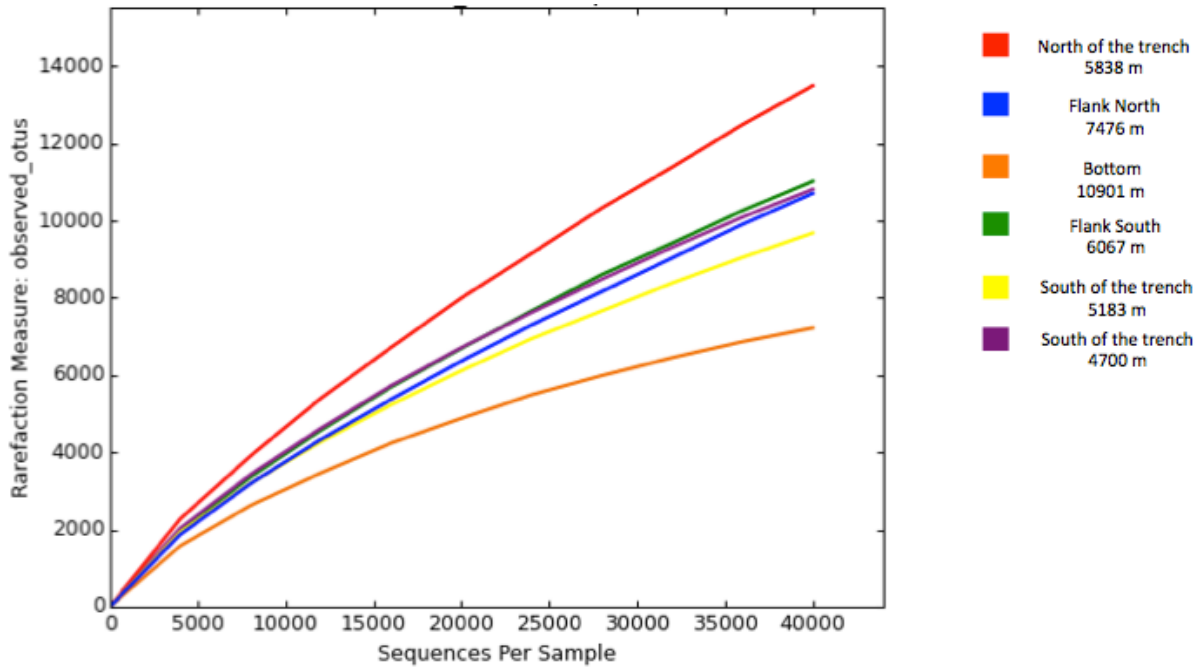

Fig. 10 Bacterial rarefaction curves based on the analysis of 40000 sequences

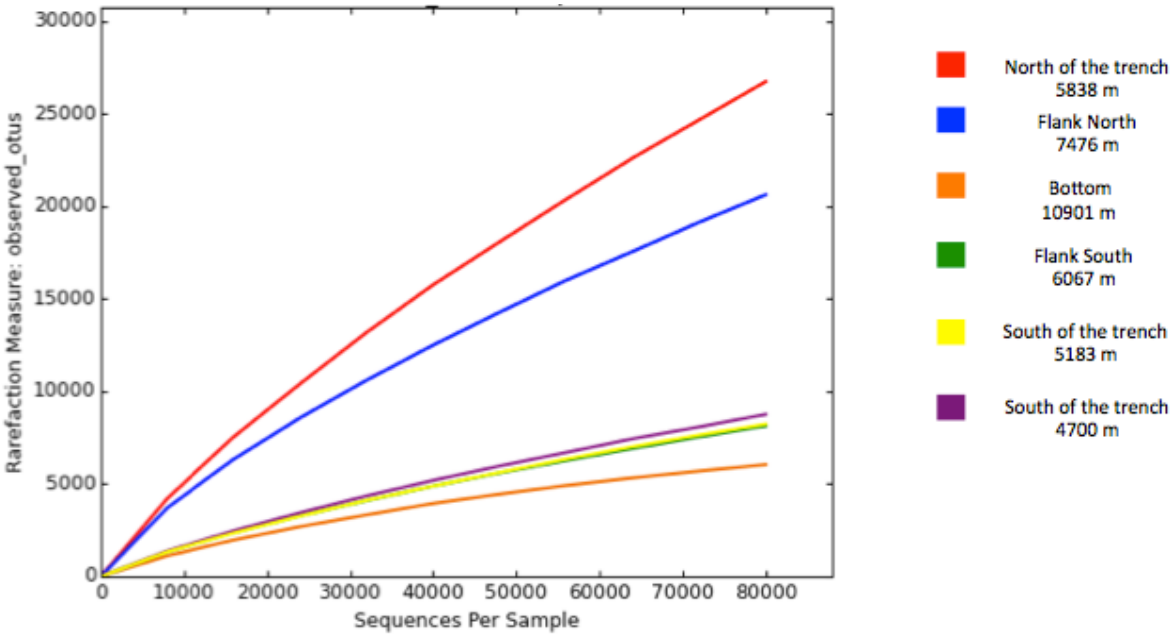

Fig. 11 Archaeal rarefaction curves based on the analysis of 80000 sequences

2.3.7 Taxonomy composition of the prokaryotic assemblages

At the bottom of the trench, Proteobacteria was the most abundant bacterial phylum, accounting for ca. 42% of all bacterial sequences. Other mostly represented phyla were Planctomycetes (12%), Chloroflexi (11%), Bacteroidetes (9%), Firmicutes (7%) and Gemmatimonadates (6%). Bacteria belonging to the order *Thiotrichales* (class Gammaproteobacteria) represented the dominant taxa in the sediment of the bottom trench, (accounting for 11% of all sequences), followed by *Phycysphaerales* (8%, class Phycyspaerae), *Clostridiales* (6%, class Clostridia), *SAR202* (6%, Chloroflexi), *Flavobacteriales* (6%, Flavobacteriia) and *Burkholderiales* (6%, class Betaproteobacteria) (Figure 12). The abyssal sediments located at the southern side were characterised by the presence of the same bacterial taxa than those inhabiting the Challenger Deep, but with a higher abundance of *Acidimicrobiales* (class Acidimicrobia), *Rhodospirillales* (class Alphaproteobacteria), and *NB1-j* (class Deltaproteobacteria). The northern sites, both along the Mariana flank and in the abyssal plain, presented differences compared to the bacteria assemblages inhabiting the bottom of the Mariana Trench, with high abundances of Tenericutes (12-18%) and

the dominance of the orders *Bacteroidales* (27-40%, class Bacteroidia), *Clostridiales* (17-26%, Clostridia) and *Entomoplasmatales* (12-17%, Mollicutes).

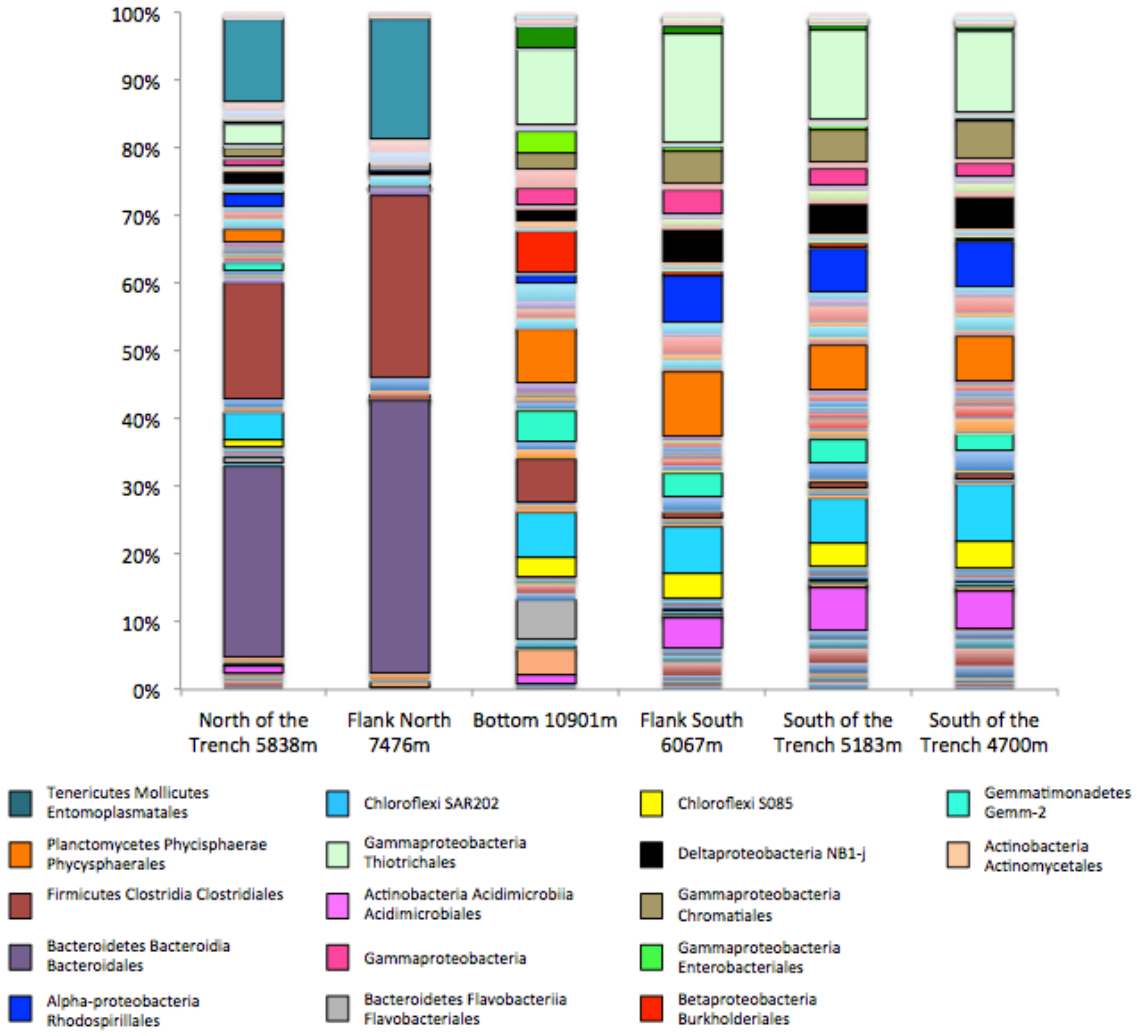

Fig. 12 Taxonomy composition of bacterial assemblages at the order level.

The most abundant archaeal phylum at the bottom of the trench was Thaumarchaeota and almost completely represented by the genus *Nitrosopumilus* (more than 97%) (Figure 13). This phylum was also dominant along the southern flank of the Mariana Trench and in the southern abyssal sites, but the genus *Nitrosopumilus* accounted for a lower fraction (45-63% of all archaeal

sequences). In the northern side of the sampling transect, the majority of archaea was unclassified (54-85%).

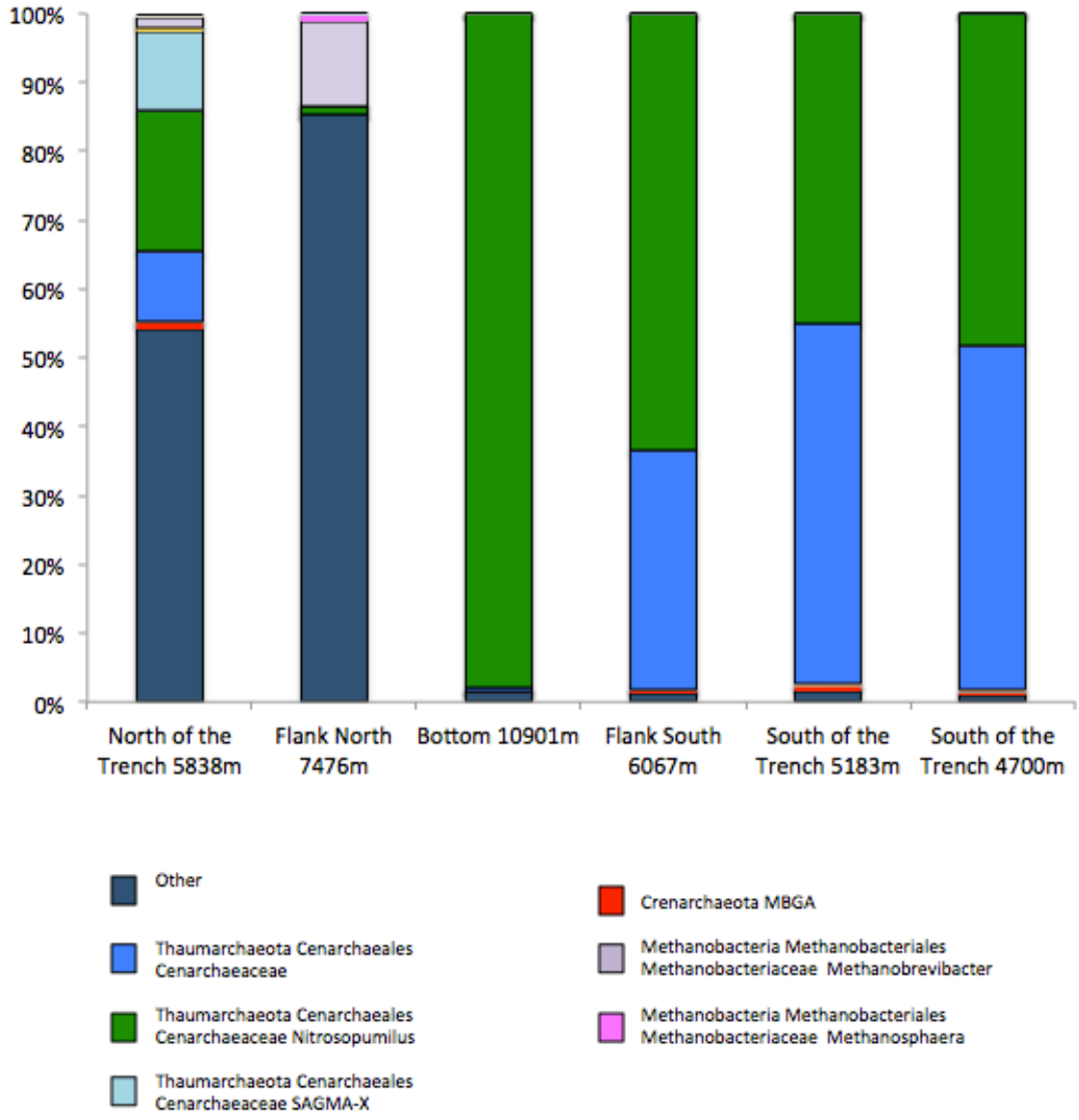

Fig. 13 Taxonomy composition of archaeal assemblages in the investigated sediments

Bacterial assemblage composition of the Mariana Trench bottom sediments displayed the highest similarity with bacterial assemblage inhabiting the southern sites (Figure 14). Also archaeal assemblage composition of the bottom of the Mariana Trench was more similar to that of the southern sites compared to the northern ones (Figure 15).

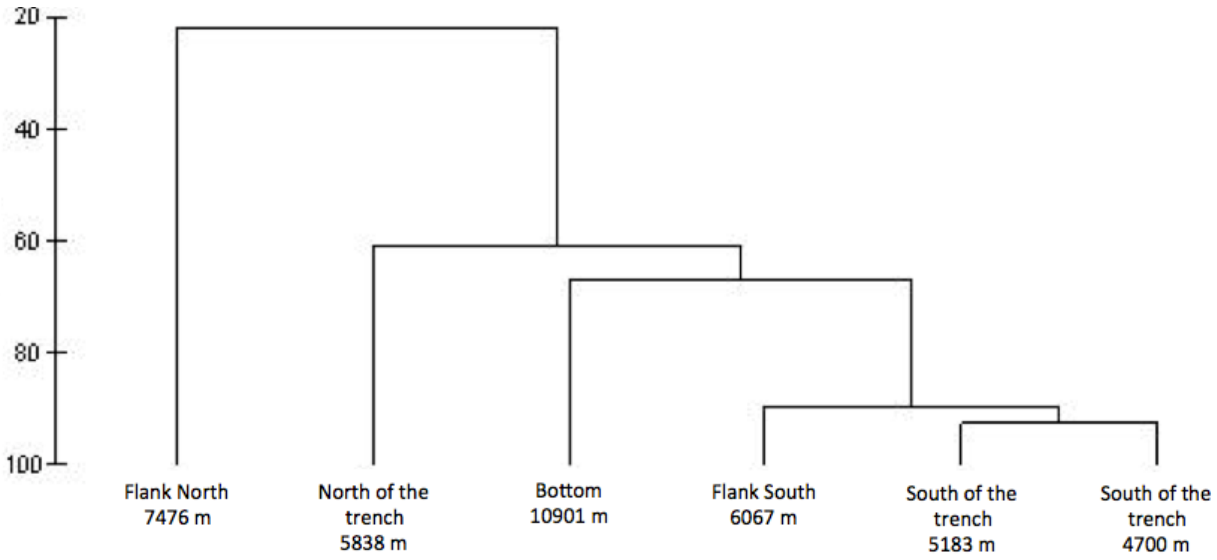

Fig. 14 Cluster analysis based on Bray Curtis similarity of bacterial assemblages inhabiting the deep-sea sediments investigated

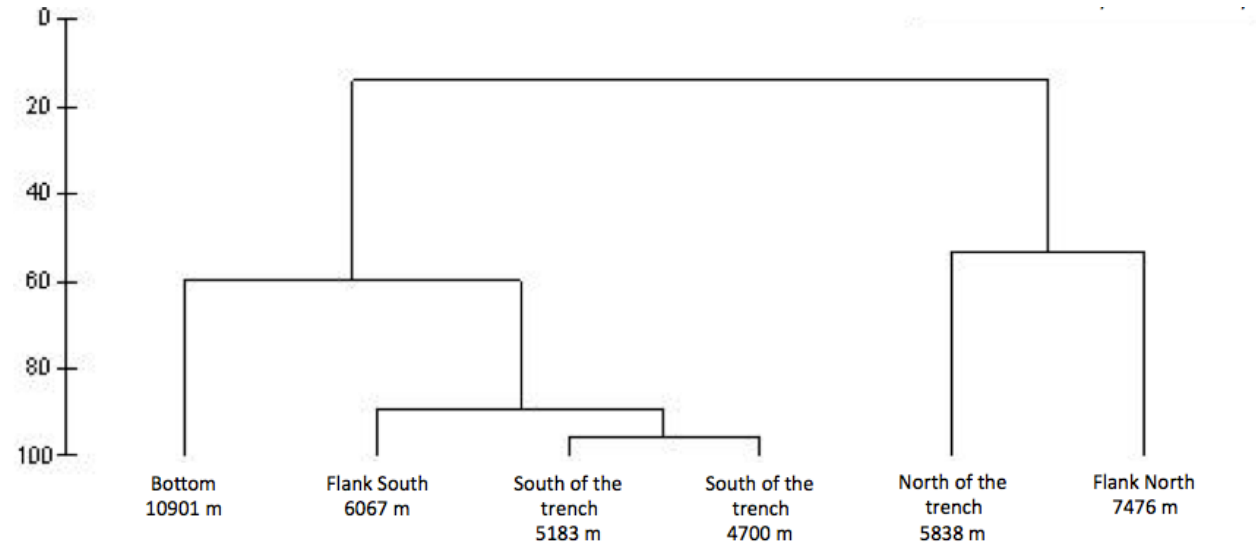

Fig. 15 Cluster analysis based on Bray Curtis similarity of archaeal assemblages inhabiting the deep-sea sediments investigated

2.4 Discussion

There is a general consensus that in open ocean ecosystems the magnitude of organic matter inputs settling to the deep seafloor is mainly dependent on the productivity of the surface waters and water column depth (Smith et al., 2008; Danovaro et al., 2014). However, this is not a general rule for benthic deep-sea ecosystems close to the land or characterised by geomorphological and hydrodynamic characteristics in which lateral transport processes of organic matter can prevail over vertical fluxes (e.g. along submarine canyons and/or trenches, Boetius et al., 1996; Danovaro et al., 2003; Canals et al., 2006; Glud et al. 2013).

The Mariana Trench is located below one of the most oligotrophic area of the world oceans, far from the continental margin (Nunoura et al., 2016), where the particulate organic carbon flux to the seafloor has been estimated to be of ≈0.1-0.5 g m^{-2} yr^{-1} (Glud et al., 2013; Turnewitsch et al., 2014). In this study we found that bottom sediments of the Mariana Trench were characterised by chlorophyll-a and biopolymeric C concentrations ca. 2-fold higher than adjacent abyssal sediments. These findings indicate that, despite the sediments investigated displayed a general oligotrophic condition (Danovaro et al., 2003; Pusceddu et al., 2009), benthic heterotrophic consumers inhabiting the bottom of the Mariana Trench are less food limited than ones of the adjacent abyssal plains. Such higher organic matter content in the bottom sediment of the Mariana Trench was associated with prokaryotic abundance and biomass, up to ca. 2 times higher than those of the adjacent abyssal plain sites. Prokaryotic abundance and metabolism in deep-sea sediments are important biotic factors influencing the distribution and dynamics of benthic viruses (Danovaro et al., 2008; Dell'Anno et al., 2015).

Viral abundances in the sediments investigated in the present study were lower than those previously reported from a variety of benthic deep-sea habitats and ecosystems (Middelboe et al., 2006; Danovaro et al., 2008; Corinaldesi et al., 2010; Corinaldesi et al., 2012) and were unrelated to the abundances of their potential hosts (i.e. prokaryotes). Indeed, the lowest viral abundances were observed at the bottom of the trench where the highest prokaryotic standing stocks were

encountered. Viral abundance in marine sediments depends on the balance between viral production and decay (Corinaldesi et al., 2010, Dell'Anno et al., 2015). In this study we found values of viral production in the sediments of the bottom trench similar or even higher to those observed in the adjacent abyssal sediments or in other benthic deep-sea ecosystems worldwide (Danovaro et al., 2008), indicating, for the first time, an intense viral replication through lytic infection in the deepest benthic ecosystem on Earth. Such an high value of viral production along with the low viral abundance observed in the bottom of the trench suggest that an important fraction of viruses produced undergoes decay processes, which have been reported to be mainly dependent upon viral capsids degradation due to extracellular proteolytic activity (Dell'Anno et al., 2015). The high extracellular aminopeptidase activity we found in the sediments of the bottom of the trench could thus explain the apparent paradox between low values of viral abundance and high values of viral production. Overall, these results indicate that viruses of the Mariana Trench are a highly dynamic component of the benthic food webs, as also highlighted by their fast turnover rates.

Benthic viruses in the Mariana Trench determine a major impact on prokaryotic assemblages. This was particular evident in the sediments of the northern flank of the Mariana trench where virus-induced mortality was very high. Although to a lower extent, also the viral impact in the sediments of the bottom of the Mariana Trench was high and comparable to that of adjacent abyssal sediments or of benthic deep-sea ecosystems worldwide previously investigated (Danovaro et al., 2008; Danovaro et al., 2016). Since viral lysis of prokaryotic cells by releasing of labile organic C can stimulate benthic metabolism of un-infected prokaryotes (Danovaro et al., 2008; Danovaro et al., 2016), these results can contribute to explain the high oxygen consumption rates previously reported in the sediments of the bottom of the Mariana Trench (Glud et al., 2013). The relevance of viral infections in controlling prokaryotic assemblages in the investigated sediments was further confirmed by multivariate multiple regression analysis. Indeed a high fraction of the variance of prokaryotic distribution was explained by virus-mediated prokaryotic mortality, followed by trophic availability. These findings suggest that prokaryotic dynamics in hadal ecosystems can be controlled by the interplay between bottom up (i.e. trophic resources) and top down (i.e. viral

predatory pressure) factors, which could have also an important role in shaping prokaryotic assemblage composition.

In this study we found a lower bacterial and archaeal OTU richness as well as lower values of Shannon's diversity index in the deepest sediments investigated compared to the other sampling sites. The deepest sediments were also characterised by a different assemblage composition. In particular, one of the most striking differences relies on the dominance of archaea affiliated to *Nitrosopumilus*, phylum Thaumarchaeota, which in the sediments of the bottom trench represented 97% of the archaeal assemblages. Since Archaea belonging to *Nitrosopumilus* are aerobic chemosynthetic producers which fix inorganic C coupled with ammonia oxidation (Nakagawa et al., 2007; Junier et al., 2010; Nitahara et al., 2011), these findings suggest that chemoautotrophic processes in the sediment of the bottom trench can be partially responsible of the higher metabolic activity, as oxygen consumption, reported in this hadal ecosystem when compared to adjacent abyssal site (Glud et al., 2013). In the sediments of the bottom trench we also found differences in terms of bacterial assemblage composition. Indeed, we found an important fraction of sequences affiliated to the class Gammaproteobacteria mostly represented by the order *Thiotrichales*, and to the class Betaproteobacteria dominated by the order *Burkholderiales. Thiotrichales* contains chemoautotrophic bacterial taxa involved in sulphur oxidation (Preisler et al., 2007), whereas *Burkholderiales* can be involved in nitrate and nitrite reduction processes (Freitag et al., 2006; Saito et al., 2008). Thus, besides microbial taxa involved in C cycling, the sediments of the bottom of the Mariana Trench host an important fraction of bacterial and archaeal taxa involved in N and S cycling. Previous findings reported that the bottom of the Mariana Trench harbours novel viromes, distinct from the viral genotypes reported in other oceanic environments (Yoshida et al., 2013). This aspect along with the specific assemblage composition reported in the present study lead hypothesising preferential viral infections on the dominant taxa (i.e. *Nitrosopumilus* belonging to Thaumarchaeota). To this regards, recent studies provided evidence that the viral impact in surface deep-sea sediments can occur primarily on different clades belonging to Thaumarchaeota (Danovaro et al., 2016). Overall findings reported in the present study suggest that, despite the

extreme conditions, benthic hadal viruses by infecting and killing their hosts can have important consequences on chemoautotrophic C production and N cycle.

2.5 Conclusions

Results reported here indicate that viruses inhabiting the bottom sediments of the Marina Trench are a dynamic component of the microbial food webs. In particular, despite the extreme environmental conditions, virus-induced prokaryotic mortality in the deepest ecosystem on Earth is relatively high and comparable to that reported in bathyal and abyssal sediments worldwide. Such viral predatory pressure is likely to be preferentially exerted on the dominant microbial taxa, i.e. the ammonia oxidizer *Nitrosopumilus*, thus influencing the structure of the microbial food webs and C and N cycling and contributing to explain the high microbial metabolism previously reported in this ultra abyssal ecosystem.

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Chapter 3.

Patterns of microbial biodiversity in abyssal and hadal systems

3.1 Introduction

The deep-sea, defined as water and seafloor deeper than 200 m (Gage & Tyler, 1991; Thurber et al. 2014), represents one of the less explored ecosystem on Earth (Danovaro et al., 2014), and even less is known for the hadal trenches (Gallo et al., 2015; Jamieson & Fuji, 2011).

There is a general consensus that in benthic deep-sea ecosystems prokaryotes play a key role in biomass production, C cycling and nutrient regeneration processes, thus contributing to the maintenance of essential ecosystem's goods and services (Whitman et al., 1998). Deep-sea prokaryotes are also highly diversified, thus contributing to the whole genetic diversity of the world oceans (Sogin et al., 2006; Huber et al., 2007; Venter et al., 2004; DeLong et al., 2006; Brown et al., 2009; Zinger et al., 2011). Recent studies have pointed out that, conversely to what reported for shallow marine ecosystems, an important fraction of prokaryotic assemblages in the deep sea is represented by archaea (Lipp et al., 2008; Takano et al., 2010; Danovaro et al., 2016), and that this component is actively involved in N and C cycles (Dekas et al., 2009; Corinaldesi et al., 2015; Orcutt et al, 2011; Molari et al., 2013; Martiny et al., 2011; Molari et al., 2012).

Also patterns of microbial assemblages distribution and diversity in deep-sea environments have recently been explored in order to determine potential factors influencing prokaryotic diversity and shaping assemblage composition. Danovaro et al. (2016) revealed that, in different deep-sea benthic ecosystems, bacteria are mainly controlled by food availability while archaea are mainly influenced by temperature. On the basis of wide scale investigations it has been reported a decrease of similarity among microbial assemblages with increasing geographic distance (Bienhold et al.

2016; Auguet et al., 2009). It is also pointed out that the prokaryotic assemblage composition can depend from a wide variety of factors related to environmental factors, specific biological (being generalists rather than specialists), and physiological features (having or not dormant stages during their life cycle; Fierer et al., 2007; Mou et al., 2008; Lindström & Langenheder, 2012). However, although the small size, the potential dispersal rates, the high abundances and the low extinction rates of microorganisms that suggest a scant influence of geographic barriers on them (Martiny et al., 2006), other studies showed that physical boundaries can limit the distribution of microorganisms in deep-sea sediments (Staley and Gosink, 1999; Beja et al., 2002; Finlay, 2002; Ramette and Tiedje, 2007; Schauer et al., 2010).

Hadal trenches are recognized for their geographical isolation, their particular topography, elevated pressures and cold temperatures, systems that can favour speciation (Jamieson et al., 2010; Levin and Dayton, 2009; Blankenship-Williams and Levin, 2009).

This study analysed the microbial community structure and the diversity of benthic bacteria and archaea inhabiting three of the deepest known hadal trenches located in the North Pacific Ocean, the Japan, the Ogasawara and the Mariana trenches, and six abyssal plain sites, in order to provide new insights on benthic microbial distribution and biodiversity in one of the less explored ecosystem on Earth.

3.2 Materials and Methods

3.2.1 Sampling areas

The sampling area covered a wide portion of the North Pacific Ocean, from North-West to the Northern Equatorial zone. Surface sediment samples were collected from three hadal trenches: Ogasawara (29°09.00’ N, 142°48.12’ E), Japan (36°04.00’ N, 142°44.00’ E), and Mariana (11°22.0533’ N, 142°25.4486’ E) trenches. Moreover, six abyssal plain sites were sampled both

close to and far from each trench. Within the Ogasawara Trench sediment samples were collected by using the lander system of the ROV *ABISMO* at 9776 m depth as also in the adjacent abyssal plain at 5747 m depth (29°16.79' N, 143°46.04' E; hereafter referred as Ogasawara T-OUT) during the KR11-11 cruise on board of the research vessel R/V Kairei in collaboration with the Japan Agency for Marine-Earth Science & Technology (JAMSTEC) (December 2011). Sediment samples from the Japan Trench at 8000 m depth as also samples from an abyssal site relatively close to this trench (5253 m depth, 39°00.0' N, 146°00.1' E; hereafter referred as Japan T-OUT) were collected during the R/V Kairei KR12-19 cruise (December 2012) by using the same lander system. Within the Challenger Deep of the Mariana Trench at 10901 m depth and in the surrounding abyssal plain, sediment samples were collected from one site at the North and one site at the South of the trench (5838m depth, 11°44'7785 N, 142°06.521' E and 5183 m depth, 10°38'9897 N, 142°33.0738' E; hereafter referred as Mariana T-OUT1 and Mariana T-OUT2) by using the lander system of the ROV *ABISMO,* during the JAMSTEC R/V Kairei KR14-01 cruise (January 2014). Finally, sediment samples were collected from two abyssal plain sites far from the trenches, one close to the Equator at 4277 m depth (01°15.2' N, 163°14.6' E; September 2013; hereafter referred as AS2) during the JAMSTEC R/V Yokosuka YK13-09 cruise, and one at 4278 m depth (12°00'0 N, 154°00.0' E; December 2013; hereafter referred as AS1), during the JAMSTEC R/V Yokosuka YK13-12, by using the lander system of the ROV *SHINKAI 6500*. Each sampling site was sampled in three replicates and surface sediments (0-2 cm) were used for analysis of the biochemical composition of the organic matter and for microbiological parameters. The samples were frozen immediately after the collection and stored at -20°C without the addition of any preservative until molecular analysis.

Fig. 1 Sampling sites

3.2.2 Environmental and trophic characteristics and virus-induced prokaryotic mortality

Bottom water temperature, salinity, and depth were measured by using a conductivity, temperature and depth (CTD) sensor. All the analysis of the biochemical composition of the organic matter and for microbiological parameters were carried out on surface sediments in three replicates. The concentrations of proteins, carbohydrates, and lipids in the sediment were determined spectrophotometrically and expressed as bovine serum albumin, glucose, and tripalmitine equivalents, respectively, and biopolymeric carbon concentrations were obtained by the sum of the concentrations of these three components converted into carbon equivalents (using the conversion factors of 0.40, 0.49, and 0.75 mg C mg^{-1}, respectively; Pusceddu et al., 2009). The number of prokaryotes killed by viruses were estimated from the ratio between viral production (analysed as previously described by Dell'Anno e al., 2009) and burst size, which was extrapolated by applying the equation: BS = 30.2 + (261.4 × cell biovolume μm^3) (Weinbauer & Hofle, 1998) for each samples. VIPM was estimated as the ratio between the number of prokaryotes killed by

viruses and the total number of prokaryotes present in the samples and expressed as percentage (data of viral production and prokaryotic abundance and biomass are found in Chapter 1 and 2).

3.2.3 Bacteria and Archaea abundances

The analysis of the abundance of both archaea and bacteria was performed by adopting the CARD-FISH technique on surface sediments in three replicates (Ishii et al., 2004; Taira et al., 2004). As first, cells were detached by 2 ml of 1:5 sediment slurry (0.25-0.5 g wet sediment) by adding pyrophosphate at a final concentration of 5mM and sonicating the samples by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W). The supernatants were then filtered properly diluted onto 0.2 μm pore-size white polycarbonate filters under low vacuum pressure (<100 mmHg). The filters were washed with 1 ml PBS and with 1 ml of a 1:1 mixture of PBS/ethanol 96%. After drying, the filters were dipped in 0.2% low gelling point agarose and left to dry at room temperature for 10-30 min. The filters were then washed with ethanol 96% (1 min at room temperature) and were incubated with proteinase K solution (4 U/ml in 0.05 M EDTA, pH 8; 0.1 M Tris-HCl, pH 8) for 30 minutes at 37°C. Filters were washed two times in reagent-grade water, then in 0.01 M HCl for 10 minutes (Proteinase k inactivation step), again in reagent-grade water, then in PBS, and finally in ethanol 96% (1 min at room temperature). The hybridization step were performed by incubating the samples on a rotation shaker (10 rpm) in humidity chamber for at least 3h at 35°C for Bacteria and 46°C for Archaea in accordance with the probes used (EUB338 for Bacteria and ARCH915 for Archaea, Amann et al., 1990a; Manini et al., 2008; Molari & Manini, 2012). For stringent washing, filters were washed for 10 minutes with buffer 3mM NaCl, 5mM EDTA, pH 8; 20 mM Tris-HCl, pH7.5, 0.01% [p/v] SDS, preheated at 37°C for Bacteria and at 48°C for Archaea, and then with reagent-grade water. To equilibrate the probe-HRP, the filters were incubated in PBS amended with 0.05% Triton X-100 for 15 min at room temperature. The filters were then incubated in substrate mix (1 part Cy3-tyramide and 40 parts of amplification diluent; TSA™ Fluorescence system PerkinElmer) for 10-30 min in the dark at 37°C (both for Bacteria and Archaea). Filters were washed in PBS amended with Triton X-100 for 15 min

at room temperature in the dark, in reagent-grade water (1 min at room temperature), and in ethanol 96% (1 min at room temperature). All analyses were performed in triplicate. Archaea and Bacteria cells counting were performed under epifluorescence microscopy. The data were normalized to sediment dry weight after dessication (60°C, 24 hours).

3.2.4 DNA extraction

The microbial DNA used for molecular analyses was recovered from surface sediment samples (0-2 cm) in each explored sampling site and purified by using the kit PowerSoil DNA isolation kit (MoBio Laboratories Inc., CA, USA). Before the extraction of DNA by in situ cell lysis, sediment samples were pre-treated with a chemical–physical procedure to remove as much as possible the extracellular DNA (Fortin et al., 2004; Danovaro, 2010).

3.2.5 Sequencing and bioinformatics

Superficial sediment samples from all the investigated sites were used to analyse the genetic diversity of the prokaryotic 16S rDNA sequences associated with the microbial DNA pools. Analyses were conducted by sequencing hypervariable regions V3 and V4 for Bacteria and V4-5-6 for Archaea. Amplicons were generated using the universal primers 314F-805R, (5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3') for Bacteria and 519F-1017R (5'- CAGCMGCCGCGGTAA-3' and 5'- GGCCATGCACCWCCTCTC) for Archaea (Klindworth et al., 2013; Gonzalez-Gil et al., 2015) and were sequenced by applying the Illumina MiSeq, V3 technology, 2x300 paired-end sequencing.

Raw bacterial and archaeal sequences were first subjected to paired-end merging with the FLASH software (Magoč & Salzberg, 2011), and joined pairs were then quality-trimmed by means of the PRINSEQ tool (Schmieder & Edwards, 2011) by removing sequences with an average minim quality of 20 and trimming ends with a quality score <20. The high-quality amplicon reads were

subsequently analysed by the QIIME pipeline (Caporaso et al., 2010), aligning them against the GreenGenes database (DeSantis et al., 2006) using the open-reference strategy at 97% of similarity (Rideout et al., 2014). Sequences from each primer set were analysed separately and OTUs not belonging to either archaea or bacteria were removed from the dataset. Each dataset was normalized to the lower number of sequences available and alpha-diversity indices (i.e. Shannon index, Pielou's index and the phylogenetic diversity) were calculated by the same pipeline on this normalized subset.

3.2.6 Statistical analyses

To assess differences between sampling stations in bacteria and archaea abundances a one-way univariate analysis of variance was carried out. The cluster analysis was used to determine the similarity level of the prokaryotic taxonomical composition related to each sampling site, separately for bacteria and archaea. The analysis was applied on two distinct Bray-Curtis similarity matrices (one for bacteria and one for archaea) based on the percentage contribution of sequences at the order level. The relationships between bacteria and archaea diversity composition of the three explored trenches and the predictable variables (environmental factors and viral infection) were investigated applying the distance-based redundancy analysis (dbRDA) on two separated Euclidean distance matrices. The PRIMER 6+ software was used for all these analysis.

3.3 Results

3.3.1 Environmental variables and virus-induced prokaryotic mortality

Temperature ranged from 1.3 to 2.5°C showing higher values within the trenches compared to the abyssal sites (Table 1). Biopolimeric carbon concentrations were higher at the bottom of the trenches compared their relative close abyssal sites and ranged from 0.6±0.2 mg g^{-1} to 7.5±0.8 mg g-

[1]. Data on virus-induced prokaryotic mortality did not present a clear pattern among sites and ranged from 0.6±0.1 to 4.8±0.5 %.

Table 1. Environmental factors and virus-induced prokaryotic mortality of all the investigated sites.

Site	Depth (m)	latitude (N)	longitude (E)	T (°C)	BPC (mg/g)	sd	VIPM (%)	sd
Japan Trench	8000	36°04.00’	142°44.00’	1.8	7.5	0.8	2.8	1.3
Ogasawara Trench	9776	29°09.00’	142°48.12’	2.3	2.0	0.1	0.8	0.1
Mariana Trench	10901	11°22.0533’	142°25.448’	2.5*	1.3	0.1	1.3	0.2
Japan T-OUT	5253	39°00.0’	146°00.1’	1.5	4.1	0.2	4.8	0.5
Ogasawara T-OUT	5747	29°16.79’	143°46.04’	1.6	1.0	0.1	0.2	0.0
Mariana T-OUT1	5838	11°44'7785	142°06.521'	1.6*	0.6	0.2	0.7	0.2
Mariana T-OUT2	5183	10°38'9897	142°33.073’	1.6*	0.8	0.1	2.3	0.3
AS1	4278	12°00'0	154°00.0'	1.4	1.2	0.2	0.6	0.1
AS2	4277	01°15.2’	163°14.6'	1.3	1.7	0.1	1.1	0.3

* Glud et al. 2013

3.3.2 Bacterial and archaeal abundances

In Ogasawara Trench bacterial abundance was significantly ($P<0.05$) higher than that found within the sediment of its close abyssal site (Ogasawara T-OUT) and presented the highest value among all the investigated sites (4.6±0.42 x 10^7 cells g^{-1}; Figure 2). This was not true for Mariana and Japan trenches that presented bacteria number similar to those of their relative close abyssal sites (Mariana T-OUT1 and OUT2 and Japan T-OUT, respectively), but higher compared the abyssal site AS2. Archaeal cells number was significantly higher within the sediments of Japan and Ogasawara trenches compared their relative close abyssal sites (highest value 2.5±0.2 x 10^7 cells g^{-1} in

Ogasawara Trench; Figure 3), as also within the Mariana Trench compared to the abyssal site Mariana T-OUT1. Each trench presented higher archaeal abundances compared the abyssal site AS2. Data related with the abyssal site AS1 were not available.

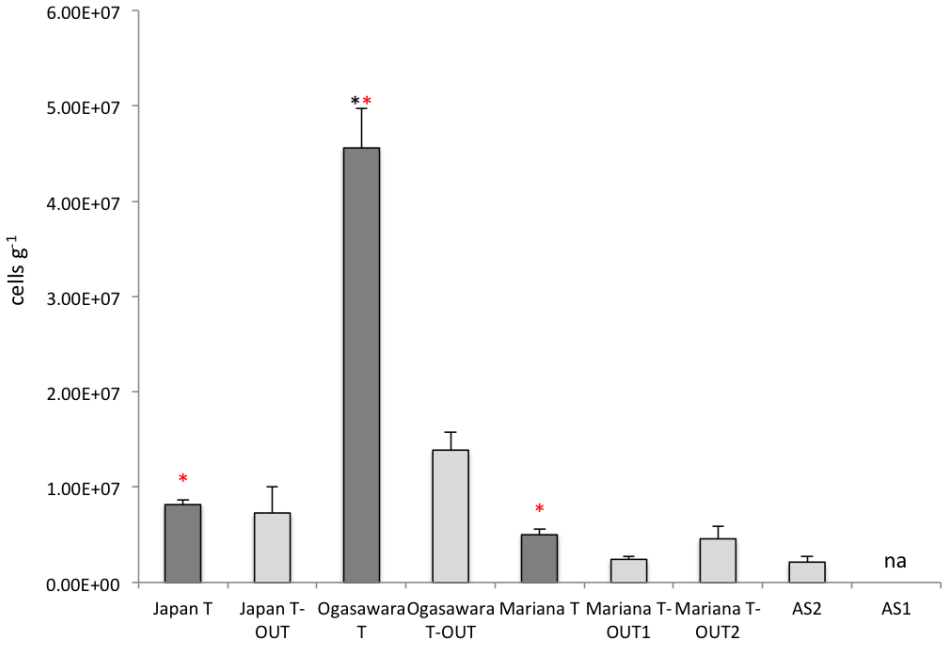

Fig. 2 Bacterial abundances detected within the surface sediments of each sampling site. * significant difference ($P<0.05$) between bottom trench and abyssal site close to the trench; * significant difference ($P<0.05$) between bottom trench and abyssal site AS2.

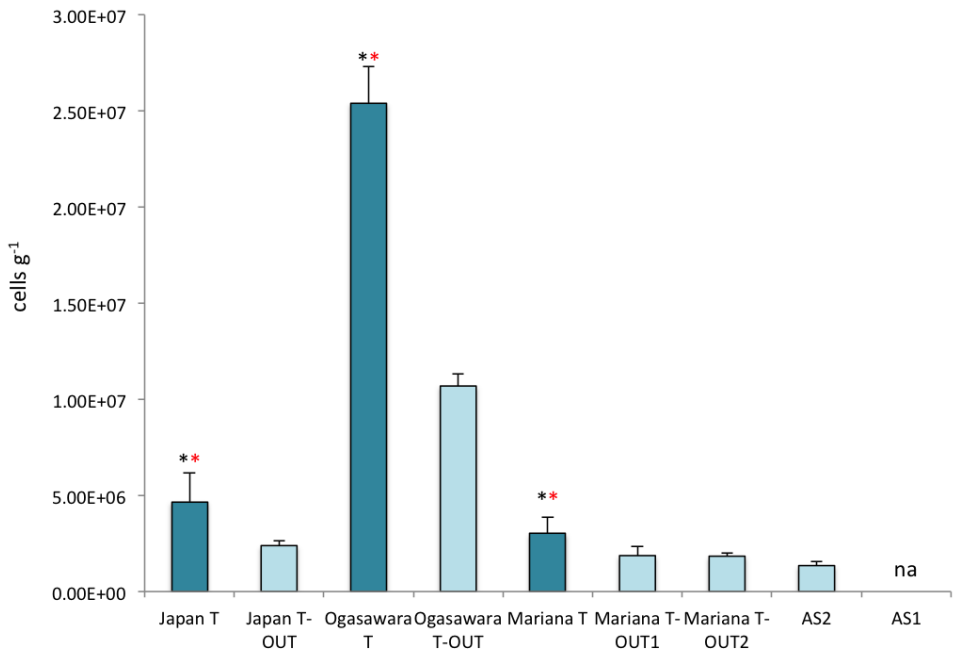

Fig. 3 Archaeal abundances detected within the surface sediments of each sampling site. * significant difference (P<0.05) between bottom trench and abyssal site close to the trench; * significant difference (P<0.05) between bottom trench and abyssal site AS2.

3.3.3 Estimates of bacterial richness and diversity and rarefaction curves

The bacterial OTUs number obtained from the sequence analysis ranged from 7317 to 71238. OTU-based alpha-diversity measurements allowed for the comparison of richness (observed OTUs number), diversity (Shannon index) and equitability (Pielou's index) among the bacterial communities inhabiting the surface sediments of each explored site, and were normalized on 38.000 sequences randomly selected from each sequenced sample.

The observed OTUs number obtained ranged from 3632 to 13191 in Ogasawara Trench and Japan Trench, respectively (Figure 4). Bacterial richness within the Japan Trench exceeded that of all the other investigated sites, while Ogasawara and Mariana trenches presented lower diversity as also the rarefaction curves confirmed (Figure 5). Especially, curves related with Ogasawara and Mariana trenches approached the plateau, while the diversity of the bacterial community of the Japan

Trench was far from the exhaustively sampled showing the highest diversity among all the investigated sites.

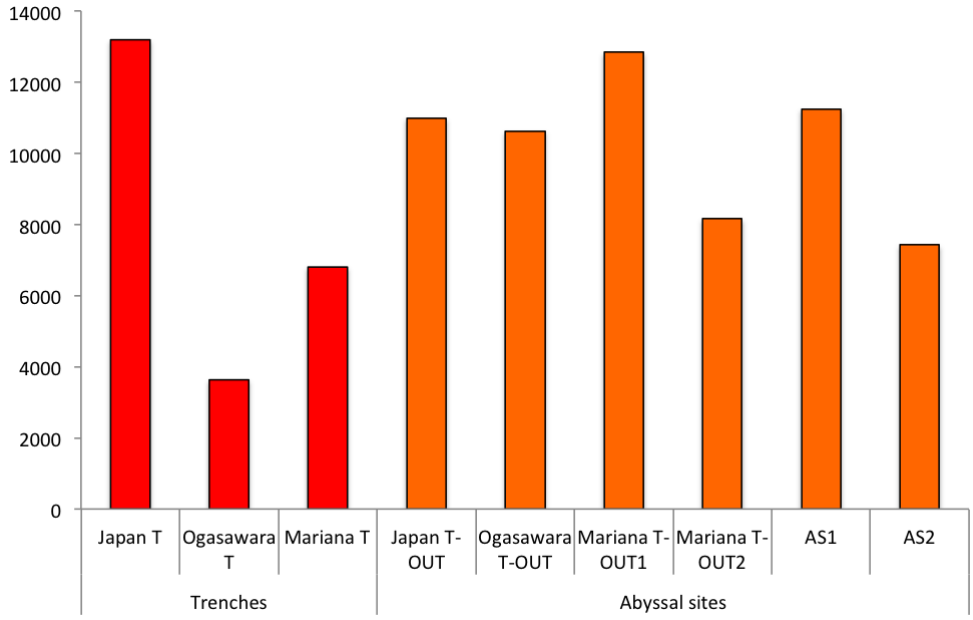

Fig.4 Bacteria observed OTUs number.

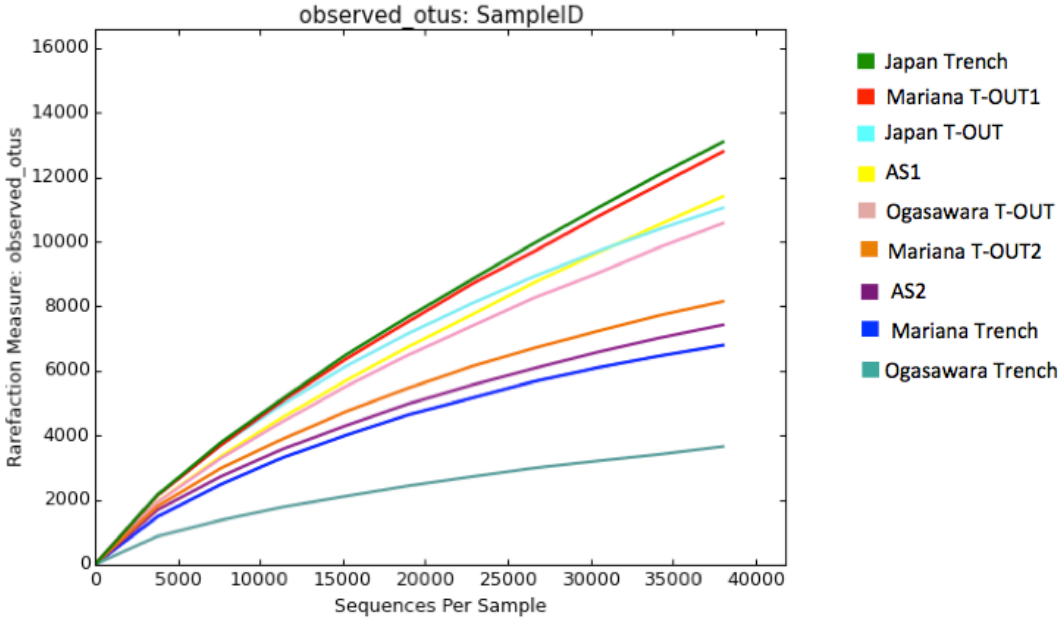

Fig. 5 Rarefaction curves for bacteria diversity based on observed OTUs.

Values of diversity (Shannon index) and equitability ranged from 8.4 to 11 and from 0.71 to 0.78 in the three explored trenches (Ogasawara and Japan Trench, respectively; Figure 6). With the exception of the Japan Trench, trenches' bacterial communities presented lower diversity and equitability compared those belonged to the abyssal sites.

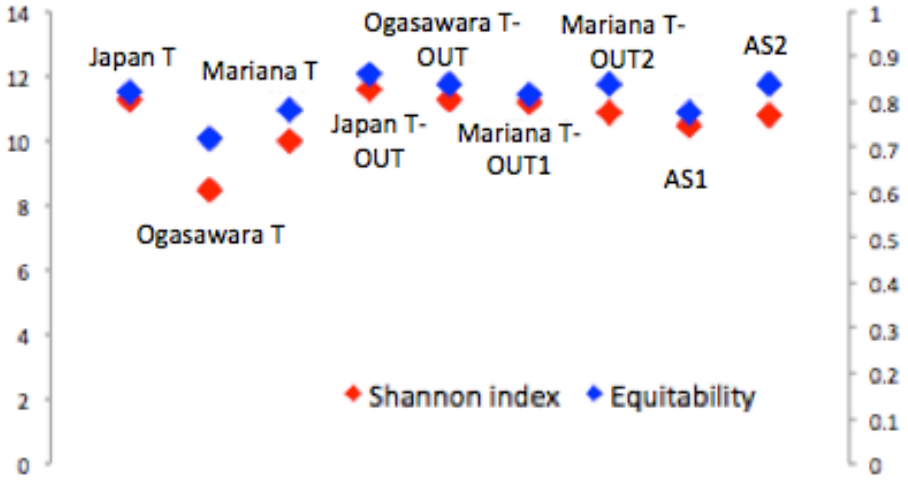

Fig. 6 Bacterial equitability and Shannon index values.

3.3.4 Taxonomy of bacterial assemblages

The number of bacterial 16SrDNA sequences obtained from the sequence analysis ranged from 38774 to 563098. The sequences percentage contribution of the predominant bacterial orders inhabiting each investigated sites is shown in Figure 7. The bacterial community belonged to the Japan Trench bottom sediments showed the predominance of the same taxa that majorly contributed to the bacterial communities inhabiting the sediments collected from the sites Mariana T-OUT1 and AS1. In these three sites the most abundant phylum was Bacteroidetes (percentage contribution between 27 and 34%), with the most contributory order *Bacteroidales* (25-33%). The other predominant orders were *Clostridiales* (phylum Firmicutes; 15-21%), and *Entomoplasmatales* (phylum Tenericutes; 11-15%). A high percentage contribution of unknown taxa was also found (ranged from 6,5% to 10%). The bottom sediments of Ogasawara and Mariana trenches as well as

the abyssal sites Japan T-OUT, Ogasawara T-OUT, Mariana T-OUT2 and AS2, were dominated by the phylum Proteobacteria (sequences percentage contribution ranging between 31 and 61%), with the predominance of the order *Thiotrichales* (γ-Proteobacteria; sequences contribution ranging between 9,5 and 17%), and also a high contribution of unclassified taxa (5%) was present. Other shared orders, differently contributory to the bacterial assemblages belonged to Ogasawara and Mariana trenches and to these four abyssal sites were *Phycisphaerales* and *Pirellulales* (Planctomycetes), *Clostridiales* (Firmicutes), *Flavobacteriales* (Bacteroidetes), *Acidimicrobiales* (Actinobacteria), *Rhodospirillales*, *Burkholderiales*, *Alteromonadales* and *NB1-j 6* (α-, β-, γ-, and δ-Proteobacteria, respectively).

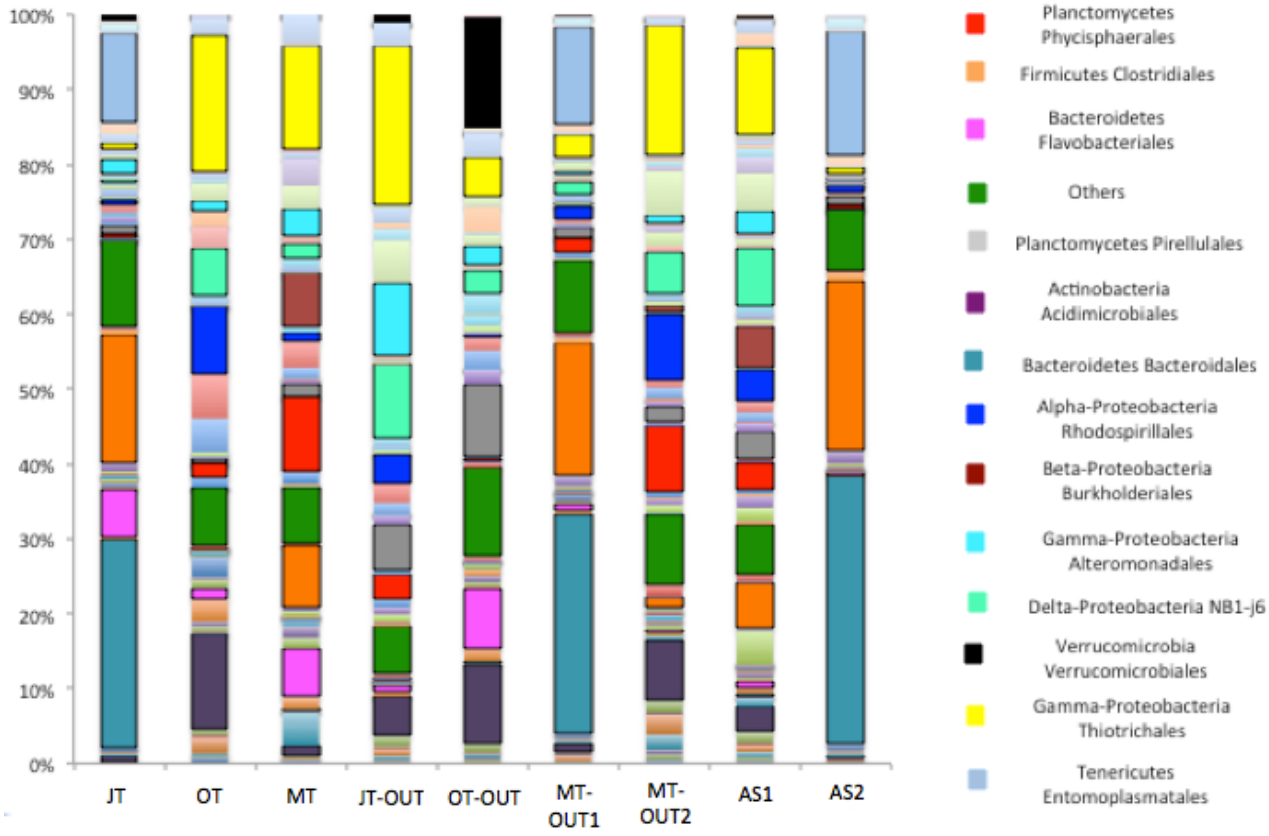

Fig. 7 Taxonomy composition of bacterial assemblages at the orders level.

The results of the cluster analysis showed that bacterial assemblages clustered in two highly divergent groups (Figure 8). Especially, the Japan Trench clustered differently respect to the Mariana and Ogasawara trenches that, on the contrary, showed higher similarity. Higher similarity was found between trenches' bacterial assemblages and those of the abyssal sites sited more distant compared those found close to the trenches.

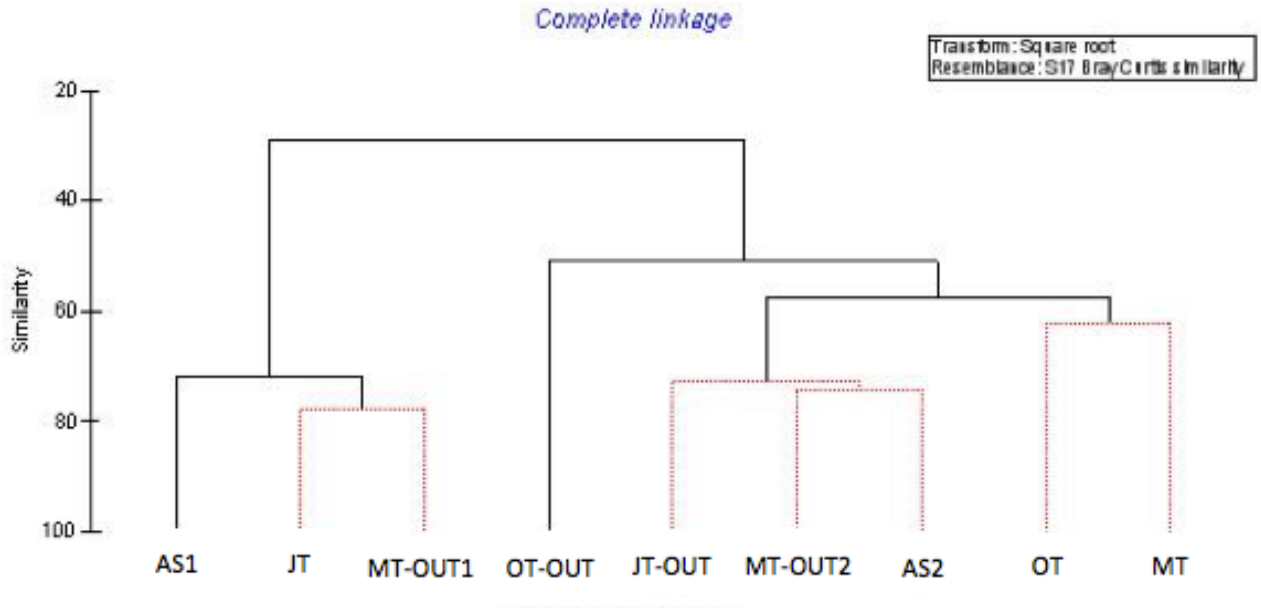

Fig. 8 Cluster analysis applied to the predominant bacterial orders.

3.3.5 Estimates of archaeal richness and diversity and rarefaction curves

The archaeal OTUs number obtained from the sequence analysis ranged from 2312 to 80481. Archaeal richness was calculated by considering 70.000 randomly selected sequences for each sample (Figure 9). The archaeal community of the Japan Trench presented the highest richness among the investigated trenches, and only sites Ogasawara T-OUT and Mariana T-OUT1 presented more observed OTUs (more than 25000), as rarefaction curves demonstrated (Figure 10).

Ogasawara and Mariana trenches' archaea presented the lowest richness among the investigated sites (1286 and 4654, respectively) and were exhaustively sampled as their rarefaction curves showed reaching the asymptote. This was true also for the abyssal sites Japan T-OUT, Mariana T-OUT2 and AS2.

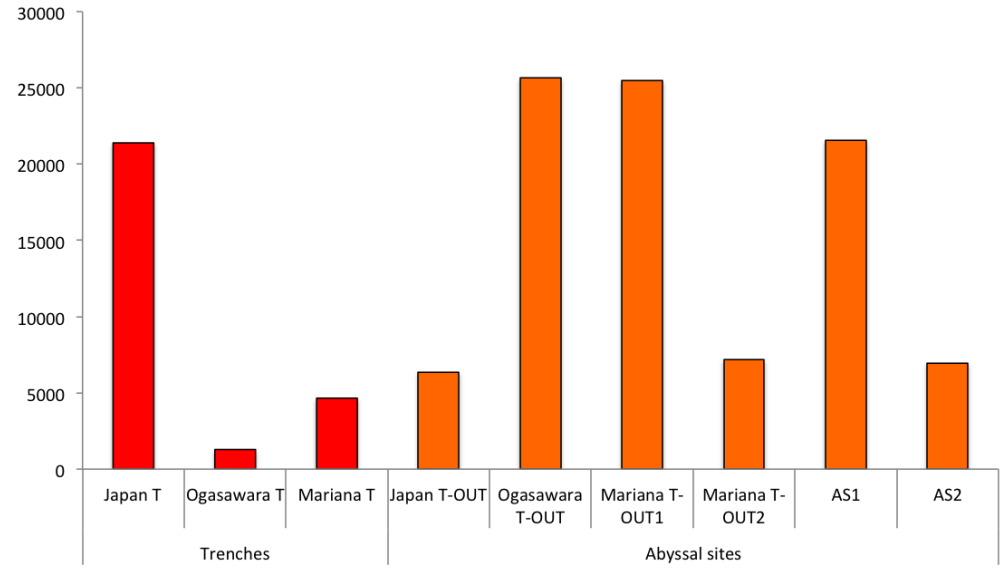

Fig. 9 Archaea observed OTUs number.

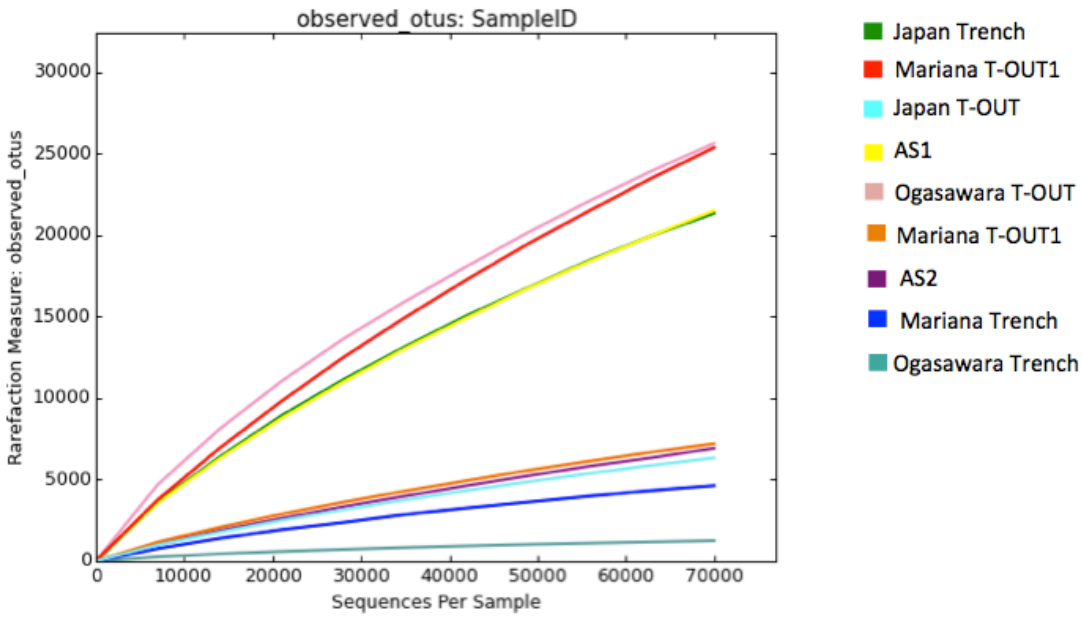

Fig. 10 Rarefaction curves for archaeal diversity based on observed OTUs.

The archaeal community of the Japan Trench showed higher diversity and equidistribution compared to Ogasawara and Mariana trenches, which presented lower values also then the abyssal sites (Figure 11).

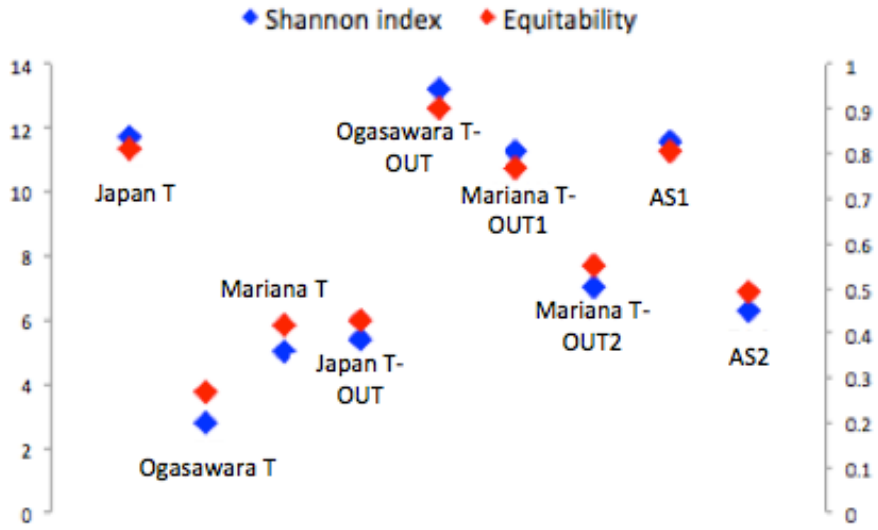

Fig. 11 Archaeal equitability and Shannon index values.

3.3.6 Taxonomy of archaeal assemblages

The number of archaeal 16SrDNA sequences obtained from the sequence analysis ranged from 82843 to 613792. The archaeal assemblages that were present in Ogasawara and Mariana trenches showed the almost complete predominance of the phylum Thaumarchaeota (93% and 98% respectively), as also in the abyssal sites Mariana T-OUT2, Japan T-OUT and AS2 with percentage contribution of 98%, 97% and 99%, respectively (Figure 12). In Ogasawara and Mariana trenches the genus *Nitrosopumilus* of the phylum Thaumarchaeota was predominant, 92% and 94% respectively, while in sites Mariana T-OUT2, Japan T-OUT and AS2 accounted for a lower fraction (39%, 71% and 60%, respectively). In the Japan Trench the archaeal assemblage was mainly unclassified (67%) and this was true also for the sites Mariana T-OUT1, AS1, and Ogasawara T-OUT

(56%, 61% and 95%, respectively). In each explored site the phyla Euryarchaeota and Parvarchaeota gave a minor contribution to the archaeal communities.

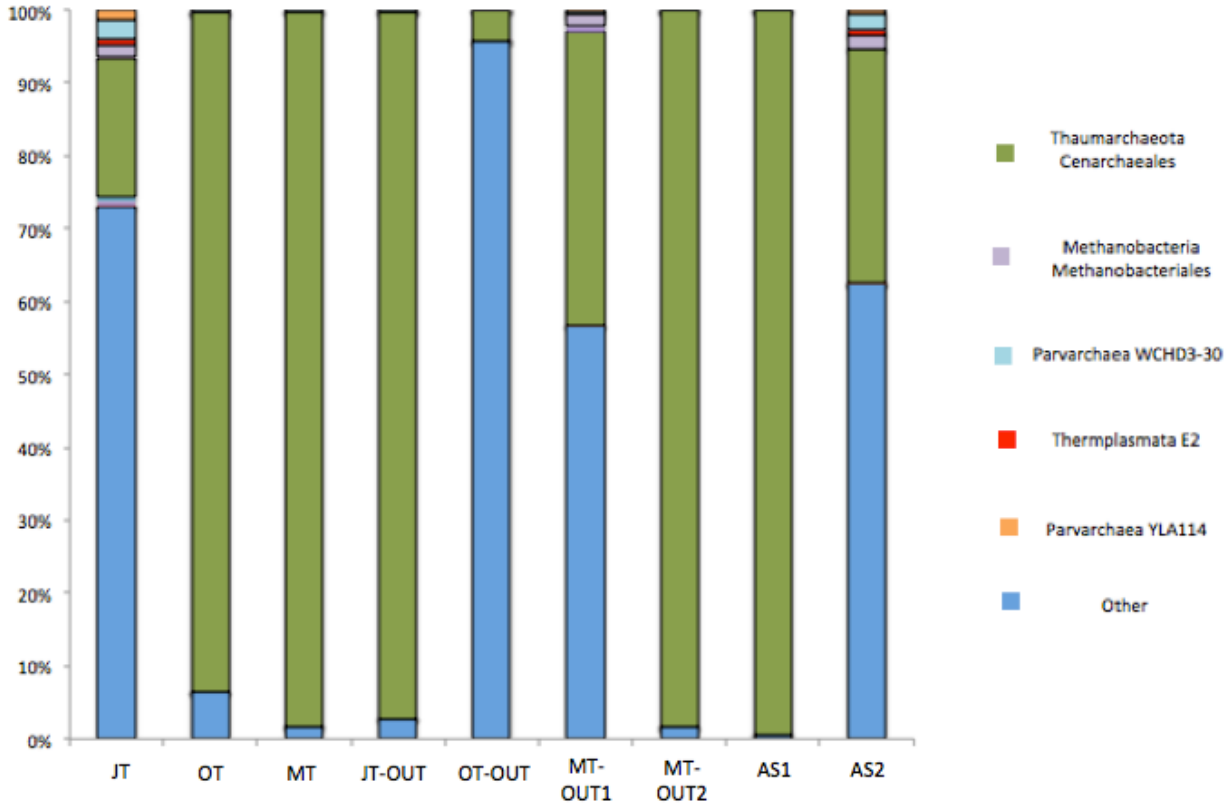

Fig. 12 Taxonomy composition of archaeal assemblages at the orders level.

The cluster analysis on archaeal taxonomy diversity showed two highly divergent groups (Figure 13). The Japan Trench clustered differently respect to the Mariana and Ogasawara trenches that, on the contrary, showed higher similarity.

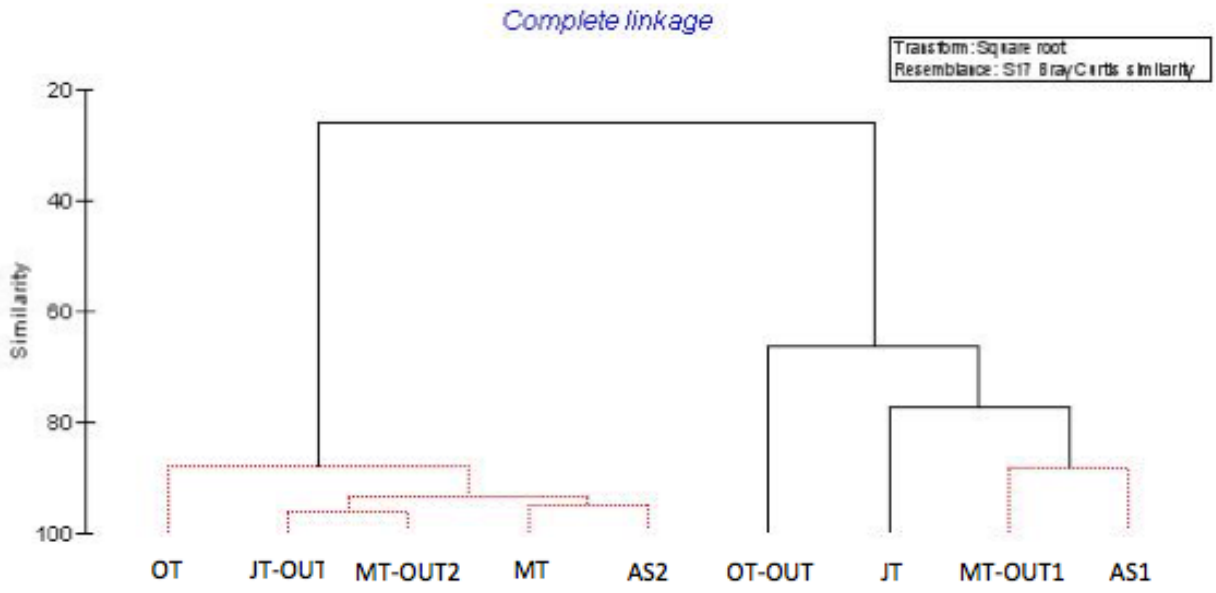

Fig. 13 Cluster analysis applied to the predominant archaeal orders.

3.3.7 Influence of environmental variables on hadal microbial assemblages

The distance-based redundancy analysis (dbRDA) was used to explore the relationships between the bacterial and archaeal community composition inhabiting the three investigated trenches and each selected predictable variable. The analysis showed that bacterial and archaeal diversity in Mariana Trench were influenced by depth (r=0.847 and r=-0.961, respectively), while in Ogasawara Trench latitude was the variable that mainly influenced both the prokaryotic assemblages (r=-0.824 and r=0.934, respectively; Figure 14-15). The bacterial and archaeal communities belonged to the Japan Trench did not show any correlation with the predictable variables and were separated from those of the other two sites.

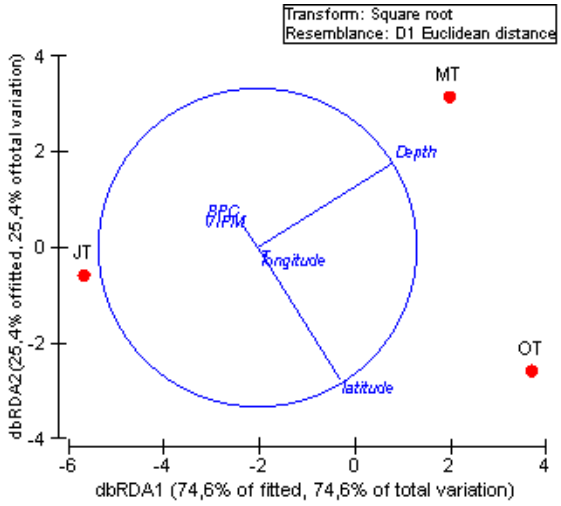

Fig. 14 dbRDA analysis of variation of bacterial taxonomic diversity.

JT=Japan Trench, MT=Mariana Trench, OT=Ogasawara Trench.

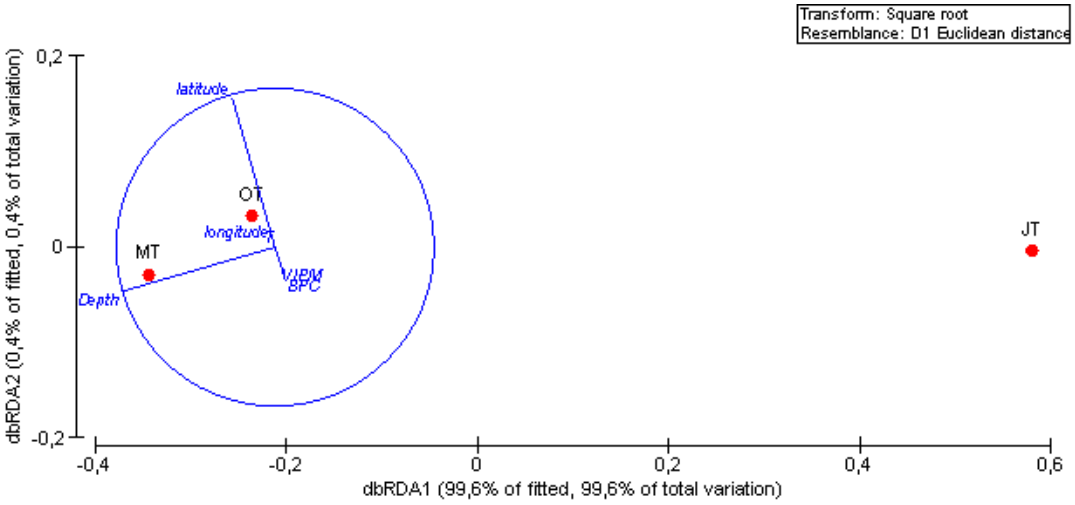

Fig. 15 dbRDA analysis of variation of archaeal taxonomic diversity.

JT=Japan Trench, MT=Mariana Trench, OT=Ogasawara Trench.

3.4 Discussion

Previous studies have revealed high prokaryotic abundances inhabiting the sediments of different hadal systems (Danovaro et al., 2003; Glud et al., 2013), but to which extent bacteria and archaea contribute to the assemblages has never been investigated.

Our study revealed at the bottom of the three explored trenches, Japan, Ogasawara and Mariana, the presence of archaea abundances exceeding up to twenty times the abundances found in the abyssal sites, as also the number of bacterial cells in these hadal systems over-passed that of the farthest abyssal site up to eighteen times. These results confirm the presence of high prokaryotic abundances within the investigated trenches, and for the first time demonstrate the high contribution that archaea give to the structure of such prokaryotic assemblages. The important presence of archaea at depths ranging from 8000 to 10900 m below the sea-surface also confirm the increase abundance of this microbial component with depth (Danovaro et al., 2015).

In order to characterize the investigated prokaryotic assemblages, besides analysing their community structure, we also explored their diversity. Both archaeal and bacterial richness detected within the Japan Trench demonstrated the presence of an extraordinary and incompletely sampled diversity, being similar or even higher than that found in the abyssal sites. We noted a high fraction of unrevealed taxa, which should have been even greater if we had not excluded the more rare species in alpha-diversity analysis (single and double-tons, sequences that occur only once or twice in the full dataset; Gobet et al., 2013). Despite the lower bacterial and archaeal richness found in Ogasawara and Mariana trenches, their values were comparable or even greater than that found in other benthic deep-sea habitats (Bienhold et al., 2016; Huber et al., 2007), suggesting hadal systems potentially able to host high microbial biodiversity. The taxonomic analysis revealed a high fraction of unknown taxa contributing to such diversity in all investigated sites ranged from 5 to 10% for bacteria and up to 95% for archaea in the Japan Trench. In comparing the taxonomic archaeal and bacterial composition of the microbial assemblages belonged to the hadal sites, we observed that both the components showed distinct prokaryotic community signatures, with

Mariana and Ogasawara Trench communities showing higher similarity, especially respect the archaeal components. In particular, the genus *Nitrosopulimus* (phylum Thaumarchaeota) was highly predominant, contributing for more than 90% to the archaeal assemblages in Ogasawara and Mariana trenches. This cluster, constituted by ammonia-oxidizing archaea (AOA), has been confirmed to be present in several habitat types (Cao et al., 2013), and thought to paly a pivotal role in favouring biomass production in hadal benthic habitats (Nunoura et al., 2013). On the contrary, very little overlap was found comparing the prokaryotic assemblages of the Mariana and Ogasawara trenches with those of the Japan Trench. This low similarity may reflect diverse local factors acting and influencing such microbial communities. To investigate relationships between hadal prokaryotic composition and environmental variables, also considering viral infection as recognized potential forcing able to shape microbial diversity (Paul, 2008; Weinbauer & Rassoulzadegan, 2004; Breitbart, 2012), a dbRDA analysis was performed. Here, we observed that within the sediments of the Ogasawara Trench both bacterial and archaeal composition was related with the latitude while in the Mariana Trench the relation was higher with the depth. On the contrary, the microbial assemblages belonged to the Japan Trench did not reveal any relation with the selected variables, thus remarking the evident separation from the other two hadal communities.

By comparing the hadal and abyssal prokaryotic communities diversity, a high similarity between trenches' prokaryotic composition and those of the abyssal sites also sited at great distances from hadal systems was observed. Especially, the order *Clostridiales* was highly contributory to bacterial communities belonged to the Japan and the Mariana Trench as well as in different abyssal sites. This order is known to comprehend termophilic species endospore-forming, typical inhabitants of deep-sea hydrothermal vents, found also in sediment samples collected from cold Arctic fjords as dormant spores and in such form passively transportable by currents even for long distances (Slobodkina et al., 2008; Hubert et al., 2010; Müller et al., 2014). The presence of this bacterial order within the investigated trenches may imply that hadal systems can host prokaryotic taxa widespread in abyssal sediments suggesting a partial level of connectivity with the abyssal systems.

3.5 Conclusions

Our study reveals higher archaeal abundances within the investigated trenches and the predominance of the ammonia oxidizer *Nitrosopumilus* both within the Mariana Trench and in Ogasawara Trench suggesting the important contribution of archaea to hadal microbial communities. The high prokaryotic richness and diversity found at the bottom of the Japan Trench put in light that hadal ecosystems can be potential hot spot of microbial biodiversity, despite their extreme environmental factors, which we discovered to differently influence each trench's microbial assemblage. The presence within the hadal sediments of prokaryotic taxa found in abyssal habitats leads to hypothesize that hadal trenches are only partially connected to the surrounding benthic ecosystems.

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Chapter 4.

Anthropogenic impact due to mining activity on virus-prokaryote interactions and prokaryotic diversity in abyssal ecosystems of the Pacific Ocean

4.1 Introduction

Due to the progressive depletion of mineral resources in terrestrial ecosystems, the exploitation of mineral deposits, such as manganese nodules, cobalt-rich manganese crusts, and polymetallic sulfide deposits on the deep ocean floor, are receiving great attention to cope the increased mineral demands (Emery & Skinner, 1977; Earney, 1990; Hyun, 2006). On the basis of this, it is expected that in the near future the anthropogenic exploitation of mineral resources in the deep oceans will represent a major threat for benthic deep-sea habitats. For instance, recent studies suggest that the exploitation of polymetallic nodules can have an impact over an area of 600 to 8,000 km^2 per year, and that after 15 years of mining the impact can occur over ca. 120,000 km^2 of seafloor (Smith et al., 2009). The Clarion-Clipperton Fracture Zone (CCFZ) located in the equatorial Pacific Ocean is considered one of the most commercially important nodule areas of the world oceans (Thiel, 2001; Wang, 2010). Also known as the Nodule Belt, this area consists of 5 million km^2 and it has been estimated that 5-10 billion tons of nodules could be exploited (Baker et al., 2001). Previous studies conducted in this oceanic zone after a simulated mining activity demonstrated that the disturbance created by the dredge profoundly modifies the distribution of different chemical species in the pore water of the sediment and that such an effect can remain for an unknown period of time being not restored through diffusive or bioturbation processes (Khripounoff et al., 2006). Other investigations pointed out that the recovery of benthic deep-sea ecosystems following mining

impacts can occur at very low rates, requiring decades or more for the soft-sediment fauna and thousands to hundreds of thousands of years for the biota associated to manganese nodules (Glover and Smith, 2003; Hannides and Smith, 2003; Smith et al., 2008). This probably will imply severe modifications of biodiversity, community assemblage composition and functioning of the benthic deep-sea ecosystems of the CCFZ, whose living components, their interactions and related ecological processes are still scantly known (e.g. due to the technical and logistic difficulties in collecting data and the great heterogeneity of the habitat characteristics; Glover et al., 2016).

It is now recognised that prokaryotes and viruses and their interactions have an important role in the functioning and biogeochemical processes of benthic deep-sea ecosystems (Danovaro et al., 2008; Corinaldesi, 2015; Dell'Anno et al., 2015; Danovaro et al., 2016). Viruses indeed, by infecting and killing prokaryotes, can accelerate biogeochemical processes and can reduce the transfer of energy and material to the higher trophic levels (Fuhrman, 1999; Wommack, 2000; Weinbauer, 2004; Suttle, 2005; Suttle, 2007; Dell'Anno, 2015). However, information on the interactions between prokaryotes and viruses in abyssal ecosystems is still limited and absent for the CCFZ. Viruses by killing their hosts can also modify the prokaryotic diversity and assemblage composition (Weinbauer & Rassoulzadegan, 2004; Bouvier & Del Giorgio, 2007; Weitz et al., 2015). Benthic prokaryotic diversity in the Clarion-Clipperton Fracture Zone and the Pacific nodule province was discovered to be high with a general predominance of Proteobacteria, especially α- and γ-Proteobacteria, possibly involved in sulfur and nitrogen cycling, and a good representation of Taumarchaeota MG-1 group likely supporting ammonia oxidation and carbon fixation processes (Xu et al., 2005; Xu et al., 2007; Wang et al., 2010). By comparing the microbial communities inhabiting nodules and the surrounding sediments it was also observed that nodules host different microbial communities (Wu et al., 2013; Tully & Heidelberg, 2013). A recent study aiming at performing a taxonomical and functional analysis of a microbial community inhabiting ores-rich stream sediments highlighted the occurrence of rare and highly adapted prokaryotic taxa to metal-rich habitats (Reis et al., 2016). All these findings suggest the presence of a high prokaryotic diversity in nodule-rich areas, which can contribute significantly to the whole microbial diversity of

benthic deep-sea ecosystems. However, no information is available on the impact of deep-sea mining activity on virus-prokaryote interactions and prokaryotic diversity. The specific goal of this study was to investigate the distribution of prokaryotes and viruses in superficial sediments of different exclusive economic areas of the CCFZ subjected to simulated mining activities carried out at different time scales. In particular, we explored for the first time how deep-sea mining can modify virus-prokaryote interactions and the microbial diversity with downstream effects on the functioning of the benthic food webs and biogeochemical cycles.

4.2 Materials and Methods

4.2.1 Study area and sampling

The study area is located within the Clarion and the Clipperton Fracture Zones (CCZ) in the equatorial Pacific Ocean (between 11° 50' 18.60" N 117° 3' 16.20" W and 14° 2' 7.20" N 130° 5' 31.20" W). This region has been licensed for seabed exploration by the International Seabed Authority (ISA) that granted contracts to twelve separate entities with the aim to establish the guidelines on the base of which minerals exploitation activity will be managed. Sediment samples were collected on board of the R/V Sonne (March-April 2015) in four exclusive economic zones, the French, the German, the Belgian, and the Interoceanmetal Joint Organization (IOM) areas (Figure 1), in the framework of the European Project MIDAS (Managing Impacts of Deep Sea Resources Exploitation). Sampling strategy included the collection of sediment samples in both undisturbed and disturbed sites that were subjected to simulated mining activities carried out at different time scales. Sediment samples were collected in the German area (11° 50' N, 117° 03' W) from six undisturbed sites and two impacted sites after three years from the impact, in the IOM area (11°04' N, 119°39' W) from nine undisturbed sites and two impacted sites 20 years after the mining activity, in the Belgian area (13°52' N, 123°15' W) in five undisturbed sites and in two impacted

sites both after few days and after eight months from the impact, and in the French area (14°02' N, 130°07' W) in seven undisturbed sites and in two impacted sites 3 and 37 years after the impact. The sediments of the impacted sites were collected by using push-corers mounted on ROV, while the sediment samples of undisturbed areas (hereafter referred as control sites) were collected through multiple-corer deployments. Surface sediment samples of the top 1 cm were used for the analysis of the biochemical composition of the organic matter, prokaryotic abundance, biomass and diversity, viral abundance and production and extracellular enzymatic activities. Samples for the determination of extracellular enzymatic activities and viral production were processed immediately after the collection of the sediments. For all other variables investigated samples were stored at -20°C till laboratory analyses.

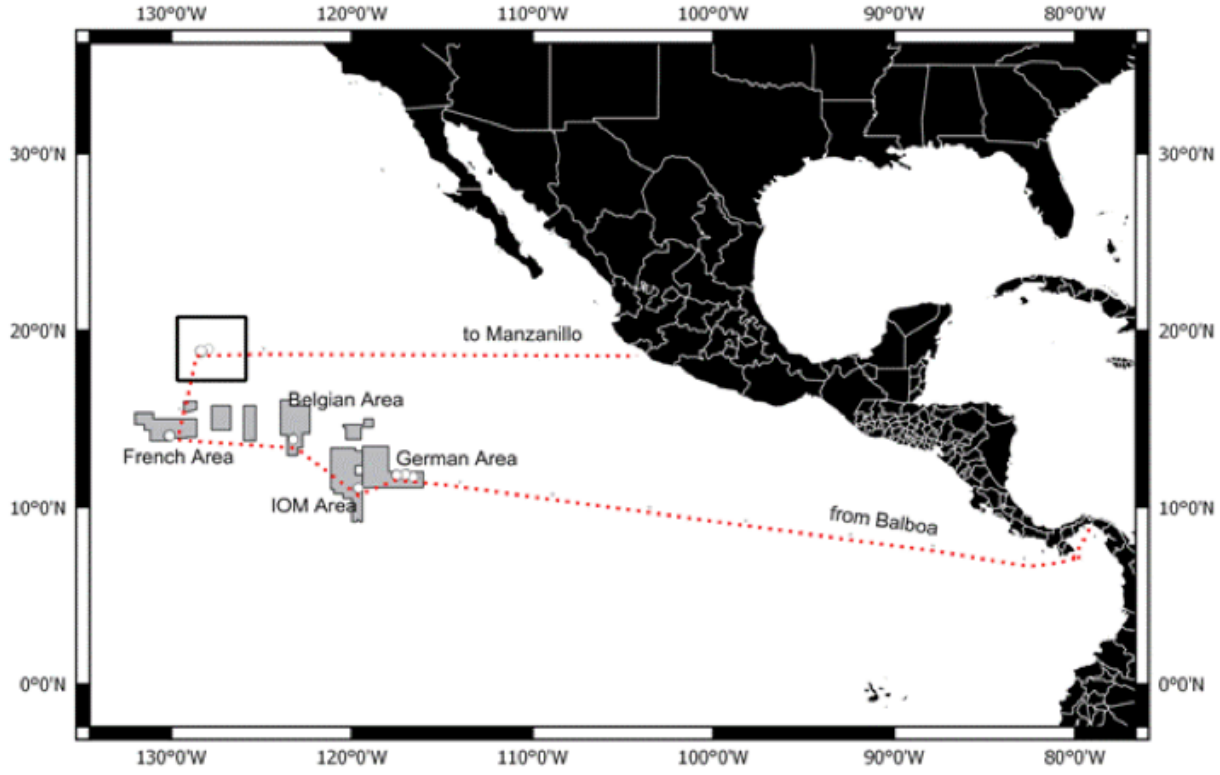


Fig. 1 Sampling areas in the Clarion-Clipperton Fracture Zone.

4.2.2 Biochemical composition of organic matter

The biochemical composition of organic matter was analysed in terms of chloroplastic pigment, protein, carbohydrate and lipid concentrations. All analyses were carried out on the first 0-1 cm of sediment from each sediment core.

Chloroplastic pigments (chlorophyll-a and phaeopigments) were analysed fluorometrically according to Lorenzen and Jeffrey (1980). Pigments were extracted with 90% acetone (24 h in the dark at 4°C). After centrifugation (800 X g), the supernatant was used to determine the functional chlorophyll-a and acidified with 0.1 N HCl to estimate the amount of phaeopigments. Total phytopigment concentrations (CPEs) were defined as the sum of chlorophyll-a and phaeopigment concentrations (Danovaro et al., 1999a,b).

Protein content (PRT) was determined according to Hartree (1972) modified by Rice (1982) to compensate for phenol interference. Concentrations are calculated from calibration curves of standard solutions of BSA, normalized to sediment dry weight and expressed as milligram of albumin equivalents per gram of dry sediment. Carbohydrates (CHOs) were analysed according to Gerchacov and Hachter (1972) and expressed as glucose equivalents normalized to sediment dry weight. The method is based on the same principle as the widely used method of Dubois et al. (1956), but it is specifically adapted for carbohydrate determination in sediments. Lipids (LIPs) were extracted by direct elution with chloroform and methanol and analysed according to Marsh and Wenstein (1966). Lipid concentrations are reported as tripalmitine equivalents normalized to sediment dry weight. For each biochemical analysis, blanks were made with the same sediment samples as previously treated in a muffle furnace (450°C, 2 h). The sum of the carbohydrate, protein and lipid concentrations converted into carbon equivalents (using the conversion factors of 0.40, 0.49 and 0.75 mg C mg^{-1}, respectively) was defined as the biopolymeric organic carbon (Pusceddu et al, 2009).

4.2.3 Prokaryotic abundance and biomass

For prokaryotic counting, *ca.* 1 cm^3 of superficial sediments of each sediment core were analysed as previously described by Danovaro et *al.* (2002). Benthic bacteria were detached from sediment by using pyrophosphate at a final concentration of 5 mM, and were sonicated by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W) to increase the extraction efficiency. For bacterial counting, subsamples were diluted 50-fold with sterilized 0.2 µm-filtered seawater and stained with Acridine Orange (final concentration, 0.01%). After the incubation at the dark, the samples were filtered onto 0.2-µm-pore-size black Nuclepore filters with vacuum pressure of 150 mm of Hg by using sterile glass filter holders for 25 mm diameter filters with 15 ml glass funnel (Millipore). Acridine Orange was used instead of SYBR Green I for bacterial analysis because it provided similar counts but did not result in the overestimation of biovolume. All filters were analysed by epifluorescence microscopy using a Nikon ECLIPSE Ni-U microscope equipped with a micrometric scale to examine the cell dimensions and estimate the biovolume. Ten to 50 fields were viewed at a magnification of X1000, and a minimum of 400 bacterial cells was counted. The prokaryotic abundance was normalised to sediment dry weight after desiccation at 60°C for 24 h. Prokaryotic biomass was estimated on the basis of prokaryotic biovolume, using a conversion factor of 310 fg of C μm^{-3} (Fry, 1990; Corinaldesi et al., 2008).

4.2.4 Extracellular enzymatic activities

Analyses of l-aminopeptidase, β-d-glucosidase, and alkaline-phosphatase activity were carried out on surface sediment samples collected at selected sites of the German, IOM, and Belgian areas. Sediment slurries (prepared with pre-filtered sterilized seawater, 1:1 v/v) were incubated for 2 h (enzymatic activity increased linearly with time) in the dark and at in situ temperature with l-leucine-4-methylcoumarinyl-7-amide (MCA), 4- methylumbelliferone β-d-glucopyranoside (MUF), and 4-methylumbelliferone phosphate (MUF-P) respectively as substrates (final concentrations of MCA 200 µM, MUF 200 µM, and MUF-P 50 µM determined after kinetic experiments). Sediment

sub-samples incubated without fluorogenic substrates were used as blanks and processed according to the procedure followed for fresh sediment samples. After incubation, samples were centrifuged (800X g) and the supernatants were analysed fluorometrically according to Hoppe (1993). Enzymatic activities were expressed as nmol of substrates hydrolysed per hour and normalized to sediment dry weight. The specific degradation activity per prokaryotic cell was estimated as the ratio between the sum of all enzymatic activities and the total prokaryotic abundances.

4.2.5 Virus abundance

Viral counting was carried out on *ca.* 1 cm^3 of surface sediments of each sediment core as described by Danovaro et *al.* (2001). Sediment samples were diluted in 5 ml volume of virus-free seawater filtered at 0.02 μm pore size using disposable syringe filters (Anotop 25, Whatman), and treated with pyrophosphate at a final concentration of 5mM. Samples were sonicated by ultrasound for three minutes with 30 seconds interval between each minute (Branson 2200 Sonifier, 60 W) to increase the extraction yield. The samples were then diluted 25-fold in virus-free seawater and incubated with the addition of DNase I from bovine pancreas (2-5 Uml^{-1}) in order to eliminate uncertainties in virus counting due to extracellular DNA interference. After the incubation, samples were filtered onto 0.02 μm pore size filters (Anodisc Al^2 O^3; 25mm diameter, Whatman) with vacuum pressure of 150 mm of Hg by using sterile glass filter holders for 25 mm diameter filters with 15 ml glass funnel (Millipore). Then the filters were stained using SYBR Gold (10000X concentrated in anhydrous dimethyl sulphoxide; Molecular Probes-Invitrogen, Grand Island, NY, USA) at a final concentration of 2X. SYBR Gold was used instead of SYBR Green I to detect both double- and single-stranded DNA and RNA, and because its strong brightness and stability as fluorescence stain. Viral counting was performed under epifluorescence microscopy, using a Nikon ECLIPSE Ni-U microscope, by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. The viral abundance was normalised to sediment dry weight after desiccation at 60°C for 24 h.

4.2.6 Viral production, turnover and virus-induced prokaryotic mortality (VIPM)

Viral production was analysed in selected surface sediment samples collected in the German, IOM, and Belgian areas, by a dilution-based procedure specifically developed for sediment samples (Dell'Anno et *al.* 2009). Briefly, sediment samples were transferred into sterile tubes and mixed with an equal volume of 0.02 μm pre-filtered virus-free seawater and incubated at in situ temperature up to 12 hours. Aliquots of samples (0.5 ml) were collected at 0, 3, 6 and 12 hours. After the extraction of viruses from each sediment samples as described above, viral counting was performed under epifluorescence microscopy by examining at least 20 fields per slide, and counting at least 400 viral particles per filter. Viral production was calculated on the basis of the maximum increment of viral abundance occurred over time.

Viral turnover was calculated as the ratio between viral production and viral abundance and expressed as turnover time (h).

The number of prokaryotes killed by viruses were estimated from the ratio between viral production and burst size, assuming a burst size of 45, reported for deep-sea sediments worldwide (Danovaro et al., 2008). VIPM was estimated as the ratio between the number of prokaryotes killed by viruses and the total number of prokaryotes present in the samples and expressed as percentage.

4.2.7 DNA extraction suitable for molecular analysis

DNA used for the analysis of the prokaryotic diversity was extracted from two independent replicates of sediment samples collected from impacted and un-impacted sites of each investigated CCFZ area by using the kit PowerSoil DNA isolation kit (MoBio Laboratories Inc., CA, USA). Before DNA extraction, sediment samples were pre-treated with a chemical–physical procedure to remove as much as possible the extracellular DNA (Fortin et al., 2004; Danovaro, 2010).

4.2.8 Sequencing and bioinformatics

Selected superficial sediment samples from both undisturbed and impacted sites of all the four studied areas were used to analyse the genetic diversity of the bacterial 16S rDNA sequences associated with the microbial DNA pools. Analyses were conducted by sequencing hypervariable regions V3 and V4. Amplicons were generated using the universal primers 314F-805R, 5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3', respectively (Klindworth et al., 2013) and were sequenced by applying the Illumina MiSeq, V3 technology, 2x300 paired-end sequencing.

Raw sequences were first subjected to paired-end merging with the FLASH software (Magoč & Salzberg, 2011), and joined pairs were then quality-trimmed by means of the USEARCH tool (Edgar, 2010) by removing sequences with a maximum error of 1.0. High-quality amplicon reads from each sampling region (the Belgian, French, German and IOM areas) were subsequently analysed separately by the QIIME pipeline (Caporaso et al., 2010), aligning them against the GreenGenes database (DeSantis et al., 2006) using the open-reference strategy at 97% of similarity (Rideout et al., 2014). Alpha-diversity indices (i.e. Shannon index, Pielou's index and the phylogenetic diversity) were calculated by the same pipeline. Weighted normalized UniFrac distance (Lozupone & Knight, 2005) was used as a beta-diversity index and a Neighbour-Joining tree was constructed from the distance matrix created by QIIME using such index and visualized with FigTree. The program STAMP (Parks et al., 2014) was used to assess the statistically-significant differential abundance of bacterial taxa between time intervals in each region; an MDS on the percentage of reads contributing to each OTU in each sample was constructed by the PRIMER-E 6 software. The same program was also used to calculate average similarity scores between samples and average dissimilarity scores between time intervals of sampling, and such scores were subsequently added to the MDS plot.

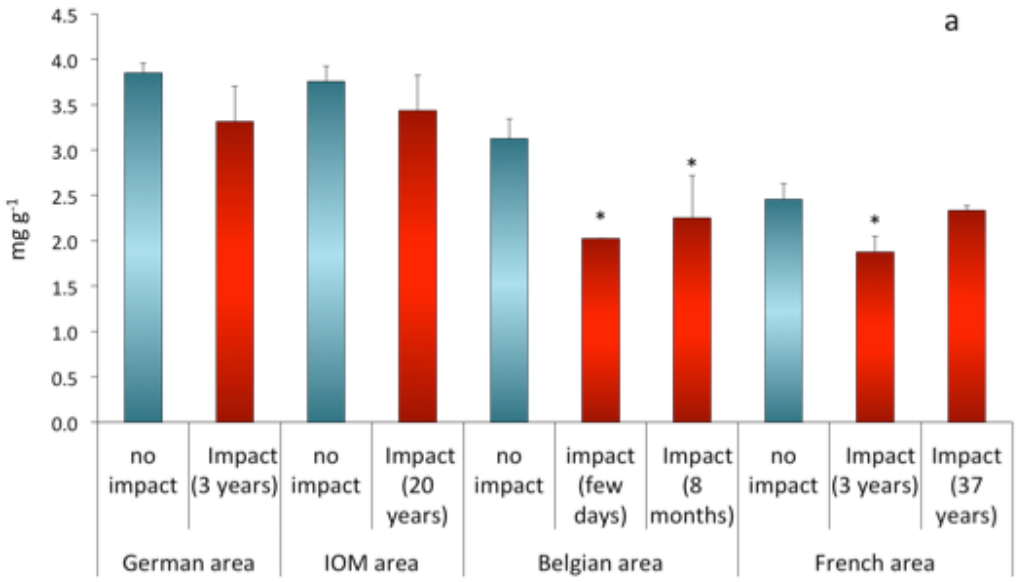
4.2.9 Statistical analyses

To test for differences in the investigated variables between impacted and un-impacted sites of each investigated CCFZ area analysis of variance (ANOVA) was carried out. To identify factors influencing prokaryotic abundance among impacted and undisturbed sites in the German, IOM and Belgian areas a DistLM analysis was performed. Biopolymeric carbon content, viral production and water column depth were used as predictor variables.

4.3 Results

4.3.1 Impact on trophic resources

Biopolimeric C and total phytopigment concentrations in the sediments of the German and IOM areas were higher than values found in the Belgian and the French ones (Figure 2a-b). BPC concentrations were significantly lower in the impacted sites of the Belgian and French areas, with the exception of the 37 years-impacted site when compared to un-impacted sites. With the exception of the German area, CPE concentrations were lower in the sediments of the impacted sites than in un-impacted sites.



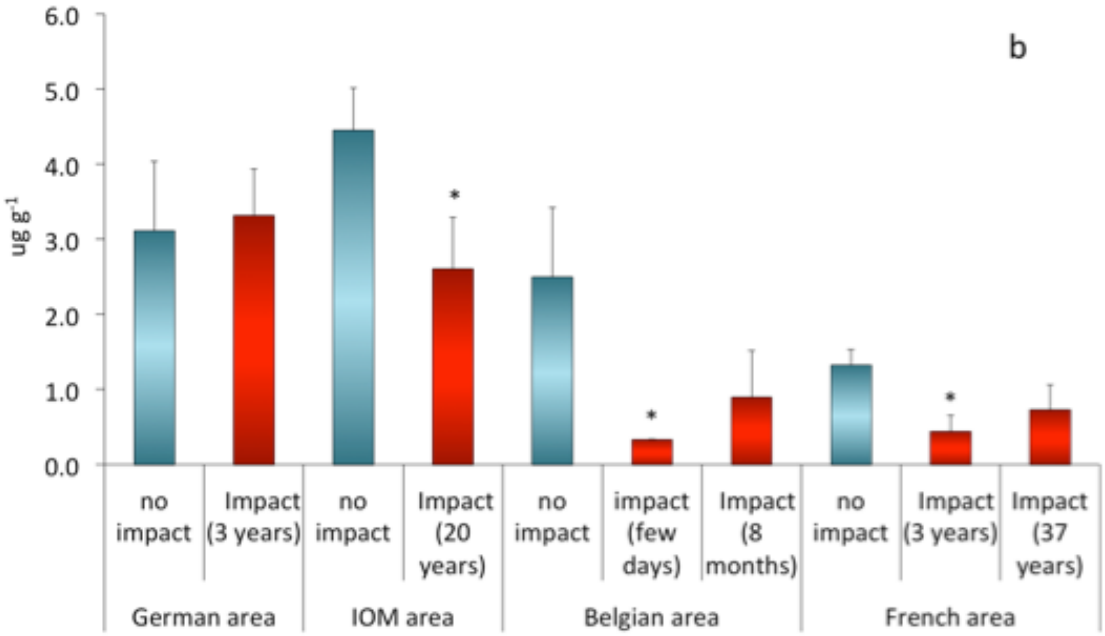


Fig. 2 Biopolymeric carbon content (a); total phytopigment content (b).

4.3.2 Impact on benthic deep-sea prokaryotes

Prokaryotic abundances were significantly higher ($P<0.05$) in un-impacted sites of all areas respect the impacted sites, with the exception of the less recently impacted site (37 years) in the French area (Figure 3a). The same pattern was observed for prokaryotic biomass, with the exception of the 8 months impacted site in the IOM area and again the 37 years impacted site in the French area (Figure 3b). Prokaryotic abundances ranged from 3.96±0.3 $\times 10^6$ cells g^{-1} in the few days-impacted site in the Belgian area to 3.25±0.9 $\times 10^7$ cells g^{-1} in the un-impacted German areas. Prokaryotic biomass followed the same pattern with the lowest value in the impacted sites (few days) in the Belgian area and the highest value in the un-impacted sites of the German area (0.23±0.0 and 2.90±0.98 µC g^{-1} respectively).

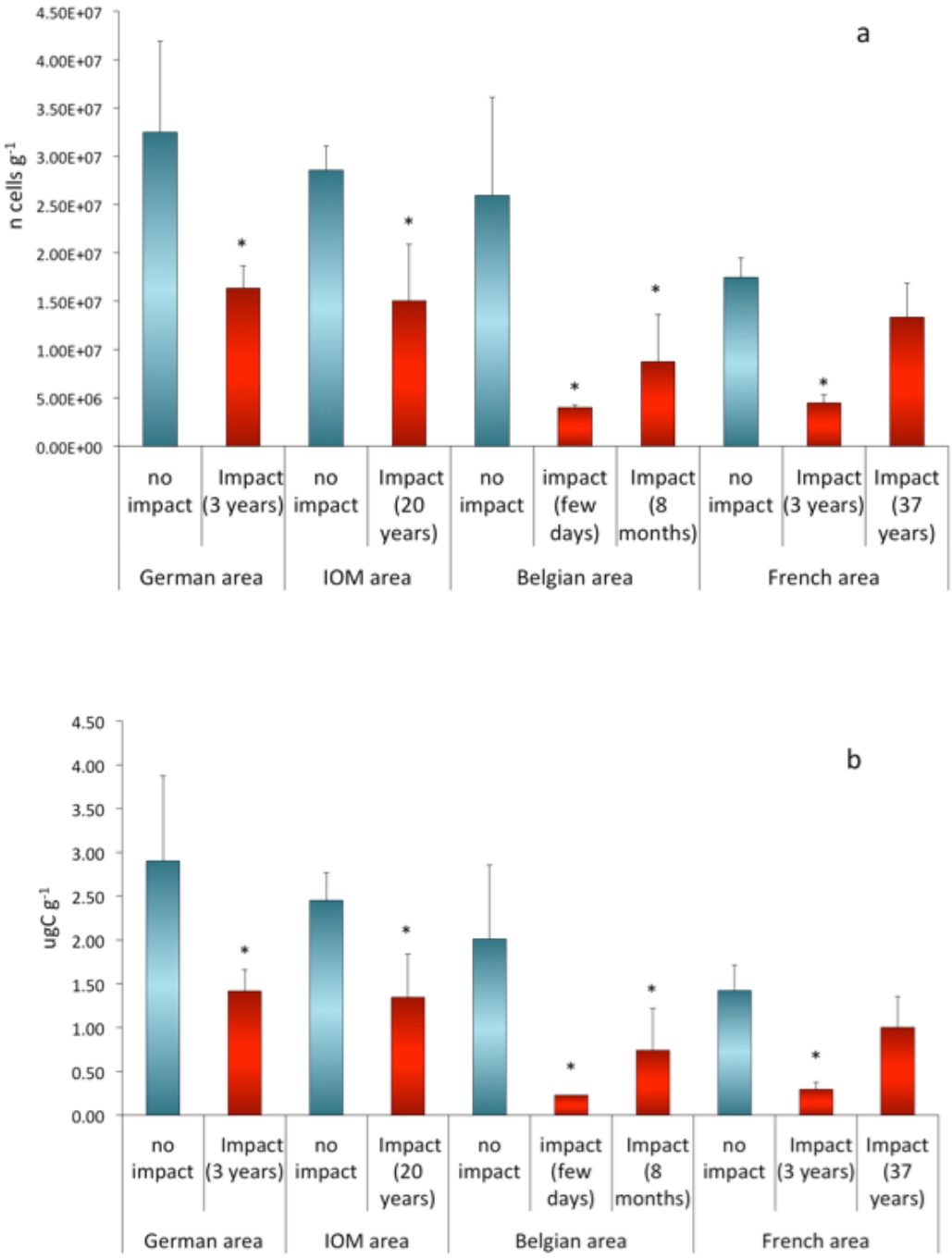


Fig. 3 Prokaryotic abundance (a); prokaryotic biomass (b).

4.3.3 Impact on extracellular enzymatic activities

In the German and Belgian areas all the enzymatic activities showed values significantly lower ($P<0.05$) in the impacted sites, while in the IOM area only the aminopeptidase activity was

significantly lower after 20 years from the impact (Figure 4a-b-c). In the IOM and Belgian areas the specific degradation activity per cell was significantly higher ($P<0.05$) in the impacted sites compared the undisturbed sites (Figure 5), while in the German area there was not a significant difference between impacted and un-impacted sites.

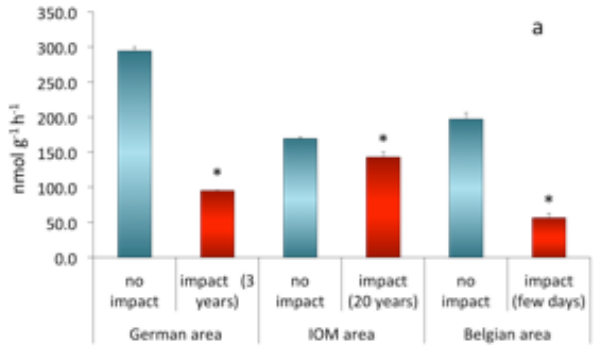


Fig. 4a Aminopeptidase activity.

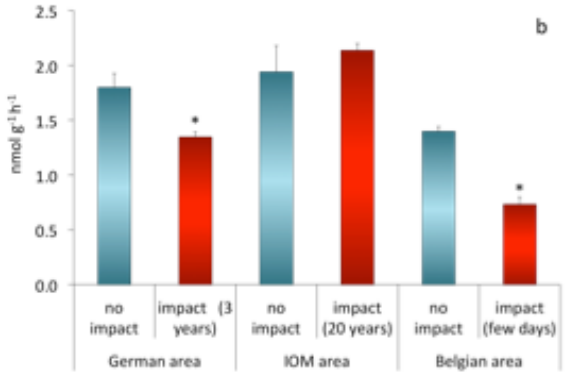


Fig. 4b β-Glucosidase acitivity.

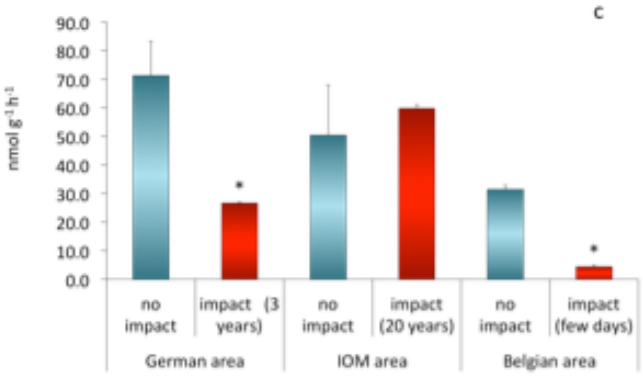


Fig. 4c Alkaline phosphatase activity.

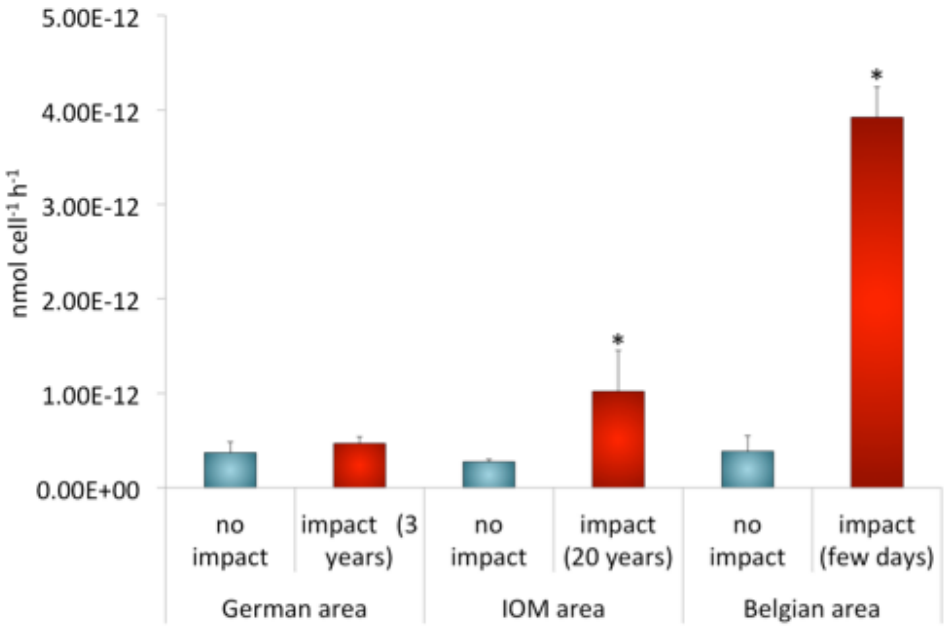


Fig. 5 Specific degradation activity per prokaryotic cell.

4.3.4 Impact on viral abundance, production and turnover times

Viral abundances were significantly lower in the sediments of the impacted sites of the German area and in the most recently impacted sites (few days) of the Belgian area (Figure 6a). However, virus abundances after 37 years from the impact in the French area were significantly higher than in control sediments. Viral production was similar between impacted and un-impacted sediments of the German and Belgian areas, while significantly higher values were observed in the 20 years-impacted site of the IOM area compared to controls (Figure 6b). In general, the viral turnover times were faster in the impacted sites than in the un-impacted sites (Figure 6c).

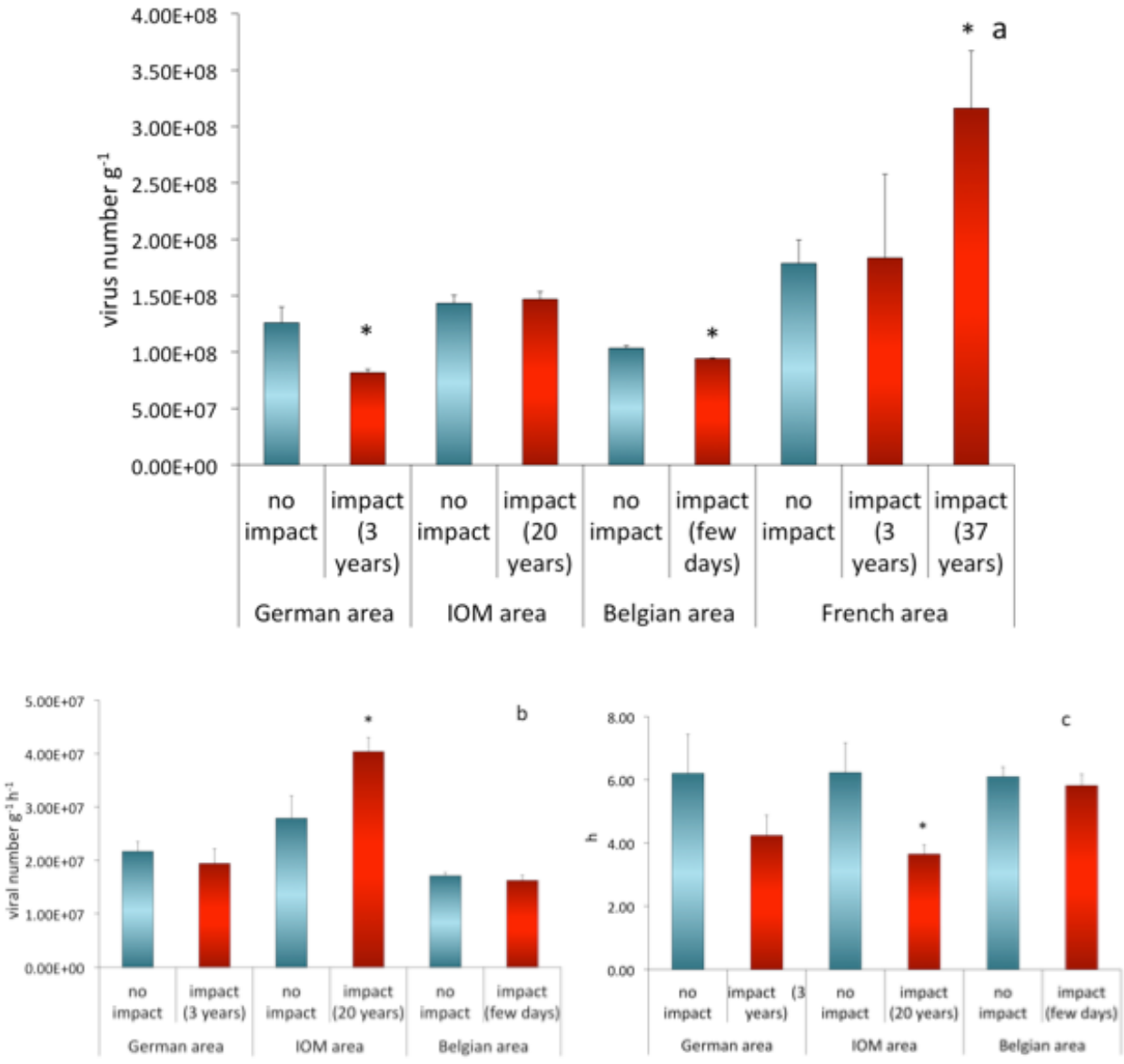


Fig. 6 Viral abundance (a); viral production (b); viral turnover expressed in hours (c).

4.3.5 Impact of deep-sea mining on virus-prokaryote interactions

The contribution of killed prokaryotes to the total prokaryotic standing stocks (up to ca. 10%) was higher in all impacted sediments of all investigated areas when compared to their respective controls (Figure 7a). Similarly, the virus to prokaryote ratio was higher in all impacted sites,

especially in the Belgian and French areas, with values up to 30 times higher than those in un-impacted sediments (Figure 7b).

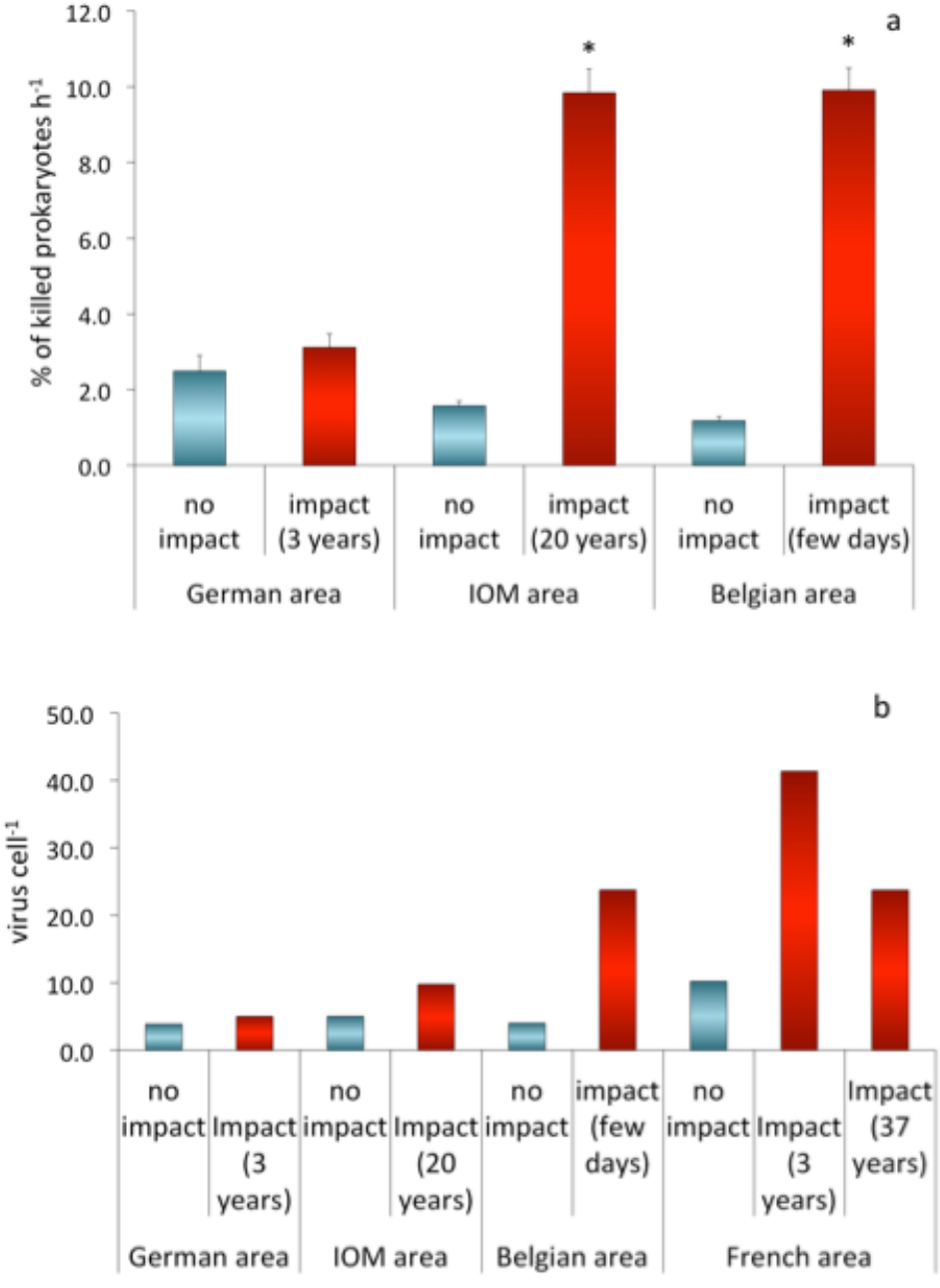


Fig. 7 Viral infection percentage rate (a); virus to prokaryote ratio (b).

4.3.6 Impact on bacterial richness and diversity

Bacterial OTU richness was characterised by a wide variability among the different investigated areas, with highest values in the impacted sites of the IOM area 20 years after the impact (9016 and 9264) and lowest values in the 3 years impacted sites of the French areas (4458 and 5972). In the Belgian area the bacterial richness strongly decreased after the impact, and the same happened in the French area after 3 years from the mining activity (Figure 8). In the French area after 37 years, in the German area after 3 years, and in the IOM area after 20 years from the impact the OTU numbers were similar to those of the controls. The lowest values of Shannon index were found in the few days impacted sites (9.16 and 9.25; Figure 9), while the highest values were observed in the sediments of the 37 years impacted site of the French area and in the impacted sites of the IOM area (10.83, 10.59 and 10.60 respectively). Values of equitability ranged from 0.73 in the impacted sites of the Belgian area (Figure 9) to 0.81-0.82 in the 37 years impacted site of the French area.

The rarefaction curves constructed considering an equal number of sequences for each samples (i.e. 50000 sequences) revealed a lower expected bacterial richness in impacted sites of the German and Belgian areas, while in the IOM area the curves of the impacted and undisturbed sites coincided (Figure 10, 11 and 12). In the French area the impacted sites showed a lower expected richness 3 years after the impact and a higher one 37 years after the impact compared to the controls (Figure 13).

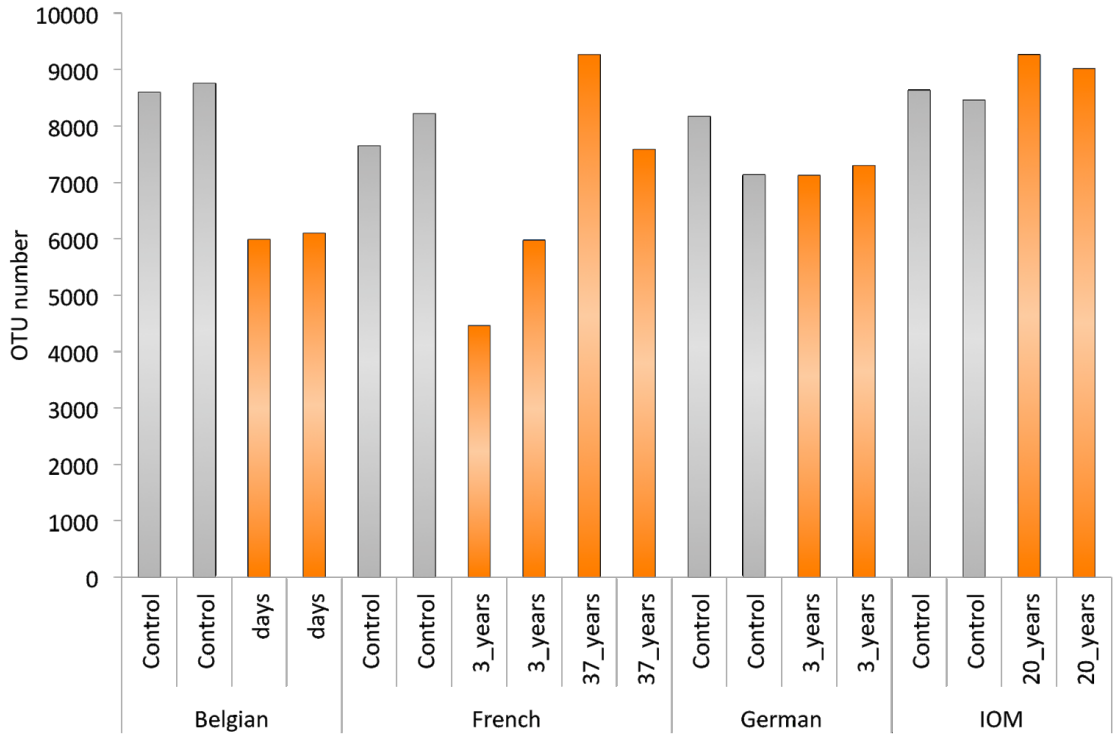


Fig. 8 OTUs number in impacted and control sites of each area.

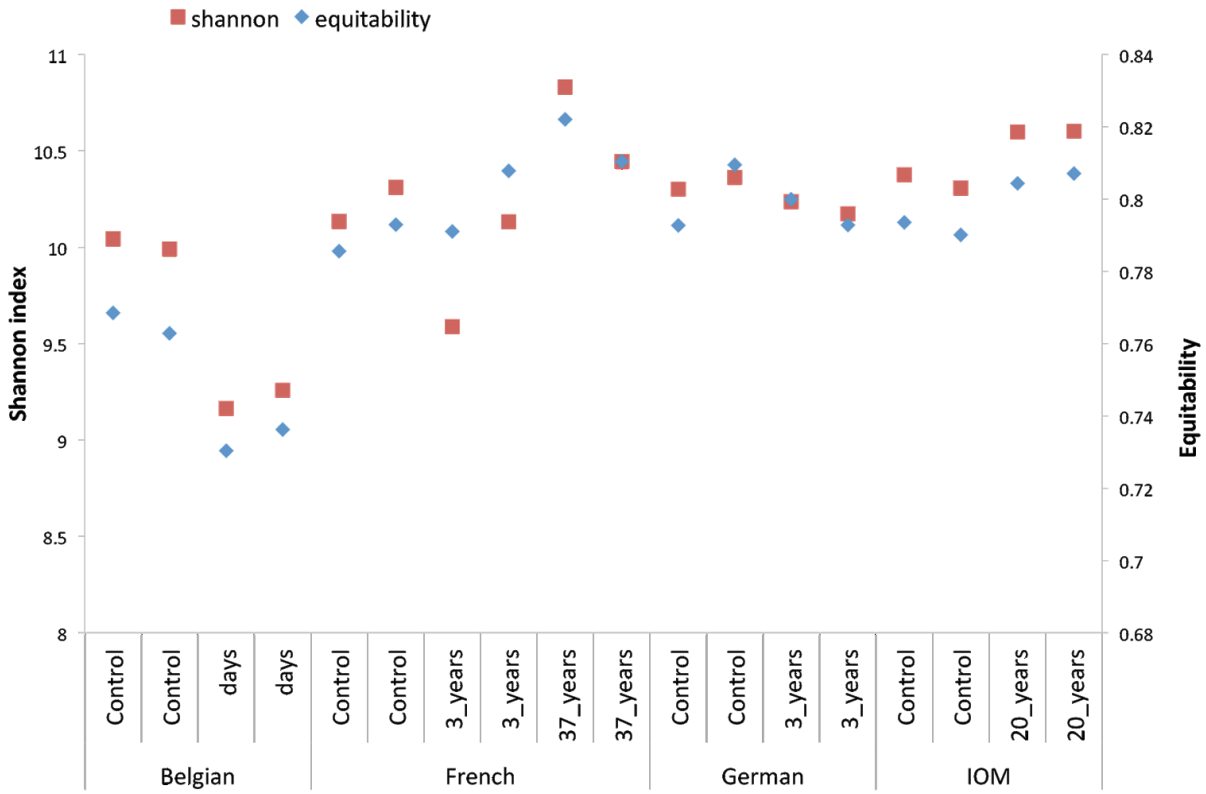


Fig. 9 Diversity estimates in terms of Shannon and Equitability indexes.

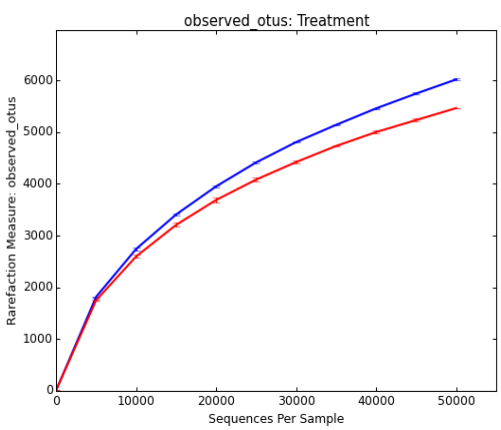


Fig. 10 German area: blue line controls, red line 3 yr impacted sites

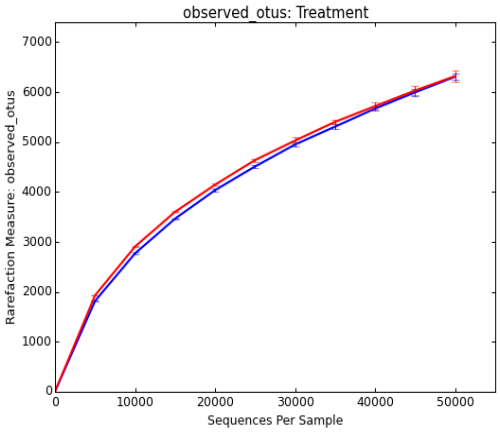


Fig. 11 IOM area: blue line controls, red line 20 yr impacted sites

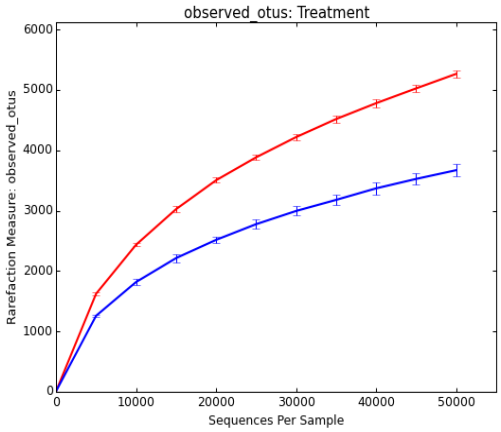


Fig. 12 Belgian area: red line controls, blue line few days impact

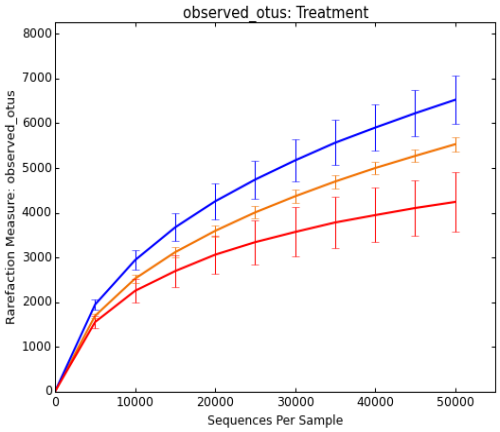


Fig. 13 French area: blue line 37 yr impact, yellow line controls, red line 3 yr impact

4.3.7 Bacterial diversity

Proteobacteria was the dominant phylum among all the sampling sites (contributing for 35-57% to all sequences), followed by Planctomycetes (10-18%), Chloroflexi (7-13%), Bacteroidetes (5-8%), Acidobacteria (4-6%), Actinobacteria (3-5%), Gemmatimonadates (2-4%) and Candidate Division OD1 (1-3%). Other less represented phyla were Aquificae, Armatimonadates, BD1-5, Caldiserica, Candidate division BRC1, KB1, OP11, OP3, TM7, WS3, Chlamydiae, Chlorobi, Chloroflexi, Cyanobacteria, Deferribacteres, Elusimicrobia, Fibrobacteres, Firmicutes, Lentisphaerae, Nitrospirae, NPL-UPA2, SBYG-2791, SM2F11, Spirochaetae, Synergistetes, TAO6, Thermodesulfobacteria, TM6 and Verrucomicrobia, which grouped together contributed about 12-15% to the bacteria assemblages. For the taxonomic analysis all the sequences contributing to prokaryotic diversity at the class level for more than 0.1% was considered. In all areas, the dominant classes of bacteria showed limited variations between impacted and un-impacted sites. In the Belgian area after few days from the impact there was a higher contribution of Alphaproteobacteria and Nitrospira, while those of the classes Planctomycetacia, Flavobacteriia and Acidimicrobiia were lower compared to the control sites (Figure 14). In the German area the contribution of Gammaproteobacteria and Planctomycetacia was lower after 3 years from the impact while that of Alphaproteobacteria, Nitrospira and SAR202 was higher compared to the controls (Figure 15). In the French area Flavobacteria were more represented in the site impacted 37 years ago, while the 3 years impacted site showed a higher contribution of Alphaproteobacteria and Chloroflexi S085 and a lower one of Planctomycetacia and Acidimicrobia respect the undisturbed sites (Figure 16). In the IOM area there were not particular differences between impacted and control sites (Figure 17). Figures 18, 19, 20, and 21 show the significant differences in terms of OTUs contribution at the family level between impacted and undisturbed sites of each area. In the impacted sites of the Belgian area there was a higher contribution of Chromatiaceae and Gemmatimonadaceae. In the German area the contributions of Rhodospirillaceae and SAR202 were higher in the impacted sites on the contrary of that of JTB255 marine benthic group. In the French area the classes whose contribution was higher after 3 years from the impact were

Nitrospinaceae and Nitrospiraceae, while after 37 years the family Flavobacteriaceae was more represented if compared to the controls. Finally, in the IOM area Anaerolinaceae and BD7-8 marine group contributions were higher in the impacted site, while the contribution of Deferribacterales and other minor group was lower. The similarity among sites (Figure 22), considering the OTUs that contribute more than 0.1% to bacterial assemblages, ranged from 80 to 90% between the replicates of each site, while among sites within each area 54-73% of similarity was observed. Only in the IOM area impacted and un-impacted sites showed a relatively higher similarity (82%). Considering the UniFrac distance-based trees (Figure 23), based on all the obtained OTUs, small distances were visible between impacted and control sites, especially in the IOM area. In the French area the bacterial assemblages of the sites impacted 37 years ago were phylogenetically closer to those of the undisturbed sites compared to the 3 years-impacted sites.

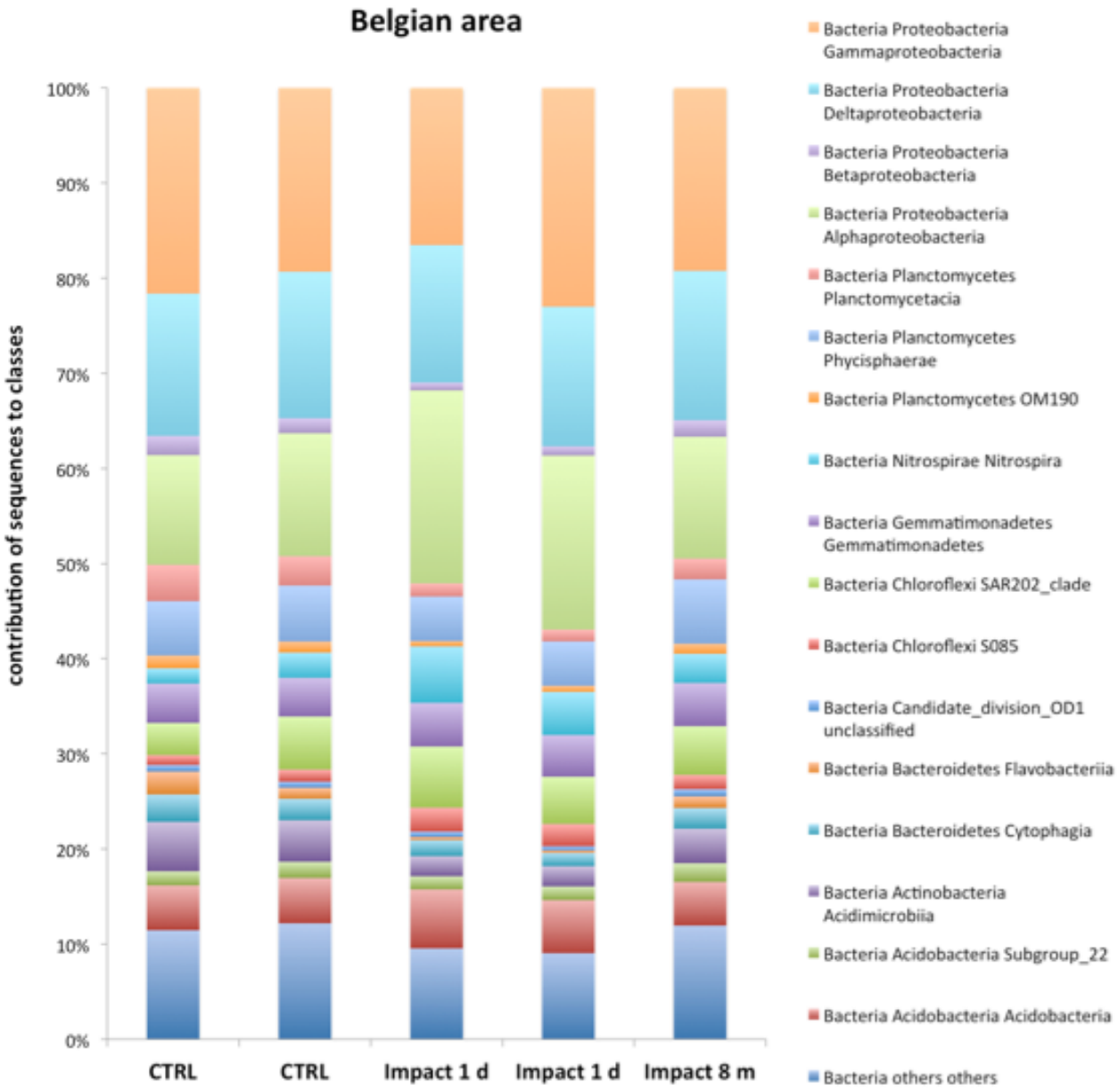


Fig. 14 Contribution of sequences to the most relevant bacterial classes in impacted and control sites of the Belgian area.

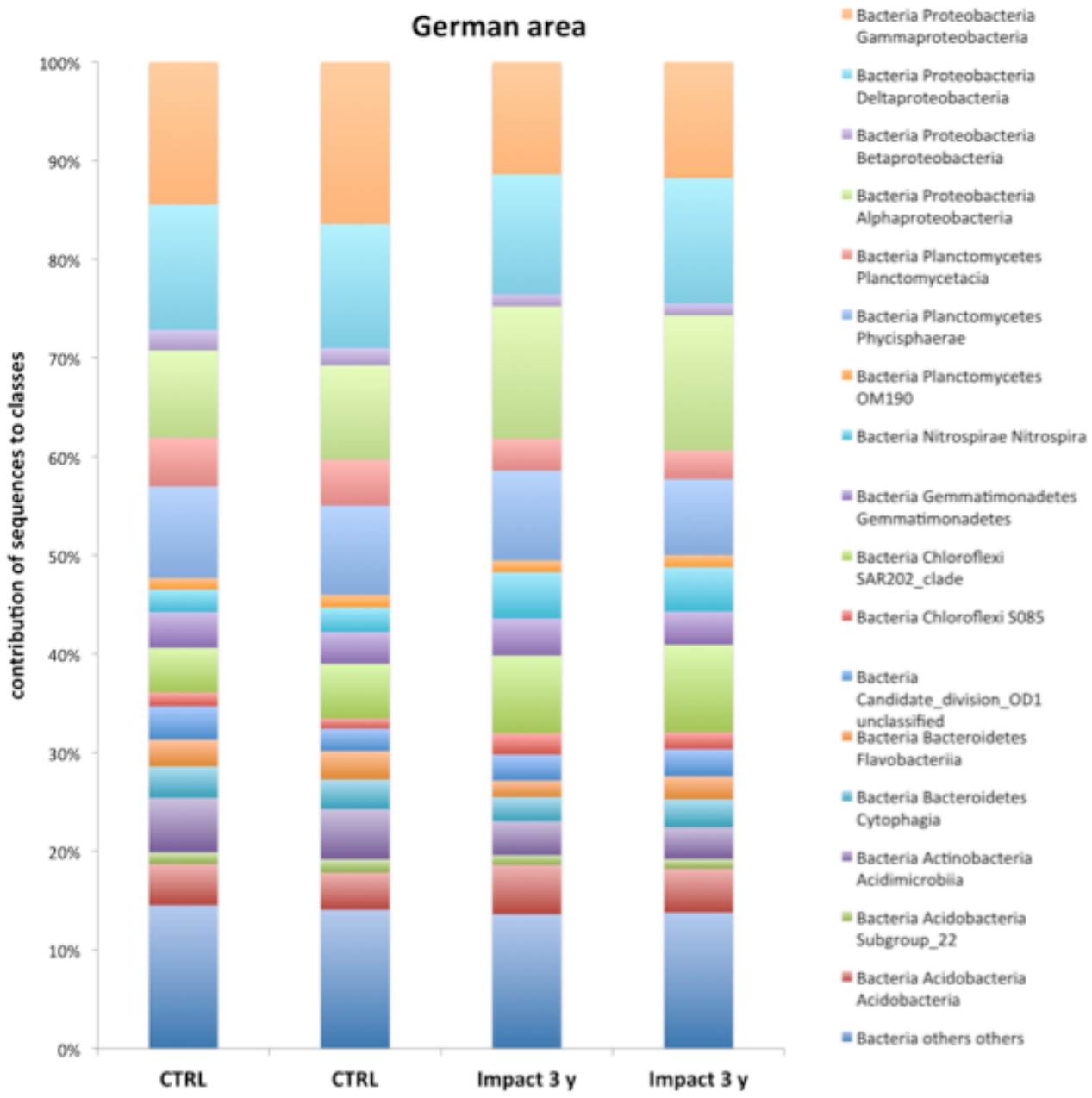


Fig. 15 Contribution of sequences to the most relevant bacterial classes in impacted and control sites of the German area.

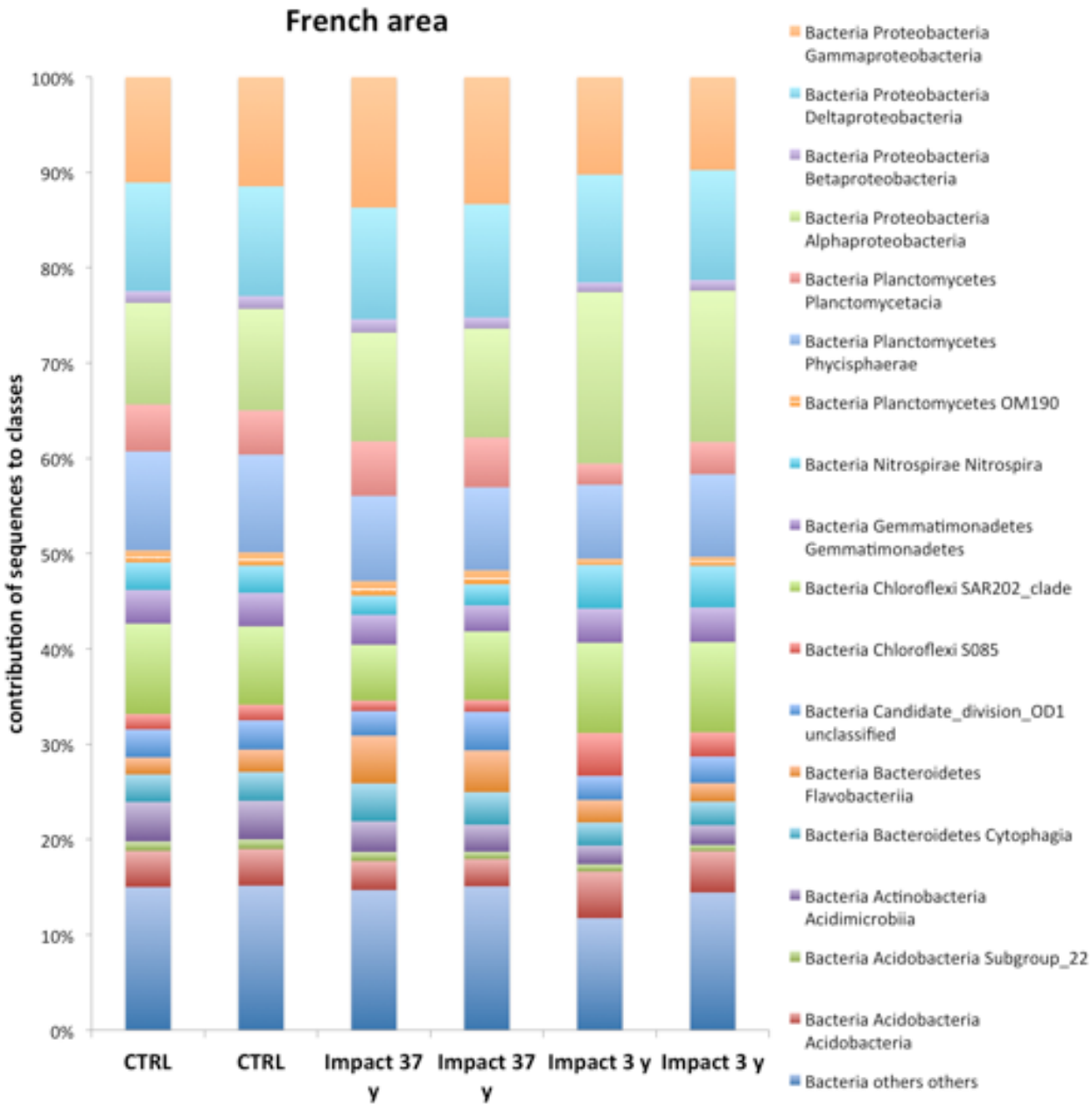


Fig. 16 Contribution of sequences to the most relevant bacterial classes in impacted and control sites of the French area.

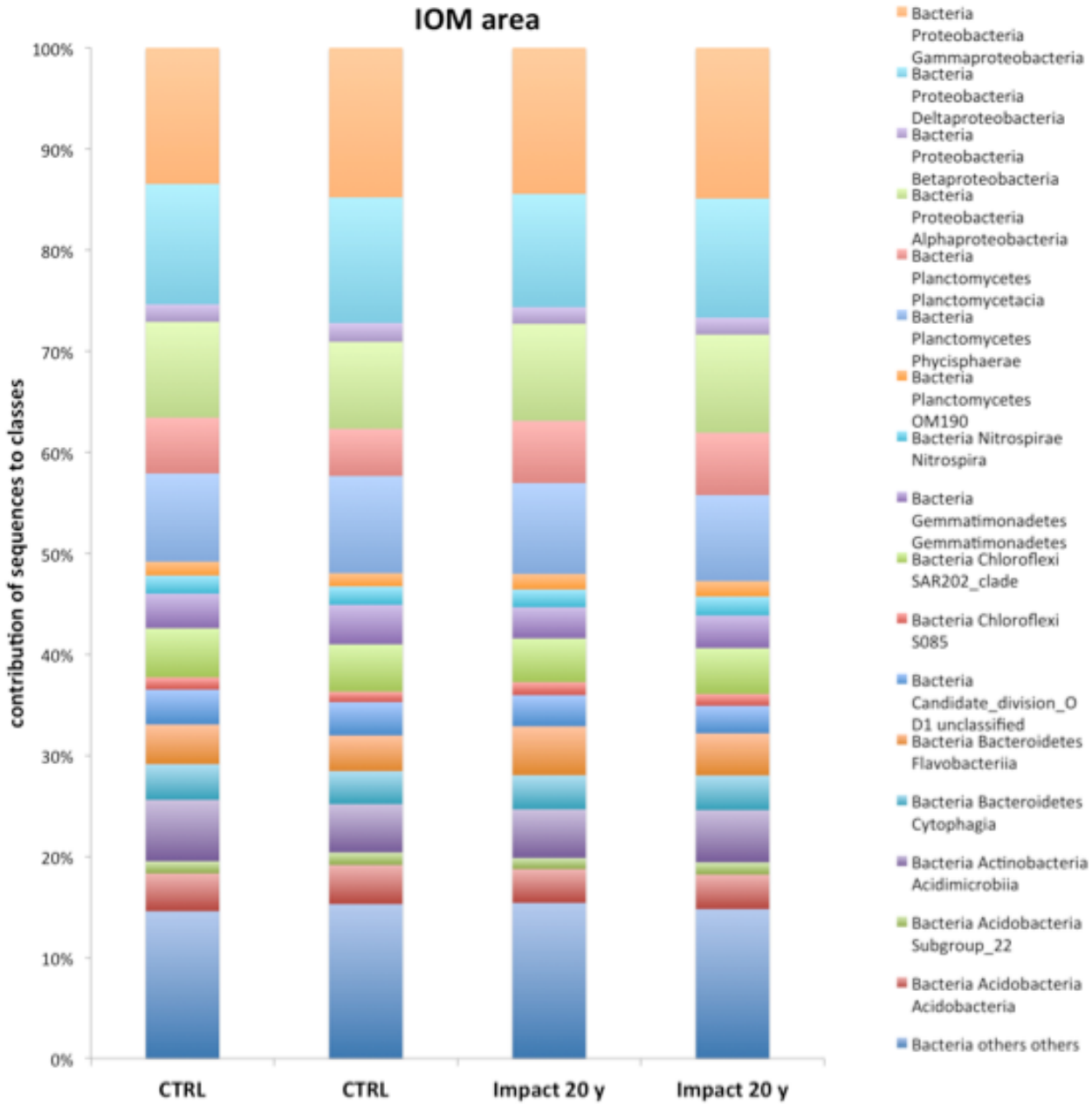


Fig. 17 Contribution of sequences to the most relevant bacterial classes in impacted and control sites of the IOM area.

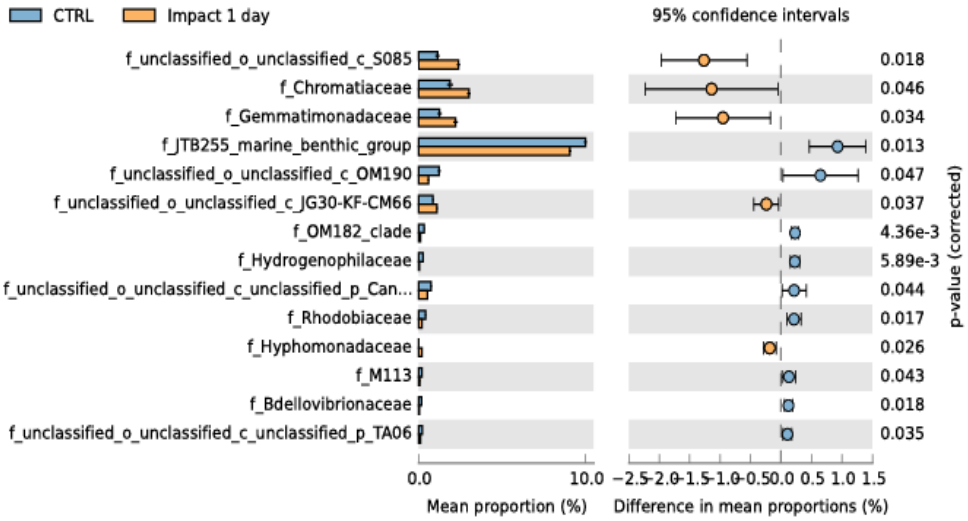


Fig. 18 STAMP graph showing the significant variations of OTUs contribution at the family level in impacted and control sites of the Belgian area.

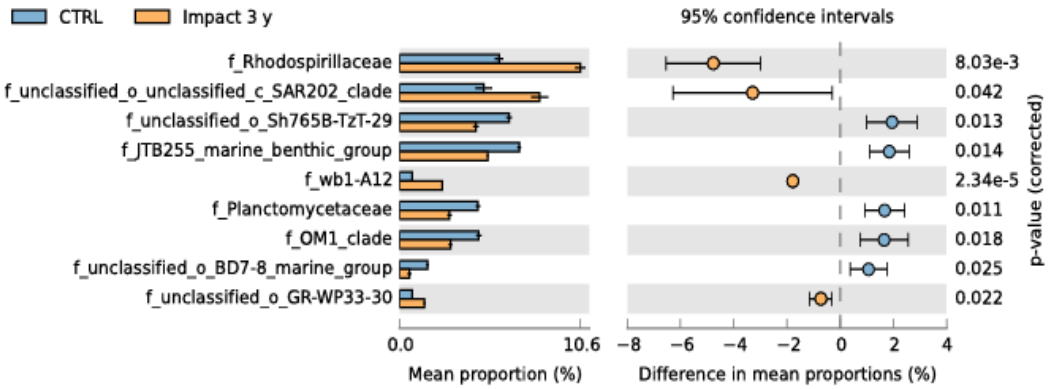


Fig. 19 STAMP graph showing the significant variations of OTUs contribution at the family level in impacted and control sites of the German area.

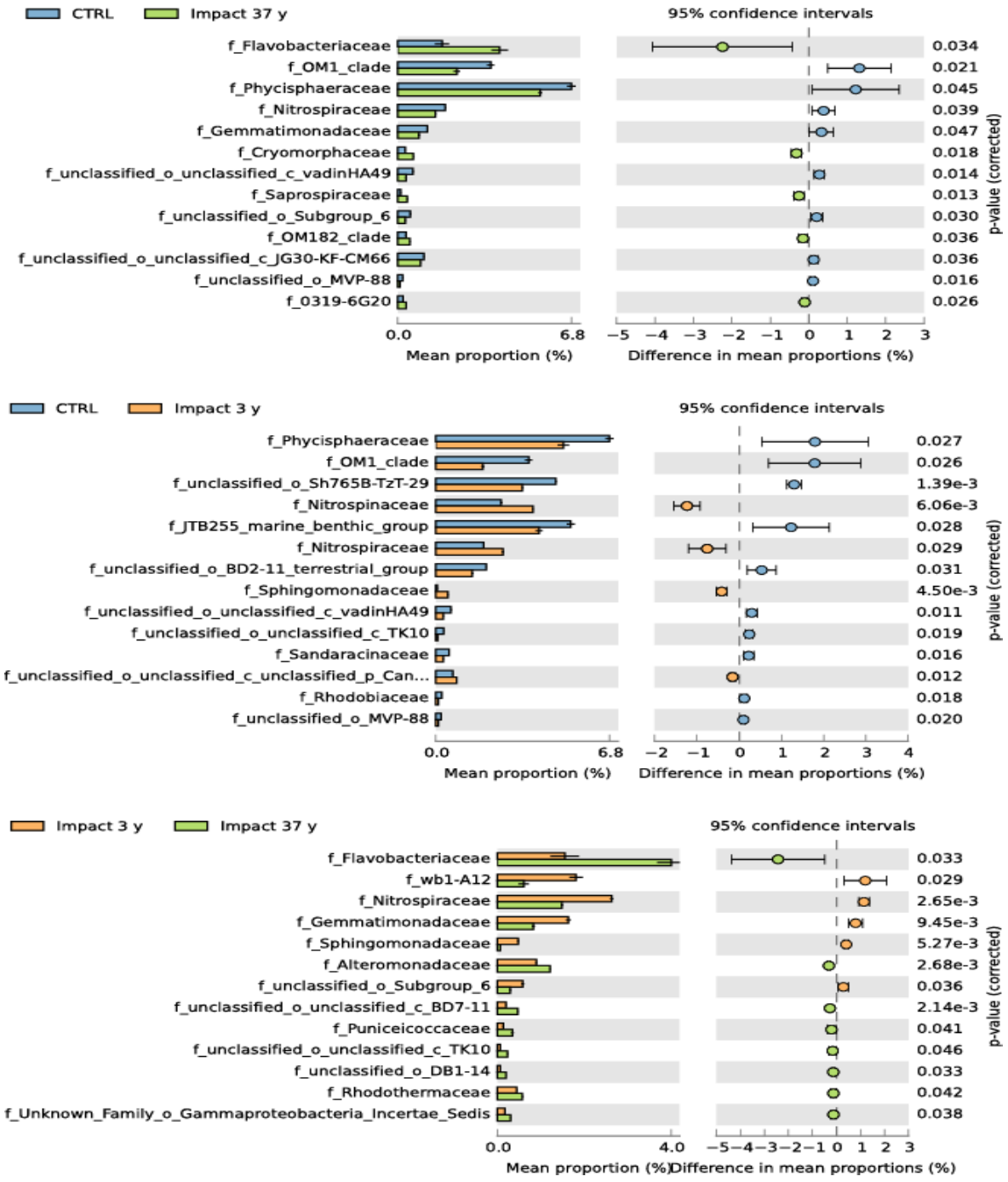


Fig. 20 STAMP graph showing the significant variations of OTUs contribution at the family level in impacted and control sites of the French area.

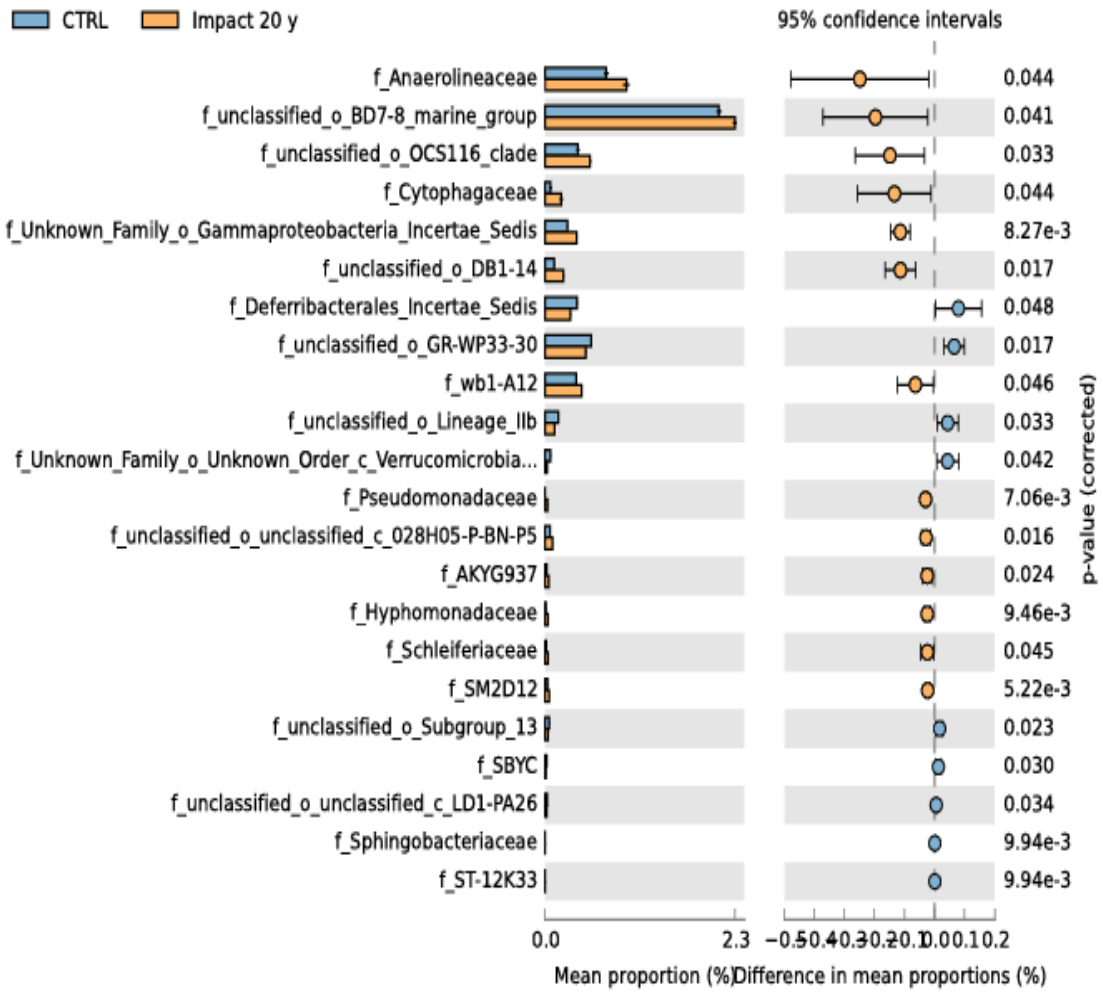


Fig. 21 STAMP graph showing the significant variations of OTUs contribution at the family level in impacted and control sites of the IOM area.

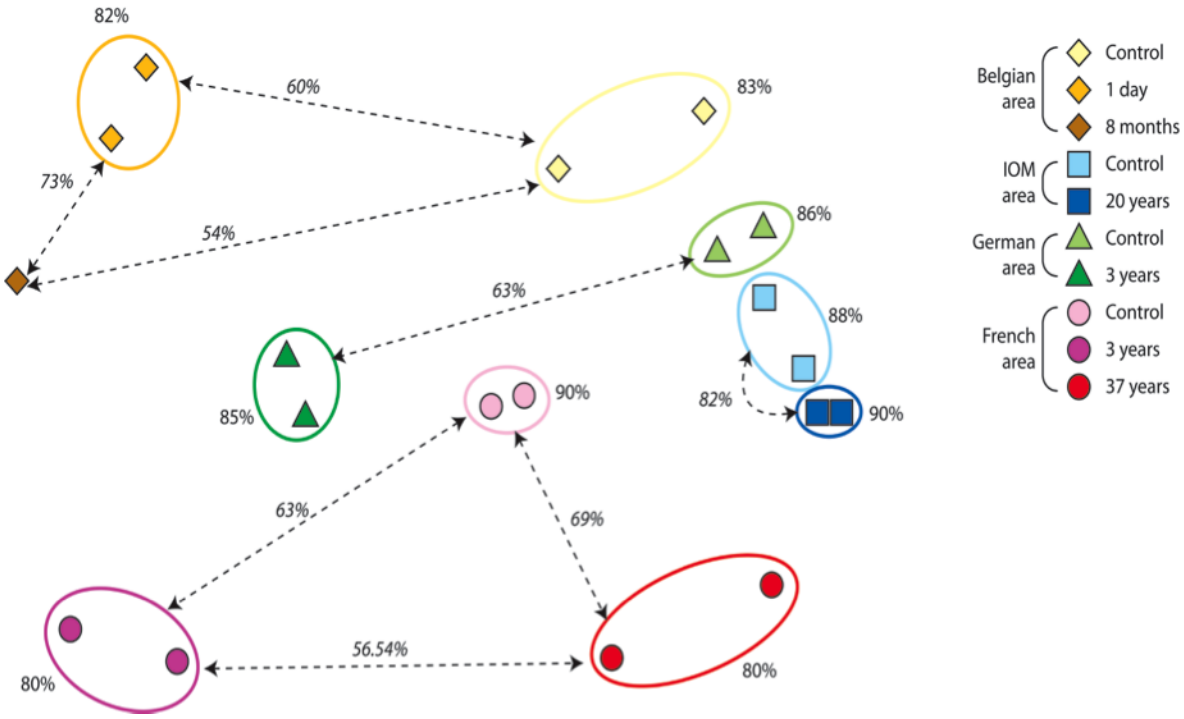


Fig. 22 MDS plot showing the OTUs percentage similarity between impacted and control sites in each area.

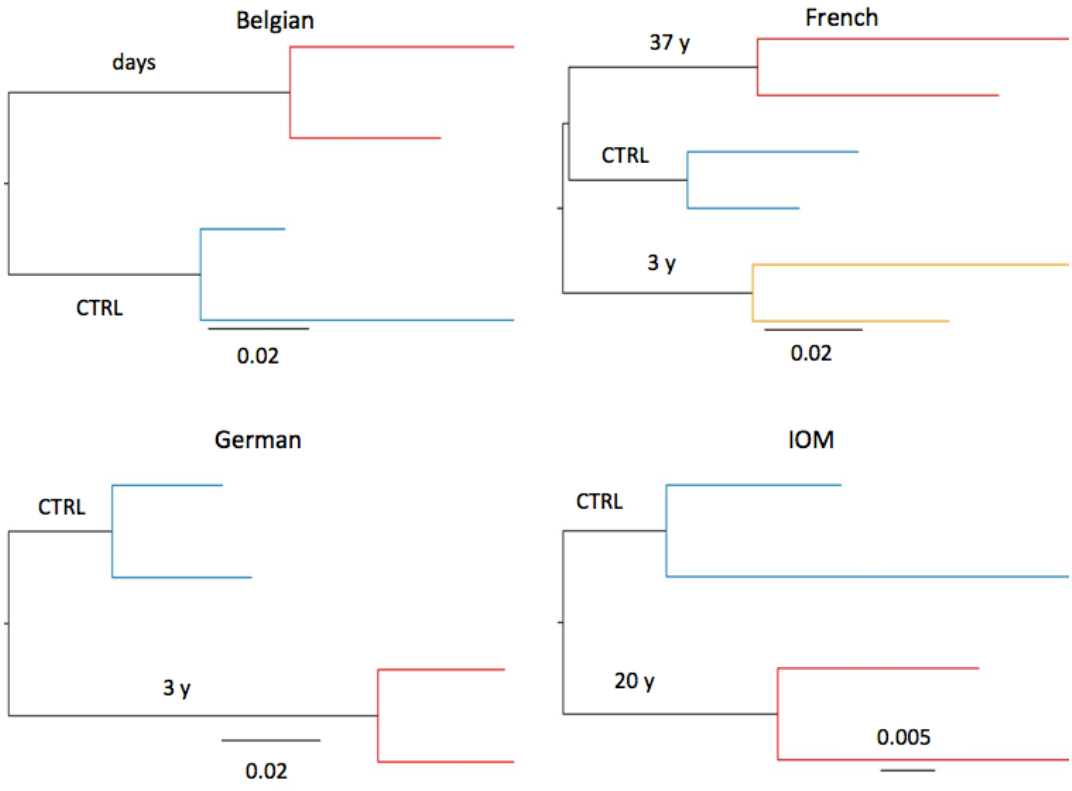


Fig. 23 UniFrac distance-based trees of impacted and control sites in each area.

4.4 Discussion

Different studies have suggested that manganese nodule exploitation can determine an important impact on benthic deep-sea ecosystems (Bluhm, 2001; Smith, 2008; Lodge, 2014; Wedding, 2015). One of the expected consequences of nodule exploitation is the disturbance of the sediment due to nodule removal. This, in turn, can determine the removal of sediment layers (up to 15-20 cm; Oebius et al., 2001), with important consequences on the food web structure and biogeochemical processes of the benthic deep-sea ecosystems. Our study revealed that mining activity can determine a significant decrease of trophic resources (in terms of biopolymeric C and total phytopigment concentrations) of the abyssal seafloor of the CCFZ. The depletion of organic matter content was evident independently from the time interval from the impact, although such effect was more evident in the sites impacted more recently and in those areas that are farer from the continent. Since deep-sea ecosystems mainly depend on the inputs of organic particles produced by photosynthesis in surface waters (Glover & Smith, 2003), it could be hypothesized that the organic matter depletion due to the mining activity can be buffered to major extent in areas subjected to higher organic C inputs (such as the German and IOM) compared to much more oligotrophic areas (such as than the French and Belgium sites).

Such changes in food availability determined by mining can have also important consequences on benthic deep-sea prokaryotes. Indeed, our results revealed a substantial decrease of prokaryotic abundance and biomass in impacted sediments, particularly evident in the most recently disturbed sediments (few days) of the Belgian area (values 7-8 times lower than those of the respective undisturbed sediments). The role of food availability in influencing prokaryotic standing stocks in the investigated areas was highlighted also by multivariate statistical analysis (i.e. trophic resources explained alone 81% of the bacterial abundance variance (Table 6, Supplementary material). Also the removal of surface sediment due to mining activities can be directly responsible of the strong decrease of the prokaryotic abundance and biomass assemblages since it is known

that prokaryotes decrease exponentially with depth in the sediment (D'Hondt et al., 2004; Parkes et al., 2005; Kallmeyer et al., 2012; Lloyd et al. 2013; Danovaro et al. 2015). The impact on prokaryotic assemblages was evident in the different investigated impacted sites, although their abundance and biomass tends to recover to values similar to those of control sites after 37 years from the impact in the French area. Also the degradation potential of organic matter estimated through the determination of extracellular enzymatic activities, were generally lower in impacted sites compared to the controls. On the contrary, the specific degradation activities (related with the cells number) were similar or even higher in impacted sites when compared to values observed in the controls, leading to hypothesize a possible shift in bacterial assemblage composition.

It is generally reported that viral abundance in benthic deep-sea ecosystems is tightly related to that of prokaryotes (Danovaro et al., 2008). Conversely in this study a different pattern of viral abundance was observed when compared to that of their prokaryotes, suggesting that mining activity can alter virus-host interactions. In particular, benthic deep-sea viruses was affected by the mining activity to a lower extent in terms of abundance compared to prokaryotes, resulting in much higher virus to prokaryote abundance ratios in all impacted sites (up to 30 times the values of the undisturbed sites). Such higher virus to prokaryote abundance ratios were also associated with higher values of virus-induced prokaryotic mortality. These findings suggest that deep-sea mining, despite it can lead to a decrease of host abundances, can increase viral infections, likely due to an increased susceptibility of prokaryotic hosts determined by changes in their biodiversity (Bouvier and Del Giorgio, 2007; Winter et al., 2009).

In this study, we observed lower values of bacterial OTU richness and Shannon index in the Belgian area few days after the mining activity compared to the non-impacted sites as well as in the French area three years after the impact. Conversely, in the other impacted areas as well as in the French area 37 years after the impact, bacterial diversity (in terms of OTU richness and Shannon index) was similar or even higher to that observed in their respective control sites. Such findings indicate that mining activity on the ocean seafloor affect mainly microbial abundances rather than their diversity.

Our results revealed also the lack of clear differences between the main bacterial phyla identified in impacted and control sites. Independent from impact and control sites, such bacterial phyla (belonging to Proteobacteria, Planctomycetes, Chloroflexi, Bacteroidetes, Acidobacteria, Actinobacteria, Gemmantimonadates and Candidate Division OD1) were widely distributed in all areas investigated and typically reported in surface deep-sea sediments worldwide (Schauer et al., 2010; Orcutt et al., 2011; Zinger et al., 2011; Parkes et al., 2014; Corinaldesi, 2015) including those from the CCFZ (Wang et al., 2010; Wu et al., 2013). Also slight differences were observed at class level between impacted and un-impacted sites, whereas significant and marked differences were found at level of different no dominant bacterial families. Indeed, in impacted sites of the Belgian area a higher contribution of *Chromatiaceae* (Gammaproteobacteria) and *Gemmatimonadaceae* was observed. In impacted sites of the German area *Rhodospirillaceae* and SAR202 clade were more represented, while the contribution of clade JTB255 (class Gammaproteobacteria, order Xanthomonadales), recognized as a cosmopolitan bacterial clade (Bienhold, 2016) decrased. In the French area after 3 years from the mining activity, the contribution of *Nitrospinaceae* and *Nitrospiraceae* known as bacteria involved in nitrite oxidation and rarely observed in dark ocean habitats (Nercessian et al., 2005; Santelli et al., 2008; Suzuki et al., 2004; Takai et al., 2008; Wegener et al., 2008; Orcutt et al., 2011; Lücker & Daims, 2014) decreased. Finally, in the impacted IOM area an increased contribution of the family *Anaerolinaceae* (class of Chloroflexi) identified as potentially metal-tolerant group (Sun et al., 2013), and BD7-8 Marine Group, was observed.

All of these changes at family level were mainly responsible of the differences of the bacterial assemblage composition between impacted and un-impacted sites as indicated by similarity analysis among the different sites within the same area. With the exception of the IOM area, impacted and un-impacted sites within the same area were characterised by phylogenetically different bacterial assemblages even after several years after the impact (i.e. French area). Overall these data suggest that deep-sea mining by influencing the 'rare' bacterial taxa (Brazelton et al., 2010; Bienhold, 2012) determine important changes of bacterial assemblage composition, which can persist over a long time scale.

4.5 Conclusions

Findings reported here indicate that deep-sea mining can affect to a large extent the benthic prokaryotic assemblages inhabiting the benthic abyssal ecosystems of the CCFZ. Deep-sea mining affect also virus-prokaryote interactions by determining an increase of viral pressure on prokaryotic hosts, potentially due to shifts of bacterial diversity. In particular, despite the dominant bacterial families, widely distributed in all sediment areas were largely un-affected by mining, important changes occurred for the less representative taxa leading to variations in prokaryotic community structure which persists also several years after the impact. Overall results obtained in this study suggest that changes in virus-prokaryote interactions and shifts in the composition of bacterial assemblages caused by deep-sea mining can affect biogeochemical cycles and the functioning of the benthic microbial food webs over a long time scale.

This study point out

Dominant prokaryotic families are relatively unaffected to deep-sea mining, but rare taxa change significantly

Virus-induced mortality rates increase after mining impact, potentially due to shifts in bacterial assemblage composition with potential consequences on biogeochemical cycles and benthic ecosystem functioning.

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Supplementary material

Table 1 Content of total biopolimeric carbon, total phytopigments, and prokaryotic abundances and biomasse

AREA	Depth (m)	latitudine	longitudine		CPE µg/g	std	BPC mg/g	std	Prok. Ab. cells g^{-1}	std	Prok. Biomass µgC g^{-1}	std
German area	4141	11° 50.68' N	117° 03.53' W	CTRL	5.89	1.13	4.22	0.31	3.10E+07	4.20E+06	2.74	0.112
	4120	11° 51.06' N	117° 03.46' W	CTRL	1.45	0.03	3.71	0.21	1.39E+07	8.77E+05	0.89	0.041
	4138	11° 51.28' N	117° 03.38' W	CTRL	6.17	1.24	3.95	0.29	na	na	na	na
	4133	11° 50.46' N	117° 03.23' W	CTRL	2.11	0.41	3.63	0.18	2.63E+07	5.65E+06	2.39	0.087
	4126	11° 51.09' N	117° 03.05' W	CTRL	1.48	0.02	3.52	0.33	5.87E+07	5.85E+06	5.58	0.190
	4076	11° 50.60' N	117° 03.41' W	CTRL	1.56	0.58	4.06	0.34	na	na	na	na
	4098	11°50.31 N	117°03.27' W	Impact 3 y	2.69	0.62	2.92	0.03	1.39E+07	2.72E+06	1.17	0.167
	4099	11°50.30' N	117°03.27' W	Impact 3 y	3.94	0.62	3.70	0.24	1.87E+07	5.26E+06	1.66	0.187
IOM area	4430	11° 04.73' N	119° 39.48' W	CTRL	2.57	0.25	3.33	0.25	2.83E+07	1.08E+06	2.12	0.098
	4433	11° 04.63' N	119° 39.06' W	CTRL	4.22	1.78	3.61	0.09	2.88E+07	6.17E+06	2.77	0.154
	4439	11° 04. 52' N	119° 39.81' W	CTRL	3.71	0.59	3.38	0.61	na	na	na	na
	4418	11° 04.39' N	119° 39.34' W	CTRL	6.02	0.60	3.35	0.31	na	na	na	na
	4422	11° 04.38' N	119° 39.35' W	CTRL	5.79	0.80	3.93	0.67	na	na	na	na
	4413	11° 04.42' N	119° 39.33' W	CTRL	4.89	0.87	4.49	0.37	na	na	na	na
	4427	11° 04.29' N	119° 39.33' W	CTRL	3.07	0.52	3.46	1.32	na	na	na	na
	4424	11° 04.30' N	119° 39.32' W	CTRL	7.36	2.96	4.68	0.72	na	na	na	na
	4424	11° 03.89' N	119° 39.18' W	CTRL	2.38	0.10	3.56	0.68	na	na	na	na
	4386	11°04.23' N	119°39.23' W	Impact 20 y	3.29	0.66	3.83	0.31	9.12E+06	7.00E+05	0.84	0.111

	4388	11°04.23' N	119°39.24' W	Impact 20 y	1.90	0.14	3.04	0.47	2.09E+07	3.62E+06	1.84	0.182
Belgium area	4516	13° 51.24' N	123° 15.29' W	CTRL	6.09	0.94	3.98	0.78	4.40E+07	7.57E+06	3.44	0.168
	4510	13° 51.28' N	123° 14.69' W	CTRL	0.90	0.00	2.72	0.46	2.50E+07	5.94E+06	2.08	0.134
	4512	13° 51.06' N	123° 14.22' W	CTRL	1.35	0.02	3.04	0.35	8.69E+06	3.95E+05	0.51	0.014
	4514	13° 50.80' N	123° 04.66' W	CTRL	1.96	0.17	2.98	0.17	na	na	na	na
	4511	13° 50.74' N	123° 15.10' W	CTRL	2.19	0.34	2.89	0.25	na	na	na	na
	4477	13°52.23' N	123°15.06' W	Impact 1 day	0.32	0.05	2.03	0.13	3.64E+06	6.73E+05	0.23	0.036
	4477	13°52.23' N	123°15.05' W	Impact 1 day	0.34	0.04	2.01	0.32	4.28E+06	7.96E+05	0.23	0.016
	4478	13°51.57' N	123°15.19' W	Impact 8 months	1.52	0.67	2.72	0.08	1.36E+07	6.21E+05	1.22	0.027
	4476	13°51.57' N	123°15.21' W	Impact 8 months	0.27	0.04	1.77	0.27	3.74E+06	3.08E+05	0.25	0.024
French area	4889	14° 03.00' N	130° 08.32' W	CTRL	0.76	0.25	2.40	0.05	1.55E+07	1.86E+06	0.97	0.105
	4940	14° 02.97' N	130° 07.85' W	CTRL	1.99	0.04	3.32	1.18	1.53E+07	4.25E+06	1.31	0.148
	4954	14° 03.00' N	130° 07.42' W	CTRL	1.21	0.22	2.49	0.16	na	na	na	na
	4918	14° 02.62' N	130° 08.32' W	CTRL	1.45	0.27	2.74	0.34	na	na	na	na
	4948	14° 02.60' N	130° 07.82' W	CTRL	0.60	0.10	2.21	0.32	na	na	na	na
	5008	14° 02.45' N	130° 05.11' W	CTRL	1.19	0.21	2.04	0.19	2.15E+07	5.22E+06	1.97	0.080
	5012	14° 02.54' N	130° 05.13' W	CTRL	2.07	0.45	1.98	0.12	na	na	na	na
	4998	14°02.13' N	130°05.52' W	Impact 3 y	0.21	0.02	1.70	0.14	3.51E+06	5.02E+05	0.21	0.034
	4954	14°02.12' N	130°05.52' W	Impact 3 y	0.66	0.17	2.05	0.25	5.34E+06	1.30E+06	0.37	0.031
	4944	14°02.03' N	130°07.13' W	Impact 37 y	1.06	0.32	2.28	0.14	1.69E+07	2.33E+06	1.36	0.223
	4944	14°02.03' N	130°07.13' W	Impact 37 y	0.38	0.13	2.39	0.39	9.74E+06	2.53E+06	0.64	0.076

. **Table 2** Data on viral abundances, production, infection, virus to prokaryote ratio and turnover rate,

AREA		Viral Abund.	std	Viral Prod.	std	Virus/Prok	viral infection	std	viral
		Virus g^{-1}		Virus g^{-1} h^{-1}			% killed prok h^{-1}		turnover h
German area	CTRL	1.52E+08	2.85E+07	2.22E+07	6.72E+06	5	1.6	0.5	6.86
	CTRL	9.91E+07	3.25E+06	2.11E+07	2.10E+06	7	3.4	0.3	4.70
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	Impact 3 y	8.14E+07	3.58E+06	1.94E+07	2.33E+06	6	3.1	0.4	4.19
	Impact 3 y	na	na	na	na	na	na	na	na
IOM area	CTRL	1.48E+08	1.60E+07	2.00E+07	6.03E+06	5	1.6	0.5	7.37
	CTRL	1.53E+08	1.16E+07	2.03E+07	2.28E+06	5	1.6	0.2	7.53
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	CTRL	1.29E+08	3.33E+07	4.33E+07	7.96E+06	na	na	na	2.97
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	Impact 20 y	1.47E+08	6.81E+06	4.04E+07	2.62E+06	16	9.8	0.6	3.63
	Impact 20 y	na	na	na	na	na	na	na	na
Belgium area	CTRL	1.02E+08	8.85E+06	1.79E+07	1.58E+06	2	0.9	0.1	5.71
	CTRL	1.04E+08	1.78E+06	1.63E+07	8.78E+05	4	1.4	0.1	6.40
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	CTRL	na	na	na	na	na	na	na	na
	Impact 1 day	9.41E+07	8.75E+05	1.62E+07	9.66E+05	26	9.9	0.6	5.80
	Impact 1 day	na	na	na	na	na	na	na	na
	Impact 8 months	na	na	na	na	na	na	na	na
	Impact 8 months	na	na	na	na	na	na	na	na
French area	CTRL	2.25E+08	6.68E+06	na	na	14.5	na	na	na
	CTRL	1.32E+08	1.50E+07	na	na	8.6	na	na	na

CTRL	na	na	na	na	na	na	na	na
CTRL	na	na	na	na	na	na	na	na
CTRL	na	na	na	na	na	na	na	na
CTRL	na	na	na	na	na	na	na	na
CTRL	na	na	na	na	na	na	na	na
Impact 3 y	1.30E+08	4.84E+06	na	na	37.1	na	na	na
Impact 3 y	2.36E+08	2.83E+07	na	na	44.1	na	na	na
Impact 37 y	4.28E+08	2.19E+07	na	na	25.4	na	na	na
Impact 37 y	2.03E+08	2.76E+07	na	na	20.8	na	na	na

Table 3 Extracellular enzymatic activities.

		MCA	sd	MUF-P	sd	β-GLU	sd
		nmol g^{-1} h^{-1}		nmol g^{-1} h^{-1}		nmol g^{-1} h^{-1}	
GERMAN AREA	Control	294.2	5.9	71.1	12.2	1.8	0.1
GERMAN AREA	Impact (3 years)	94.8	0.9	26.5	0.4	1.3	0.0
IOM AREA	Control	169.0	2.4	50.3	17.7	1.9	0.2
IOM AREA	Impact (20 years)	142.8	7.7	59.7	1.4	2.1	0.1
BELGIUM AREA	Control	197.1	9.0	31.3	1.6	1.4	0.0
BELGIUM AREA	Impact (1 day)	56.3	6.4	4.2	0.6	0.7	0.1
BELGIUM AREA	Impact (8 months)	na		na		na	
FRENCH AREA	Control	na		na		na	
FRENCH AREA	Impact (3 years)	na		na		na	
FRENCH AREA	Impact (37 years)	na		na		na	

Table 4 Extracellular enzymatic activities per cell.

		MCA/PA	sd	MUF-P/PA	sd	β-Glu/PA	sd
		nmol g^{-1} h^{-1} $cell^{-1}$		nmol g^{-1} h^{-1} $cell^{-1}$		nmol g^{-1} h^{-1} $cell^{-1}$	
GERMAN AREA	Control	9.06E-06	4.48E-07	2.19E-06	9.17E-07	5.54E-08	9.73E-09
GERMAN AREA	Impact (3 years)	5.82E-06	5.78E-08	1.62E-06	2.74E-08	8.26E-08	2.85E-09
IOM AREA	Control	5.92E-06	2.57E-07	1.76E-06	6.19E-07	6.80E-08	8.43E-09
IOM AREA	Impact (20 years)	9.52E-06	5.14E-07	3.98E-06	9.29E-08	1.42E-07	4.12E-09
BELGIUM AREA	Control	7.60E-06	8.49E-07	1.21E-06	1.50E-07	5.39E-08	4.41E-09
BELGIUM AREA	Impact (1 day)	1.42E-05	1.60E-06	1.07E-06	1.51E-07	1.84E-07	1.69E-08
BELGIUM AREA	Impact (8 months)	na		na		na	
FRENCH AREA	Control	na		na		na	
FRENCH AREA	Impact (3 years)	na		na		na	
FRENCH AREA	Impact (37 years)	na		na		na	

Table 5 Values of the DistLM analysis of prokaryotic abundance variance

SEQUENTIAL TESTS							
Variable	R^2	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
VP	0.05	283.90	0.89	0.374	0.05	0.05	16
BPC	0.86	4383.60	88.59	0.001	0.81	0.86	15
Depth	0.91	262.54	7.66	0.003	0.05	0.91	14

Final conclusions

In the present study, for the first time, the interactions between viruses and prokaryotes in the sediments of three of the World's deepest hadal trenches, the Japan, Ogasawara and Mariana trenches, and the diversity of the benthic prokaryotic communities inhabiting these extreme and remote ecosystems were explored.

Overall, our findings reveal that all the hadal trenches investigated support high prokaryotic abundances and biomasses. These elevate biomasses favour highly dynamic viral components, enhancing the rates of viral lysis that are relatively high and comparable to those of the abyssal sediments. The role of viruses appears to be relevant independently of the hadal trench considered. Especially, in the Japan Trench, the virus-induced prokaryotic mortality can stimulate the release of highly bioavailable organic matter for benthic metabolism playing a fundamental role in funnelling organic C within the microbial food webs and in favouring prokaryotic biomass production of the non-infected cells.

Results obtained from the study of the virus-prokaryote interactions in the Mariana Trench reveal that viruses are a highly dynamic component allowing us to hypothesise a preferential viral infection of the dominant taxa inhabiting this trench with influence on the structure of hadal microbial food webs and hence on biogeochemical cycles.

Despite the high prokaryotic enzymatic activities found within the investigated trenches, the efficiency of degradation of organic matter per cell appears to be low, potentially due to non-heterotrophic pathways of biomass production. In fact, our study on trench's prokaryotic community structure and diversity confirms the presence of higher abundances of archaea in hadal sediments compared those found in abyssal systems, and the predominance of the ammonia oxidizer *Nitrosopumilus,* belonged to the phylum Thaumarchaeota, both within the Mariana Trench and in the Ogasawara Trench. These results suggest the key role that chemoautotrophic prokaryotes play on C and N cycling in these ultra abyssal ecosystems.

Beyond the high contribution of the archaeal component to the investigated microbial assemblages, we found a high prokaryotic richness in all trenches, especially in the Japan Trench. This result highlights that hadal ecosystems can be potential hot spot of microbial biodiversity although their extreme environmental conditions. However, such conditions influence trench's microbial assemblage differently. The presence within the hadal sediments of prokaryotic taxa found in abyssal habitats leads to hypothesize that hadal trenches are only partially connected to the surrounding benthic ecosystems.

In this study, we have also investigated the responses of microbial assemblages (virus-prokaryote interactions and bacterial diversity) to anthropogenic impact due to mineral exploitation in deep-sea benthic ecosystems, which will represent a major future threat for deep-sea habitats. The studied impact was exerted on the seabed of four zones belonging to an area of major commercial interest, the Clarion-Clipperton Fracture Zone (equatorial Pacific Ocean), and at different time scales.

Our results indicate that deep-sea mining can highly affect the benthic prokaryotic assemblages inhabiting the impacted seafloor as also virus-prokaryote interactions by determining an increase of viral pressure on prokaryotic hosts. These altered interactions are likely due to changes in bacterial assemblage composition among both different investigated areas and different temporal scales. In particular, despite the dominant bacterial families, widely distributed in all sediment areas were not affected by mining, important changes occurred for the less representative taxa leading to variations in prokaryotic community structure, which persists also several years after the impact. The results obtained in this study suggest that changes in virus-prokaryote interactions and shifts in the composition of bacterial assemblages caused by deep-sea mining can affect biogeochemical cycles and the functioning of the benthic microbial food webs over a long time scale.