Free-Living and Symbiotic Bacterial Communities in Contrasting Hydrothermally Active Habitats

by

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B.A., Université du Québec à Montréal, 2004
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Supervisory Committee

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Abstract

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Prokaryotic microorganisms, which are at the base of deep-sea hydrothermal vent food webs, adapt rapidly to environmental fluctuations. This study aimed at comparing bacterial communities in contrasting hydrothermal habitats to better understand compositional adaptations to local conditions. I first used small subunit (SSU) ribosomal RNA (rRNA) gene sequences to compare mat-forming bacterial communities associated with iron oxides at two hydrothermal vent sites on the Tonga Arc, southwest Pacific. Operational taxonomic units (OTUs), defined at 97% sequence similarity, were affiliated to a great diversity of autotrophic and heterotrophic groups. Metabolically diverse *Gammaproteobacteria* dominated the sample from Volcano 19, collected at 992 m depth. The sample from Volcano 1, collected at 197 m depth, was dominated by iron-oxidizing bacteria from the class *Zetaproteobacteria*. The depth of the sampling sites was proposed to explain clone library dissimilarities.

In the following studies, I compared bacterial communities associated with the vestimentiferan tubeworm *Ridgeia piscesae*, a foundation species at the Juan de Fuca Ridge. Samples of the polychaete were collected from tubeworm habitats in contrasting flow regimes that influenced temperature and hydrogen sulphide concentrations. Free-living bacteria were analyzed using both sequencing and 454 pyrosequencing of the SSU rRNA gene. Statistical analyses suggested a predictable pattern of bacterial community composition for the two habitats, with higher proportions of sulphur and hydrogen oxidizers in High Flow and more heterotrophic groups in Low Flow environments. Temperature, available energy for metabolism, and stability of the habitat were suggested to explain these distinctive bacterial communities. Symbiotic assemblages were investigated using the same sequencing methods together with catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH). *Gammaproteobacteria* dominated all sequence libraries, followed by *Epsilonproteobacteria*. CARD-FISH confirmed the co-occurrence of these groups within *R. piscesae* trophosomes. Statistical analyses indicated distinctive membership and structure of trophosome assemblages between sampling sites. Analysis of *R. piscesae* juvenile showed distinctive structural properties when compared to adult individuals, but similar membership, within sampling sites. These results suggested that the composition of trophosome assemblages might be affected by specific physical and chemical conditions at each vent site and that a selection process might occur during *R. piscesae*'s development.

Table of Contents

List of Tables

List of Figures

Acknowledgments

Destiny is no matter of chance; it is a matter of choice.
It is not a thing to be waited for; it is a thing to be achieved.
William Jennings Bryan

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Dedication

For my parents, who taught me that all dreams can come true.

Chapter 1 Introduction

Circulation of seawater through the seafloor and the presence of thermal springs at mid-ocean ridges had been proposed by geologists before the discovery of hydrothermal vents to explain differences between measured conductive heat flow from young ocean crust and thermal models of crustal cooling (Talwani *et al.*, 1971; Lister, 1972). However, the presence of a dense exotic fauna associated with the discharge of hydrothermal fluids, first seen in photographs at the Galápagos Spreading Centre, was completely unexpected (Lonsdale, 1977). The fauna of the deep sea was previously known to be sparse and slow growing, limited by the availability of organic matter produced photosynthetically in the surface ocean (Rowe, 1971). Lonsdale (1977) proposed two hypotheses to explain the surprisingly high biomass of deep-sea hydrothermal vent communities in the absence of light: the first one involved convection currents driven by the rising of warm waters and bringing organic matter to filter-feeders, while the second implied the role of chemoautotrophic bacteria supplying the production of new organic matter. As opposed to photoautotrophic organisms, using solar radiation as a source of energy to fix carbon dioxide into organic matter, chemosynthetic bacteria derive their metabolic energy from the oxidation of reducing inorganic substrates.

Measurements of high concentrations of metabolically active bacteria and hydrogen sulphide in hydrothermal fluids soon confirmed Lonsdale's second hypothesis: this alternative type of primary production by microorganisms represents the base of food webs at deep-sea vents, allowing a mostly endemic and specifically adapted fauna to colonize this extreme environment (Corliss *et al.*, 1979; Jannasch *et al.*, 1979; Karl *et al.*, 1980). Although hydrogen sulphide is often the major source of energy available

(Edmond *et al.*, 1979), chemolithoautotrophy at vents can also be fuelled by other oxidation states of sulphur, as well as ammonium, nitrogen, hydrogen, iron, methane and manganese (Karl, 1995). The discovery of hydrothermal vents triggered a renewed interest in chemosynthetic bacteria, which had previously been considered as unusual organisms with no major contribution to primary production (except for nitrifying bacteria extensively studied at the end of the 19th century (Winogradsky, 1890)), and therefore, with a minor role in the functioning of any ecosystem.

Free-living microbial communities

In addition to a focus on chemosynthetic bacteria, microbiologists have been attracted to hydrothermal vents because of the wide range of physical and chemical conditions available for microbial growth and metabolism. Free-living (not associated with a symbiotic host) chemolithoautotrophic microorganisms thrive in mixing zones between hydrothermal fluids and cold, oxygenated seawater, where both oxidants and reductants are the most accessible (Felbeck *et al.*, 1982; Jannasch, 1985). The first microbiological studies aimed to prove the presence and importance of sulphur-oxidizing bacteria. Using a combination of morphological and physiological analysis, such as isolation of pure cultures, quantification of radio-labelled CO_2 uptake, as well as measurements of ATP and other molecules related to metabolic activities, the prominence of these microorganisms was quickly demonstrated in visible flocculent material discharged with hydrothermal fluids (Jannasch *et al.*, 1979; Karl *et al.*, 1980), in neutrally-buoyant hydrothermal plumes (Cowen *et al.*, 1986; Winn *et al.*, 1986; Dick *et al.*, 2010), in microbial mats covering surfaces in the vicinity of vents (Jannasch *et al.*, 1981; Ruby *et al.*, 1981) and on sulphide mineral edifices (Jones *et al.*, 1983).

A first investigation of the relationship between the diversity of microbial communities and physico-chemical conditions of the environment was undertaken by Jannasch and Wirsen (1981) only a few years after the discovery of hydrothermal vents. Their study, based on morphological observations of microorganisms attached to both natural and artificial surfaces, showed a variety of structures characteristic of previously described organisms, allowing the authors to discuss the possible ecological significance of the different metabolic types suspected. Subsequently, research on hydrothermal vent microbiology focused mainly on specialized groups, such as sulphur bacteria (Ruby *et al.*, 1981; Felbeck *et al.*, 1982; Wirsen *et al.*, 1986; Ruby *et al.*, 1987) and thermophilic microorganisms (Baross *et al.*, 1983; Jones *et al.*, 1983; Belkin *et al.*, 1986; Jannasch *et al.*, 1988). However, morphological observations, carbon isotope ratios and enzyme activity assays provided only limited information on the taxonomic composition of vent microorganisms.

Molecular techniques overcame the limitations of culture enrichments and are now permitting a more thorough portrayal of microbial diversity, from which general patterns are emerging. For example, *Epsilonproteobacteria* often dominate microbial mats, sediments, fluids, chimneys, *in situ* growth chamber and colonization devices exposed to vent emission (Moyer *et al.*, 1995; Reysenbach *et al.*, 2000; Corre *et al.*, 2001; Teske *et al.*, 2002; López-García *et al.*, 2003; Alain *et al.*, 2004; Moussard *et al.*, 2006; Perner *et al.*, 2007; Opatkiewicz *et al.*, 2009; Zhou *et al.*, 2009; Huber *et al.*, 2010; Lanzen *et al.*, 2011; Jaeschke *et al.*, 2012). In addition, the use of high-throughput sequencing techniques has revealed a surprisingly high diversity of microorganisms associated with different vent environments (Sogin *et al.*, 2006; Huber *et al.*, 2007; Flores *et al.*, 2011; Jorgensen *et al.*, 2012; Sylvan *et al.*, 2012a; Zhang *et al.*, 2012).

Symbiotic bacterial assemblages

Shortly after the discovery of hydrothermal vents, primary production by free-living microbial communities was recognized as an important source of nutrition for suspension- and deposits-feeders, but it did not explain the presence of large populations of the giant white clam *Calyptogena magnifica* and the gutless tubeworm *Riftia pachyptila* (Jannasch *et al.*, 1979). In parallel, a study of *Siboglinum fiordicum*, a frenulate siboglinid common in Norwegian fjords at depths between 25 and 560 m and closely related to *R. pachyptila*, showed that the uptake of dissolved organic matter alone could not account for high metabolic rates (Southward *et al.*, 1979). Morphological observations and analyses of lipopolysaccharides, enzymes and carbon isotope ratios soon confirmed the presence of chemoautotrophic bacteria in the clam gill tissue and in the tubeworm trophosome (Cavanaugh, 1980; Cavanaugh *et al.*, 1981; Felbeck, 1981; Felbeck *et al.*, 1981; Jones, 1981; Rau, 1981; Southward *et al.*, 1981; Southward, 1982). This kind of association will hereafter be referred to as symbiosis, which is used to describe a long-term relationship in which all partners gain benefits (mutualism).

This new concept of an ecosystem where the bulk of the biomass is supported by symbiotic associations between invertebrates and chemolithoautotrophic microorganisms stimulated the discovery of chemosynthetic symbioses in other reducing environments, such as cold seeps (Paull *et al.*, 1984; Childress *et al.*, 1986; Kulm *et al.*, 1986), whale and wood falls (Smith *et al.*, 1989; Bennett *et al.*, 1994; Distel *et al.*, 2000), and mangrove sediments (Ott *et al.*, 1998). These associations were proposed to benefit each partner: the host bridging the oxic-anoxic interface and provide symbionts with access to dissolved oxygen and reducing substrates, while the bacterial partners furnish their host with organic carbon, assimilable nitrogen and other nutrients (Felbeck *et al.*, 1982; Bright *et al.*, 2000; Stewart *et al.*, 2005; Cavanaugh *et al.*, 2006; Duperron *et al.*, 2006; Woyke *et al.*, 2006; Bates, 2007; Newton *et al.*, 2007; Dubilier *et al.*, 2008; Goffredi, 2010; Watsuji *et al.*, 2010; Tsuchida *et al.*, 2011; Ponsard *et al.*, 2013).

Chemosynthetic symbioses have been found in seven invertebrate phyla (Dubilier *et al.*, 2008), and six major families represent the greater part of the biomass at vents, cold seeps, and whale and wood falls: Vesicomyidae and Mytilidae bivalves, Provannidae gastropods, Alvinocarididae decapods, and Alvinellidae and Sibloglinidae polychaetes (Vrijenhoek, 2010). The chemosynthetic partners are also phylogenetically and metabolically diverse. For example, the vesicomyid clam *C. magnifica* hosts the sulphur-oxidizing *Candidatus Ruthia magnifica*, a gammaproteobacterial intracellular endosymbiont (Newton *et al.*, 2007). Some mytilid mussels, such as *Bathymodiulus* sp., can simultaneously harbour two different gammaproteobacterial intracellular symbionts: one thiotrophic and one methanotrophic (Fisher *et al.*, 1993; Duperron *et al.*, 2006). A study from Urakawa et al. (2005) showed that two provannid snails, *Alviniconcha hessleri* and *Ifremeria nautilei*, collected from the Manus Basin had different intracellular symbionts. While both are sulphur-oxidizing bacteria, *I. nautilei* hosts a member of the *Gammaproteobacteria* and *A. hessleri* hosts an *Epsilonproteobacteria* (Urakawa *et al.*, 2005). A recent study of the epibiotic community of the alvinocaridid shrimp *Rimicaris exoculata* suggested the co-occurrence of iron-, sulphide- and methane-oxidizing bacteria clustering in the *Epsilon*-, *Gamma*-, *Delta*-, and *Alphaproteobacteria* (Zbinden *et al.*, 2008). The alvinellid worm *Alvinella pompejana* was shown to host both heterotrophic and autotrophic epibionts (Prieur *et al.*, 1990) belonging to the *Epsilonproteobacteria* (Haddad *et al.*, 1995; Cary *et al.*, 1997) and *Spirochaete* (Campbell *et al.*, 2001) Proteomic and metagenomic studies confirmed that the siboglinid worm *R. pachyptila*, known from vents on the East Pacific Rise and the Galapagos Ridge, hosts a single sulphur-oxidizing intracellular symbiont, *Candidatus Endoriftia persephone*, which is a member of the *Gammaproteobacteria* (Markert *et al.*, 2007; Robidart *et al.*, 2008). However, the endosymbionts of the Northeast Pacific siboglinid *Ridgeia piscesae* have been shown to be more diverse, with up to five different phylotypes belonging to the taxonomic groups *Gammaproteobacteria*, *Alphaproteobacteria* and *Bacteroidetes* (Chao *et al.*, 2007).

Research focus

Hydrothermal vent conditions are extremely variable both in time and space, and are characterized by steep thermal and chemical gradients (McCollom *et al.*, 1997), affecting the composition of faunal assemblages (e.g., Fustec *et al.*, 1987; Hessler *et al.*, 1988; Tunnicliffe *et al.*, 1990; Sarrazin *et al.*, 1997; Tunnicliffe *et al.*, 1997; Sarrazin *et al.*, 1999a; Sarrazin *et al.*, 1999b; Sarrazin *et al.*, 2002; Mullineaux *et al.*, 2003; Cuvelier *et al.*, 2009; Marcus *et al.*, 2009; Cuvelier *et al.*, 2011). While microorganisms are known to adapt rapidly to environmental conditions, early studies of microbial communities have primarily presented a static portrait of the diversity of a particular vent habitat at a particular point in time. More recently, attempts to link environmental parameters to the composition of microbial populations have started to emerge (Huber *et al.*, 2003; Schrenk *et al.*, 2003; Kormas *et al.*, 2006; Perner *et al.*, 2007; Hodges *et al.*, 2009; Kato *et al.*, 2009; Opatkiewicz *et al.*, 2009; Huber *et al.*, 2010; Perner *et al.*, 2011; Jaeschke *et al.*, 2012; Jorgensen *et al.*, 2012; Sylvan *et al.*, 2012b; Sylvan *et al.*, 2012a; Anderson *et al.*, 2013), but only one study attempted to relate the variation of the microbial communities with different faunal successional stages (Pagé *et al.*, 2004).

The main objective of my thesis was to compare the membership and structure of free-living and symbiotic bacterial populations in contrasting, hydrothermally active habitats in order to better understand the influence of hydrothermal conditions on the composition of bacterial communities. During the course of my thesis, I investigated two hydrothermal systems differentiated by the main source of energy available for microbial growth. The first one, located on a volcanic arc, was characterized by weak hydrothermal flow and the iron oxide deposits, suggesting that reducing iron was the primary source of metabolic energy. The second one, located on a spreading centre, featured typical hydrothermal vent faunal communities described in sulphide-driven systems. In the latter system, I investigated bacterial communities associated with the Northeast Pacific vestimentiferan *Ridgeia piscesae*. This tubeworm, which entirely depends on a symbiotic relationship with chemosynthetic bacteria for its nutrition, act as a foundation species, providing a colonization substrate for other species (Marcus *et al.*, 2009). A dynamic succession model developed by Sarrazin et al. (1997; Sarrazin *et al.*, 1999a; 1999b; 2002) indicated the presence of *R. piscesae* in three of the six chimney faunal assemblages described at the Main Endeavour vent field on the Juan de Fuca Ridge, illustrating the ability of the worm to live in variable hydrothermal conditions. The tubeworm, originally described as two species (Jones, 1985), exhibits corresponding phenotypic plasticity, ranging from the “short-fat” morphotype, colonizing diffuse High Flow environments in the vicinity of black smokers characterized by sulphide concentrations of 40 μM or more and temperatures up to 41.9°C (Sarrazin *et al.*, 2002), to the "long-skinny" morphotype, found in Low Flow environments where dissolved hydrogen sulphide is often undetectable and temperatures are close to those of ambient seawater (Urcuyo *et al.*, 2003). I compared free-living and trophosome bacterial assemblages between these two extreme habitats, referred hereafter as High Flow and Low Flow habitats, in order to gain a better understanding of their adaptation to the environment.

Methodological approach

All samples were collected using either the remotely operated vehicle ROPOS or the submersible *Alvin*. The comparison of the diversity and structure of bacterial communities was based on small subunit ribosomal RNA (SSU rRNA) gene sequences. In Chapter 2, I used Sanger sequencing to construct SSU rRNA clone libraries from two iron oxide samples collected on volcanoes on the Tonga Arc, Southwest Pacific. I also used this chapter to develop a workflow for analysing bacterial diversity using molecular tools, in order to identify general habitat specific trends in bacterial assemblage composition and infer energy sources for bacterial metabolism. The analysis of the clone libraries revealed a high diversity of bacterial taxa within what appeared to be a single lineage of iron oxidizers.

In the following chapters, I used increasingly sophisticated molecular and statistical methods to compare free-living and trophosome bacterial assemblages associated with *R. piscesae* in High Flow and Low Flow habitats. Since macrofaunal assemblages become more complex as hydrothermal vent conditions become less severe (Sarrazin *et al.*, 1999b), I hypothesized that High Flow bacterial populations would be dominated by fewer, thermophilic taxa involved in sulphide oxidation while Low Flow populations would be more diverse and include taxa using different sources of metabolic energy. *R. piscesae* samples were transported in sealed bioboxes to limit contamination between each other and with background seawater. In Chapter 3, Sanger sequencing was combined with pyrosequencing to investigate four particulate detritus samples from each habitat, collected from four different sites on the Endeavour Segment of the Juan de Fuca Ridge, Northeast Pacific. The number of replicates and the amount of data produced by pyrosequencing allowed powerful statistical analyses suggesting predictable patterns of the composition of the bacterial communities in High Flow and Low Flow habitats.

Adult *R. piscesae* trophosome assemblages from different vent sites on Axial Volcano and the Endeavour Segment of the Juan de Fuca Ridge were studied in Chapter 4 using both sequencing approaches previously mentioned as well as catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). Sanger sequencing, performed on the DNA extracted from the trophosome of three individuals from each habitat, was used to confidently assigned phylogenetic affiliations to the sequences detected, while pyrosequencing, performed on 20 individuals from each habitat, permitted a deeper probing of potential symbiont phylotypes and the comparison of High Flow and Low Flow habitats. CARD-FISH allowed the visualization of the co-occurrence of two taxonomic groups within twelve *R. piscesae* trophosomes. Possible contamination of trophosome tissue by environmental bacteria was controlled by comparing the results to pyrosequencing data obtained for free-living bacterial communities from the same habitats.

Chapter 5 compared the trophosome assemblages of adult and juvenile individuals collected from the same vent sites on Axial Volcano and the Endeavour Segment of the Juan de Fuca Ridge using pyrosequencing data. In addition, the comparison of the sequence libraries between sampling sites and habitats allowed the detection of patterns in the membership and structure of the trophosome assemblages.

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Chapter 2 Bacterial diversity in Fe-rich hydrothermal sediments at two South Tonga Arc submarine volcanoes

Chapter 2 is a research article published in the peer-reviewed journal *Geobiology*:

Forget, N.L., Murdock, S.A., and Juniper, S.K. (2010) Bacterial diversity in Fe-rich hydrothermal sediments at two South Tonga Arc submarine volcanoes. *Geobiology* **8**: 417-432.

Sheryl Murdock (Research Associate, Dr. Juniper's Microbial Ecology laboratory, University of Victoria) supervised the molecular work and phylogenetic analyses. Dr. Juniper (Thesis supervisor, University of Victoria) provided the resource for this work and assisted with the writing of this article.

Part of the results reported in Chapter 2 were used in another research article published in the peer-reviewed journal *Cahiers de Biologie Marine*:

Murdock, S., Johnson, H., Forget, N., and Juniper, S.K. (2010) Composition and diversity of microbial mats at shallow hydrothermal vents on Volcano 1, South Tonga Arc. *Cahiers de Biologie Marine* **51**: 407-413.

I contributed to this article by supervising the molecular work, performing part of the phylogenetic analyses, and assisting with the writing. This article appears in Appendix B.

Abstract

Seafloor iron (Fe) oxide deposits are a common feature of submarine hydrothermal systems. Morphological study of these deposits has led investigators to suggest a microbiological role in their formation, through the oxidation of reducing Fe in hydrothermal fluids. Fe-oxidizing bacteria, including the recently described

Zetaproteobacteria, have been isolated from a few of these deposits but generally little is known about the microbial diversity associated with this habitat. In this study, we characterized bacterial diversity in two Fe oxide samples collected on the seafloor of Volcanoes 1 and 19 on the South Tonga Arc. We were particularly interested in confirming the presence of *Zetaproteobacteria* at these two sites and in documenting the diversity of groups other than Fe oxidizers. Our results (small subunit ribosomal RNA gene sequence data) showed a surprisingly high bacterial diversity, with 150 operational taxonomic units belonging to 19 distinct taxonomic groups. Both samples were dominated by *Zetaproteobacteria* Fe oxidizers. This group was most abundant at Volcano 1, where sediments were richer in Fe and contained more crystalline forms of Fe oxides. Other groups of bacteria found at these two sites include known S- and a few N-metabolizing bacteria, all ubiquitous in marine environments. The low similarity of our clones with the GenBank database suggests that new species and perhaps new families were recovered. The results of this study suggest that Fe-rich hydrothermal sediments, while dominated by Fe oxidizers, can be exploited by a variety of autotrophic and heterotrophic microorganisms.

Introduction

The presence of iron (Fe) oxide deposits associated with geothermally heated fluids emerging from the seafloor has been reported often since the discovery of hydrothermal vents in 1977 (e.g., Alt, 1988; Juniper *et al.*, 1988; Emerson *et al.*, 1997; Emerson *et al.*, 2002; Kennedy *et al.*, 2003b, a; Fortin *et al.*, 2005; Langley *et al.*, 2008). These fluids undergo high-temperature reactions with the Earth's crust, in which Fe is the fourth most abundant element. Mixing of high-temperature hydrothermal fluids with crustal seawater can occur prior to venting, resulting in subseafloor cooling, pH buffering and oxidation of reducing sulphur species. Where subsurface mixing is extensive, it is not uncommon to observe venting fluids that contain reducing Fe but no dissolved sulphide. When such fluids are discharged at the seafloor, they mix with cold, oxygenated seawater at circumneutral pH, and the chemical oxidation of reducing Fe occurs rapidly. Despite this well-known spontaneous reaction, the origin of Fe oxide deposits in submarine hydrothermal systems has been the subject of many studies and is still extensively debated. Filamentous textures of many Fe oxide deposits often resemble biogenic Fe oxides produced by the neutrophilic Fe-oxidizing bacteria *Gallionella ferruginea* and *Leptothrix discophora*, which has led investigators to suggest a biological origin for Fe oxidation (Alt, 1988; Juniper *et al.*, 1988; Emerson *et al.*, 1997; Emerson *et al.*, 2002; Kennedy *et al.*, 2003b, a; Fortin *et al.*, 2005; Langley *et al.*, 2008). Understanding the role of pH-neutral, Fe-oxidizing bacteria in forming hydrothermal Fe oxide deposits has been problematical; they are difficult to grow in laboratory cultures and microorganisms can also indirectly precipitate Fe oxides on their reactive cell surfaces (Juniper *et al.*, 1995; Emerson *et al.*, 2002; Severmann *et al.*, 2006; Langley *et al.*, 2008). A study of Fe oxide mats from seafloor vents at Loihi Seamount (Hawaii) (Emerson *et al.*, 2002) finally led to the isolation and culturing of obligate, pH-neutral Fe-oxidizing bacteria (strains PV-1 and JV-1) from a marine environment, where an abundant source of reducing Fe was available. Isolate PV-1 was later described as *Mariprofundus ferrooxydans*, an obligately lithotrophic bacterium belonging to the *Zetaproteobacteria* that requires microaerobic conditions and Fe^{2+} as the energy source (Emerson *et al.*, 2007).

Fe oxidation can form substantial accumulations of Fe-rich sediments at active (and inactive) seafloor hydrothermal sites. Previous studies have focused on the presence of Fe oxidizers at these sites and their role in the formation of Fe oxides (Emerson *et al.*, 2002; Edwards *et al.*, 2003; Edwards *et al.*, 2004), but several basic questions about the microbial ecology of these deposits have yet to be addressed. Is there a typical Fe oxide sediment flora? Are these deposits colonized by monocultures of Fe oxidizers or is the diversity of microbes comparable with that of other marine sediments? Is Fe oxidation the primary process supporting microbial growth in this environment? Do venting characteristics (i.e. intensity of the flow, presence of H_2S, etc.) and/or external inputs of organic matter (from photic zone) have an impact on the bacterial diversity? In a recent study of Fe-containing microbial mats collected on submarine volcanoes, Hodges and Olson (2009) reported the dominance of *Epsilonproteobacteria*, abundant *Gammaproteobacteria* and only a few confirmed Fe oxidizers belonging to the *Zetaproteobacteria*. Here we address the above and other questions in a comparative study of the composition and diversity of bacterial communities in hydrothermal Fe oxide deposits on two seafloor volcanoes on the South Tonga Arc (South Pacific). We used small subunit (SSU) ribosomal RNA (rRNA) gene sequence analysis to examine the overall phylogenetic diversity of the bacterial community inhabiting these sediments and to provide molecular evidence for the presence of Fe oxidizers, especially *Mariprofundus*. We also used statistical methods to assess species richness and to compare microbial community structure on both volcanoes.

Previous surveys of our two study sites, Volcanoes 1 and 19, had revealed areas of diffuse hydrothermal venting and Fe oxide deposits. Langley et al. (2008) suggested a biogenic origin for the Fe oxides on Volcanoes 1 and 19, based on electron microscopy, X-ray diffraction and Mössbauer spectroscopy and the presence of structures morphologically similar to the genus *Mariprofundus*. Our molecular study used subsamples of the same material examined by Langley et al. (2008).

Methods

Site description

Fe oxide samples were collected in April–May 2007 during the joint German–Canadian–New Zealand MANGO research cruise (SO-192⁄2) on the R⁄V *Sonne*, using the Canadian remotely-operated vehicle (ROV) ROPOS (Fig. 2.1). A survey of the Western caldera structure at Volcano 19, located at the southern end of the Tonga Arc, revealed an area of diffuse, active venting, where the seafloor was covered with Fe oxyhydroxide crusts extending over more than 900 m along the base of the caldera wall and over a depth range of 985–850 m. Fe oxide chimneys up to 2–3 m in height were observed throughout the site (Fig. 2.2A). These structures were very friable, composed of unconsolidated sedimentary material. They were predominantly rust-coloured, suggestive of ferrihydrite, or possibly microcrystalline goethite (Langley *et al.*, 2008), with lighter beige-coloured tops mostly composed of dense filamentous microbial mats. No shimmering water or bubbling was visible at the site of sampling, but significant temperature anomalies were measured with a probe inserted into the sediments (13.4°C above ambient) (Table 2.1).

Volcano 1 is situated further north on the Tonga Arc. A chain of three or more explosion craters (pit craters) occurs on the outer rim of the caldera of Volcano 1. The central pit crater was hydrothermally active in 2007 and in the western portion there was

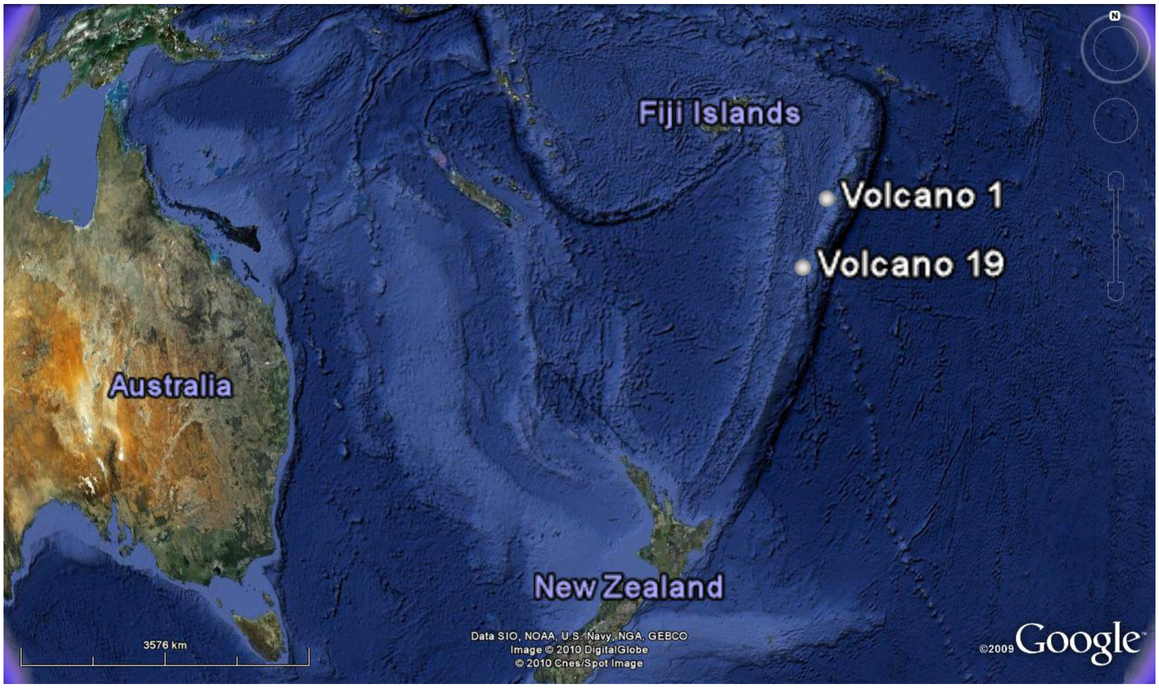


Figure 2.1

Map showing the locations of Volcanoes 1 and 19 sampled in this study. © 2009 Google; Data SIO, NOAA, U.S. Navy, NGA, GEBCO; Image © 2010 DigitalGlobe; © Cnes/SpotImage.

extensive Fe oxide-encrusted ash and abundant microbial mat material (Fig. 2.2B). The mat material was approximately of the same rust color as that seen on Volcano 19, but no chimney structures were observed in this area. Immediately below the surface layer, the Fe oxides were much darker, red-brown in color, suggestive of recrystallization from ferrihydrite with goethite to perhaps, hematite (Langley *et al.*, 2008). There were no obvious signs of venting, but temperature anomalies up to 4°C above ambient were recorded within the sediments (Table 2.1). In 2005, low- to moderate-temperature venting was observed at this site by the SITKAP cruise (Stoffers *et al.*, 2006), suggesting a recent change in flow characteristics.

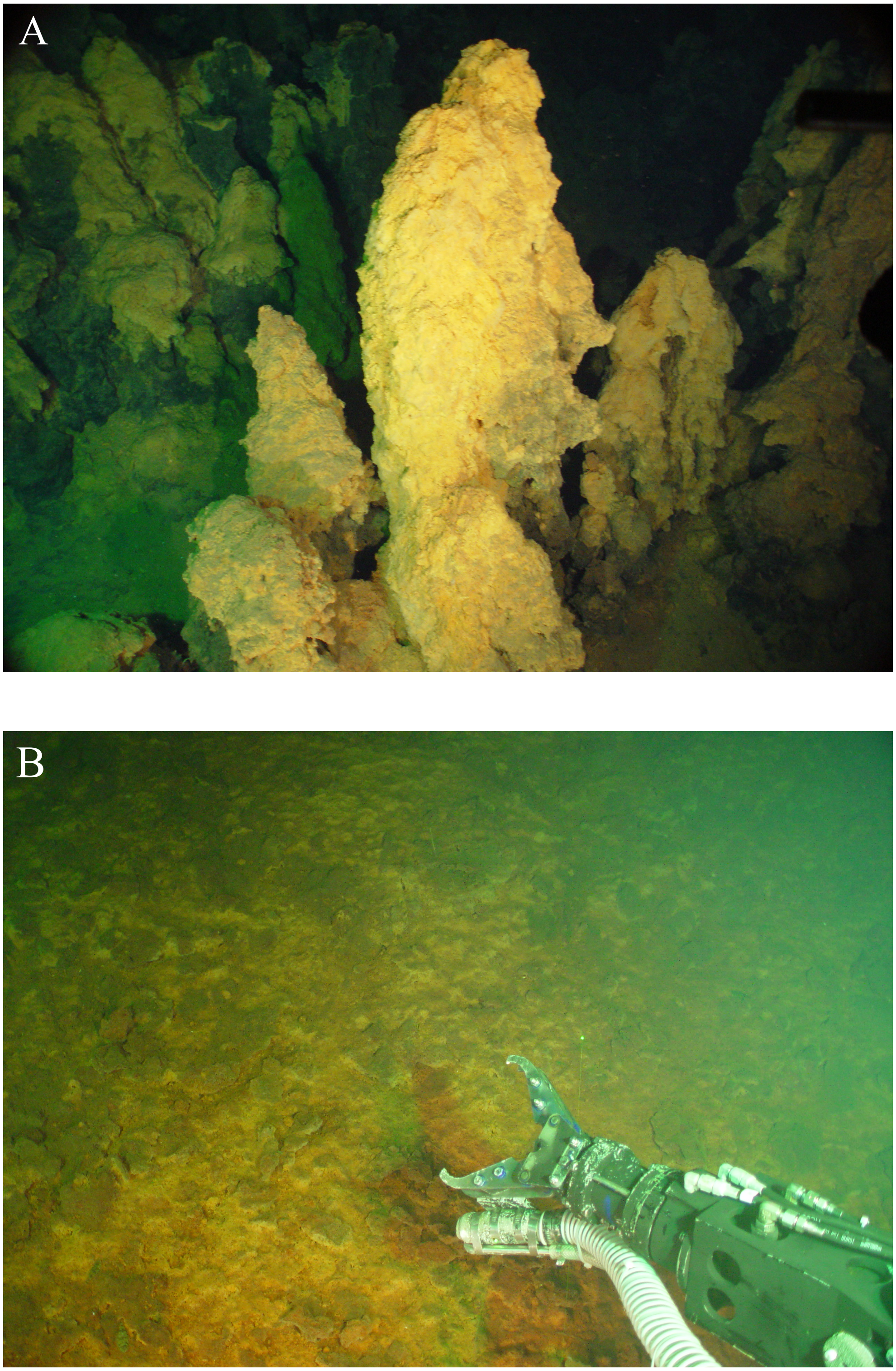


Figure 2.2

(A) Fe oxide structures in western caldera of Volcano 19, measuring 2-3 m in height. (B) Fe oxide-encrusted ash covering the floor of the western end of the central pit crater on Volcano 1.

Table 2.1 Description and location of the sampling sites.

Sampling site	Sample description	Latitude/Longitude	Depth (m)	Ambient seawater temp (˚C)	Max sample temp (˚C)
Volcano 19	Fe-oxide chimney	24°48.282’S/177°1.141’W	992	2.6	16
Volcano 1	Fe-oxide sediments	21°9.205’S/175°44.764’W	197	16	21

Sample collection

Samples were collected in 2-liter acrylic jars using the ROPOS suction sampler, where a 200-µm mesh covered the outflow from each jar. The sample from Volcano 19 (177°1.141’ W 24°48.282’ S, 992 m depth) was collected from the top of a chimney structure, where living filamentous microbial communities appeared to be present in greatest abundance. This sample was characterized by fine-grained Fe oxide particles with little clumping of the solid material and slow settling of the particles from suspension. The sample taken at Volcano 1 (175°44.764’ W 21°9.205’ S, 197 m depth) suctioned the first 5–10 cm of Fe oxide sediments (Table 2.1). This sample consisted of Fe oxides mixed with larger (5 cm) clumps of dark gray to black, brittle material, probably cemented ash. The Fe oxides at this site were coarser grained and tended to clump and settle from suspension much faster than those obtained from Volcano 19. Onboard on the research vessel, the samples were frozen at -80°C for future analyses.

Clone library construction and sequencing

Frozen sediment and Fe oxide chimney samples were thawed overnight at 5°C, then centrifuged (11.5 g, 5 min) to eliminate excess seawater, following which DNA was extracted from approximately 5 g of material (dry weight) using the UltraClean Mega Soil DNA Kit (Mo BIO, Carlsbad, CA, USA), following the manufacturer’s instructions.

The crude DNA extracts were purified with the QIAquick PCR Purification Kit (QIAGEN, Carlsbad, CA, USA) according to the manufacturer's instructions. Following a 1/10 dilution of the purified DNA extracts, SSU rRNA gene sequences were amplified by PCR using the universal bacterial primers BAC- 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and BAC- 1492R (5'-GTT TAC CTT GTT ACG ACT T-3'). A range of $MgCl_2$ concentrations and annealing temperatures were tested and the most stringent combinations with sufficient yield in the target size range were used. The final reaction mixture used (20 µL) contained 1-µL template DNA, 3.125 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 0.25 µM (each) primer, 1x PCR buffer (Invitrogen, Valencia, CA, USA) and 1 U of *Taq* DNA polymerase (Invitrogen). PCRs were performed with the following program: initialization step at 94°C, 2 min; 30 cycles of denaturation at 94°C, 30 s; annealing 54°C, 45 s; extension 72°C, 2 min; and final elongation at 72°C, 10 min. Amplification bands were visualized on 1.2% agarose gels stained with SYBR safe (Invitrogen), and reconditioned following (Thompson *et al.*, 2002) to eliminate heteroduplexes using the same reaction mixture and PCR program but with only three cycles of denaturation, annealing and extension. Products of four parallel PCRs were combined, followed by a second purification as described above. DNA was cloned into pGEM-T-Easy vector (QIAGEN) according to the manufacturer's protocol. White colonies were randomly chosen and screened for inserts by PCR reaction using the vector primers M13F (5'-GTA AAA CGA CGG CCAG- 3') and M13R (5'-CAG GAA ACA GCT ATG AC- 3') in a 50-µL reaction mixture, containing 1 µL clone culture, 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.4 µM (each) primer, 1x PCR buffer (Invitrogen), and 1.25 U of *Taq* DNA polymerase (Invitrogen). Sequencing of the inserts was completed at the High-Throughput Genomics Unit (University of Washington) using the PCR primers BAC-8F and BAC-1492R, and 519b (5’-GWA TTA CCG CGG CKG CTG-3’), 515Fb (5’-GTG CCA AGC MGC CGC GGT AA-3’), 907Rb (5’-CCG TCA ATT CMT TTR AGT TT-3’) and 926Fb (5’-AAA CTY AAA KGA ATT GAC GG-3’).

Prokaryotes belonging to the domain *Archaea* were not included in this study. Repeated PCR attempts with universal archaeal SSU primers ARCH-8F (5’-TCC GGT TGA TCC TGC C- 3’) and ARCH-1492 (5’-GGC TAC CTT GTT ACG ACT T- 3’) yielded no product for the sample from Volcano 19 and only a very weak yield for the sample from Volcano 1. This together with the fact that known neutrophilic Fe oxidizers cluster in the domain *Bacteria* led us to exclude *Archaea* from consideration and emphasize the *Bacteria*.

Phylogenetic analyses

The sequences were assembled and checked manually for errors using SEQUENCHER v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). The SSU rRNA gene sequence data set was screened for potential chimeric structures using the Bellerophon server (Huber *et al.*, 2004), available through the Greengenes website, and sequences found to be of potential chimeric origin were excluded from this study. Closely related sequences were recovered using the online BLAST tool (available through the National Centre for Biotechnology Information (NCBI)) and all sequences were aligned with alignment gap characters conserved using the Greengenes Align tool (DeSantis *et al.*, 2006). Alignments were manually checked and the percentage of similarity was obtained using BIOEDIT, v7.0.5.3 (Hall, 1999). Neighbor-joining trees were constructed using the PHYLIP software package, v3.6 (Felsenstein, 2005) with 1000 bootstrap replicates, and visualized with TREEVIEW (Page, 1996). The phylogenetic affiliation of each sequence was assigned using the Classifier tool available through the Ribosomal Database Project (RDP) (Wang *et al.*, 2007) and the ARB software (http://www.arb-home.de). For all analyses and subsequent discussion of the bacterial diversity at Volcanoes 19 and 1, sequences with less than 3% difference were considered to be the same operational taxonomic unit (OTU, i.e. sequence type). The genus, family and phylum levels were differentiated at 5%, 10% and 20% difference respectively (see Schloss *et al.*, 2004).

Diversity analyses and comparisons of clone libraries

Rarefaction curves were calculated using DOTUR (Schloss *et al.*, 2005) from the distance matrices calculated in PHYLIP. The coverage of the two clone libraries was calculated as in Ravenschlag et al. (1999) with the equation $C = [1 - (n_1/N)] \times 100$, where n_1 is the number of unique clones within the library and N is the total number of clones. Bacterial community overlap between the two samples was estimated by the non parametric maximum likelihood estimator (NPMLE) as described by Yue and Clayton (2005).

Nucleotide sequence accession numbers

The sequences representing the OTUs reported in this paper have been submitted to GenBank and assigned accession numbers FJ905616–FJ905765.

Results

Diversity analysis

In order to characterize and compare the bacterial diversity in the Fe oxide samples, two clone libraries representing each of the two volcanoes were constructed with the obtained SSU rRNA gene sequences. Within a total of 158 sequenced clones from Volcano 19, a total of 76 different OTUs were detected, while 74 different OTUs were identified out of 176 sequenced clones from Volcano 1 (Table 2.2). Neither of the two rarefaction curves reached a clear asymptote, suggesting that environmental diversity was not fully represented in our clone libraries (Fig. 2.3). This result was supported by the coverage index, which was calculated to assess the completeness of our clone libraries (Table 2.2).

Overall characteristics of the clone libraries

The DNA retrieved from the Fe oxide samples was phylogenetically affiliated with 19 major groups of the domain *Bacteria*: *Alpha*-, *Gamma*-, *Delta*-, *Epsilon*- and *Zetaproteobacteria*, *Nitrospira*, *Acidobacteria*, *Firmicutes*, *Chloroflexi*, *Fibrobacteres*, *Deferribacteres*, *Gemmatimonadetes*, *Bacteroidetes*, *Chlorobi*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, candidate division OP1 and candidate division WS6.

There was a similarly wide range of diversity in the two samples (Fig. 2.4). However, members of the *Chlorobi* and the *Gemmatimonadetes* phyla were only detected at Volcano 1, and members of the *Firmicutes* phylum, the candidate division OP1 and the candidate division WS6 were only detected at Volcano 19. Seven OTUs, belonging to

Table 2.2 Diversity analyses of the SSU rRNA gene clone libraries from two Fe-oxide samples collected on Volcanoes 19 and 1.

Sampling site	% Coverage	OTUs detected	Unique clones	NPMLE
Volcano 19	44.74	77	42	0.59
Volcano 1	39.19	74	45	

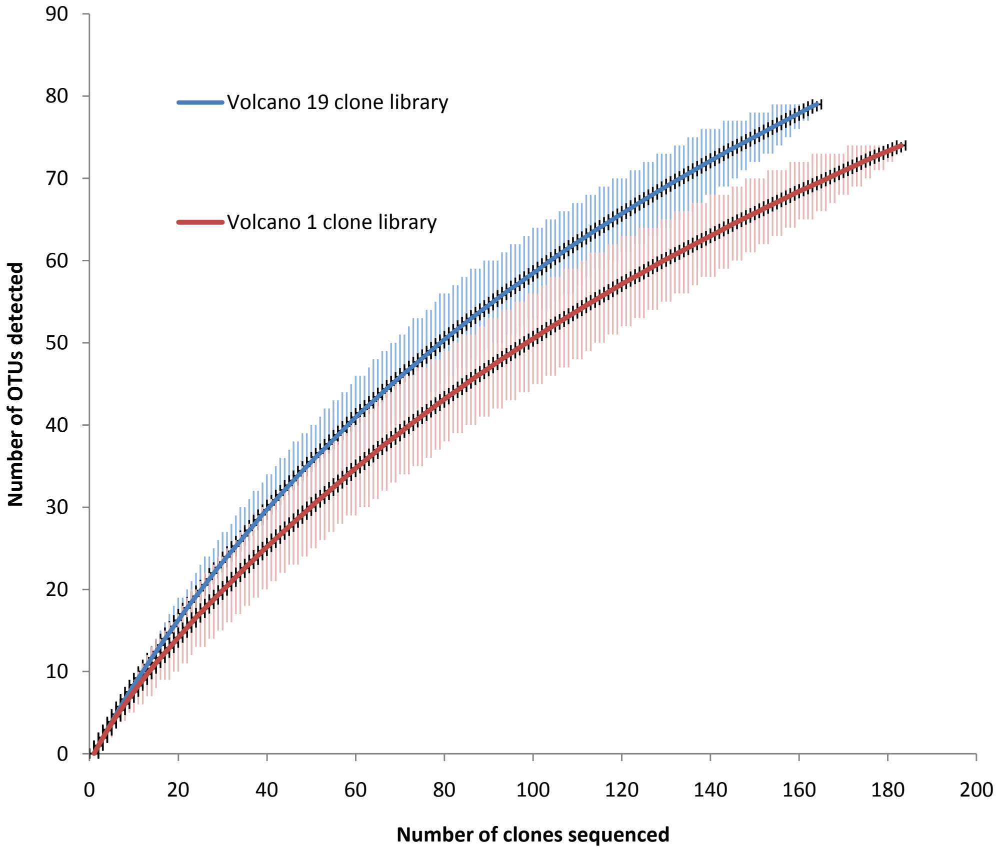
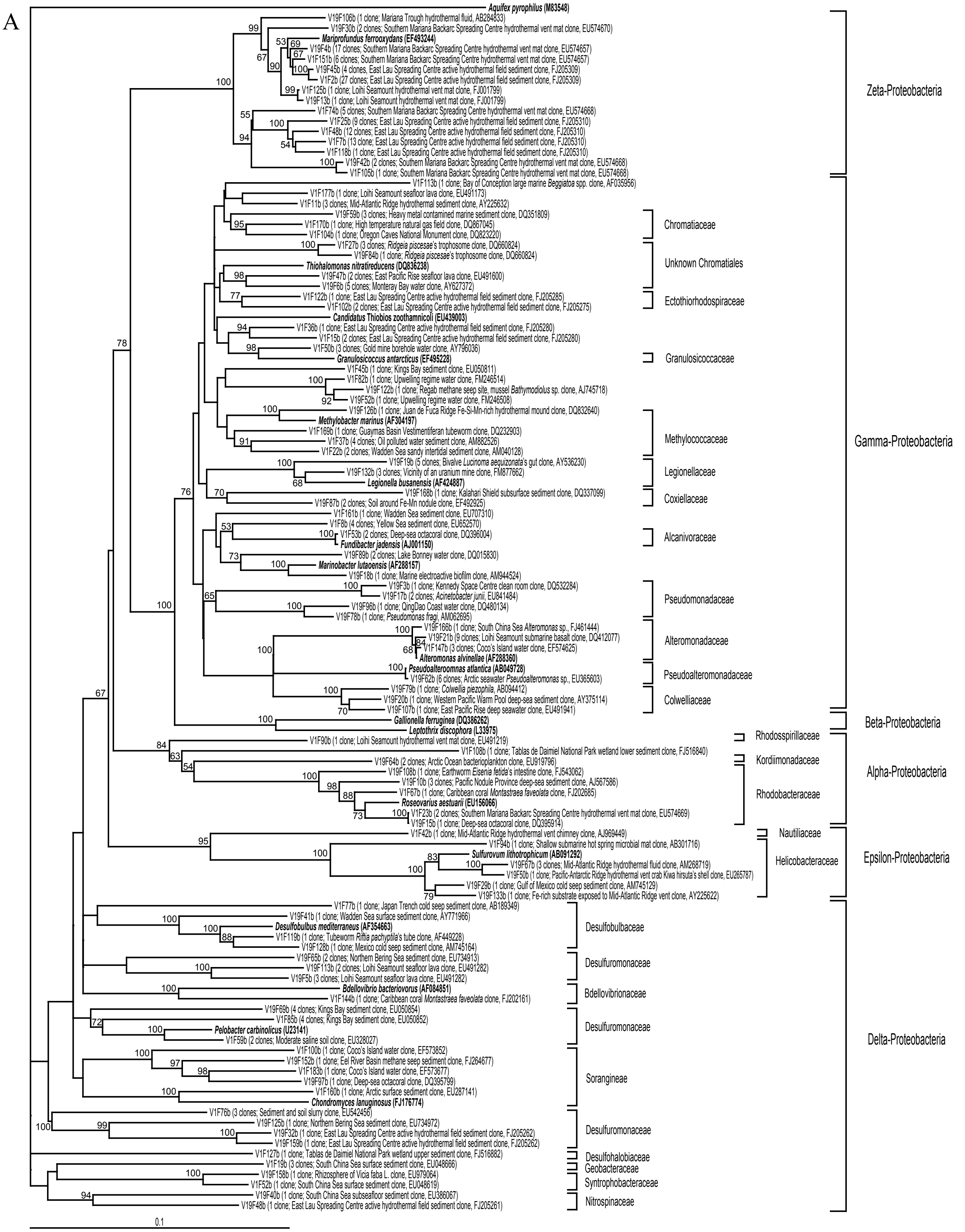


Figure 2.3

Rarefaction curves plotted with error bars representing 95% confidence internals and showing the species richness in Fe oxide samples from Volcanoes 19 and 1. The trend of the curves indicates that sequencing more clones would have increased the number of operational taxonomic units (OTUs) found in both samples. OTUs are defined at ≥ 97% sequence similarity.


A
Aquifex pyrophilus (M83548)
V19F106b (1 clone; Mariana Trough hydrothermal fluid, AB284833)
V19F30b (2 clones; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574670)
Mariprofundus ferrooxydans (EF493244)
V19F4b (17 clones; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574657)
V1F151b (6 clones; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574657)
V19F45b (4 clones, East Lau Spreading Centre active hydrothermal field sediment clone, FJ205309)
V1F2b (27 clones; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205309)
V1F125b (1 clone; Loihi Seamount hydrothermal vent mat clone, FJ001799)
V19F13b (1 clone; Loihi Seamount hydrothermal vent mat clone, FJ001799)
V1F74b (5 clones; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574668)
V1F25b (9 clones; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205310)
V1F48b (12 clones; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205310)
V1F7b (13 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205310)
V1F118b (1 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205310)
V19F42b (2 clones; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574668)
V1F105b (1 clone; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574668)
Zeta-Proteobacteria
V1F113b (1 clone; Bay of Conception large marine Beggiatoa spp. clone, AF035956)
V1F177b (1 clone; Loihi Seamount seafloor lava clone, EU491173)
V1F11b (3 clones; Mid-Atlantic Ridge hydrothermal sediment clone, AY225632)
V19F59b (3 clones; Heavy metal contamined marine sediment clone, DQ351809)
V1F170b (1 clone; High temperature natural gas field clone, DQ867045)
V1F104b (1 clone; Oregon Caves National Monument clone, DQ823220)
Chromatiaceae
V1F27b (3 clones; Ridgeia piscesae's trophosome clone, DQ660824)
V19F84b (1 clone; Ridgeia piscesae's trophosome clone, DQ660824)
Thiohalomonas nitratireducens (DQ836238)
V19F47b (2 clones; East Pacific Rise seafloor lava clone, EU491600)
V19F6b (5 clones; Monteray Bay water clone, AY627372)
Unknown Chromatiales
V1F122b (1 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205285)
V1F102b (2 clones; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205275)
Ectothiorhodospiraceae
Candidatus Thiobios zoothamnicoli (EU439003)
V1F36b (1 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205280)
V1F15b (2 clones; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205280)
V1F50b (3 clones; Gold mine borehole water clone, AY796036)
Granulosicoccus antarcticus (EF495228)
Granulosicoccaceae
V1F45b (1 clone; Kings Bay sediment clone, EU050811)
V1F82b (1 clone; Upwelling regime water clone, FM246514)
V19F122b (1 clone; Regab methane seep site, mussel Bathymodiolus sp. clone, AJ745718)
V19F52b (1 clone; Upwelling regime water clone, FM246508)
V19F126b (1 clone; Juan de Fuca Ridge Fe-Si-Mn-rich hydrothermal mound clone, DQ832640)
Methylobacter marinus (AF304197)
V1F169b (1 clone; Guaymas Basin Vestimentiferan tubeworm clone, DQ232903)
V1F37b (4 clones; Oil polluted water sediment clone, AM882526)
V1F22b (2 clones; Wadden Sea sandy intertidal sediment clone, AM040128)
Methylococcaceae
V19F19b (5 clones; Bivalve Lucinoma aequizonata's gut clone, AY536230)
V19F132b (3 clones; Vicinity of an uranium mine clone, FM877662)
Legionella busanensis (AF424887)
Legionellaceae
V19F168b (1 clone; Kalahari Shield subsurface sediment clone, DQ337099)
V19F87b (2 clones; Soil around Fe-Mn nodule clone, EF492925)
Coxiellaceae
V1F161b (1 clone; Wadden Sea sediment clone, EU707310)
V1F8b (4 clones; Yellow Sea sediment clone, EU652570)
V1F53b (2 clones; Deep-sea octacoral clone, DQ396004)
Fundibacter jadensis (AJ001150)
Alcanivoraceae
V19F89b (2 clones; Lake Bonney water clone, DQ015830)
Marinobacter lutaoensis (AF288157)
V19F18b (1 clone; Marine electroactive biofilm clone, AM944524)
V19F3b (1 clone; Kennedy Space Centre clean room clone, DQ532284)
V19F17b (2 clones; Acinetobacter junii, EU841484)
V19F96b (1 clone; QingDao Coast water clone, DQ480134)
V19F78b (1 clone; Pseudomonas fragi, AM062695)
Pseudomonadaceae
V19F166b (1 clone; South China Sea Alteromonas sp., FJ461444)
V19F21b (9 clones; Loihi Seamount submarine basalt clone, DQ412077)
V1F147b (3 clones; Coco's Island water clone, EF574625)
Alteromonas alvinellae (AF288360)
Alteromonadaceae
Pseudoalteroomnas atlantica (AB049728)
V19F62b (6 clones; Arctic seawater Pseudoalteromonas sp., EU365603)
Pseudoalteromonadaceae
V19F79b (1 clone; Colwellia piezophila, AB094412)
V19F20b (1 clone; Western Pacific Warm Pool deep-sea sediment clone, AY375114)
V19F107b (1 clone; East Pacific Rise deep seawater clone, EU491941)
Colwelliaceae
Gamma-Proteobacteria
Gallionella ferruginea (DQ386262)
Leptothrix discophora (L33975)
Beta-Proteobacteria
V1F90b (1 clone; Loihi Seamount hydrothermal vent mat clone, EU491219)
Rhodosspirillaceae
V1F108b (1 clone; Tablas de Daimiel National Park wetland lower sediment clone, FJ516840)
V19F64b (2 clones; Arctic Ocean bacterioplankton clone, EU919796)
Kordiimonadaceae
V19F108b (1 clone; Earthworm Eisenia fetida's intestine clone, FJ543062)
V19F10b (3 clones; Pacific Nodule Province deep-sea sediment clone, AJ567586)
V1F67b (1 clone; Caribbean coral Montastraea faveolata clone, FJ202685)
Roseovarius aestuarii (EU156066)
V1F23b (2 clones; Southern Mariana Backarc Spreading Centre hydrothermal vent mat clone, EU574669)
V19F15b (1 clone; Deep-sea octacoral clone, DQ395914)
Rhodobacteraceae
Alpha-Proteobacteria
V1F42b (1 clone; Mid-Atlantic Ridge hydrothermal vent chimney clone, AJ969449)
Nautiliaceae
V1F94b (1 clone; Shallow submarine hot spring microbial mat clone, AB301716)
Sulfurovum lithotrophicum (AB091292)
V19F67b (3 clones; Mid-Atlantic Ridge hydrothermal fluid clone, AM268719)
V19F50b (1 clone; Pacific-Antarctic Ridge hydrothermal vent crab Kiwa hirsuta's shell clone, EU265787)
V19F29b (1 clone; Gulf of Mexico cold seep sediment clone, AM745129)
V19F133b (1 clone; Fe-rich substrate exposed to Mid-Atlantic Ridge vent clone, AY225622)
Helicobacteraceae
Epsilon-Proteobacteria
V1F77b (1 clone; Japan Trench cold seep sediment clone, AB189349)
V19F41b (1 clone; Wadden Sea surface sediment clone, AY771966)
Desulfobulbus mediterraneus (AF354663)
V1F119b (1 clone; Tubeworm Riftia pachyptila's tube clone, AF449228)
V19F128b (1 clone; Mexico cold seep sediment clone, AM745164)
Desulfobulbaceae
V19F65b (2 clones; Northern Bering Sea sediment clone, EU734913)
V19F113b (2 clones; Loihi Seamount seafloor lava clone, EU491282)
V19F5b (3 clones; Loihi Seamount seafloor lava clone, EU491282)
Desulfuromonaceae
Bdellovibrio bacteriovorus (AF084851)
V1F144b (1 clone; Caribbean coral Montastraea faveolata clone, FJ202161)
Bdellovibrionaceae
V19F69b (4 clones; Kings Bay sediment clone, EU050854)
V1F85b (4 clones; Kings Bay sediment clone, EU050852)
Pelobacter carbinolicus (U23141)
V1F59b (2 clones; Moderate saline soil clone, EU328027)
Desulfuromonaceae
V1F100b (1 clone; Coco's Island water clone, EF573852)
V19F152b (1 clone; Eel River Basin methane seep sediment clone, FJ264677)
V1F183b (1 clone; Coco's Island water clone, EF573677)
V19F97b (1 clone; Deep-sea octacoral clone, DQ395799)
V1F160b (1 clone; Arctic surface sediment clone, EU287141)
Chondromyces lanuginosus (FJ176774)
Sorangineae
V1F76b (3 clones; Sediment and soil slurry clone, EU542456)
V19F125b (1 clone; Northern Bering Sea sediment clone, EU734972)
V19F32b (1 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205262)
V19F159b (1 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205262)
Desulfuromonaceae
V1F127b (1 clone; Tablas de Daimiel National Park wetland upper sediment clone, FJ516882)
Desulfohalobiaceae
V1F19b (3 clones; South China Sea surface sediment clone, EU048666)
Geobacteraceae
V19F158b (1 clone; Rhizosphere of Vicia faba L. clone, EU979064)
V1F52b (1 clone; South China Sea surface sediment clone, EU048619)
Syntrophobacteraceae
V19F40b (1 clone; South China Sea subseafloor sediment clone, EU386067)
V19F48b (1 clone; East Lau Spreading Centre active hydrothermal field sediment clone, FJ205261)
Nitrospinaceae
Delta-Proteobacteria
0.1

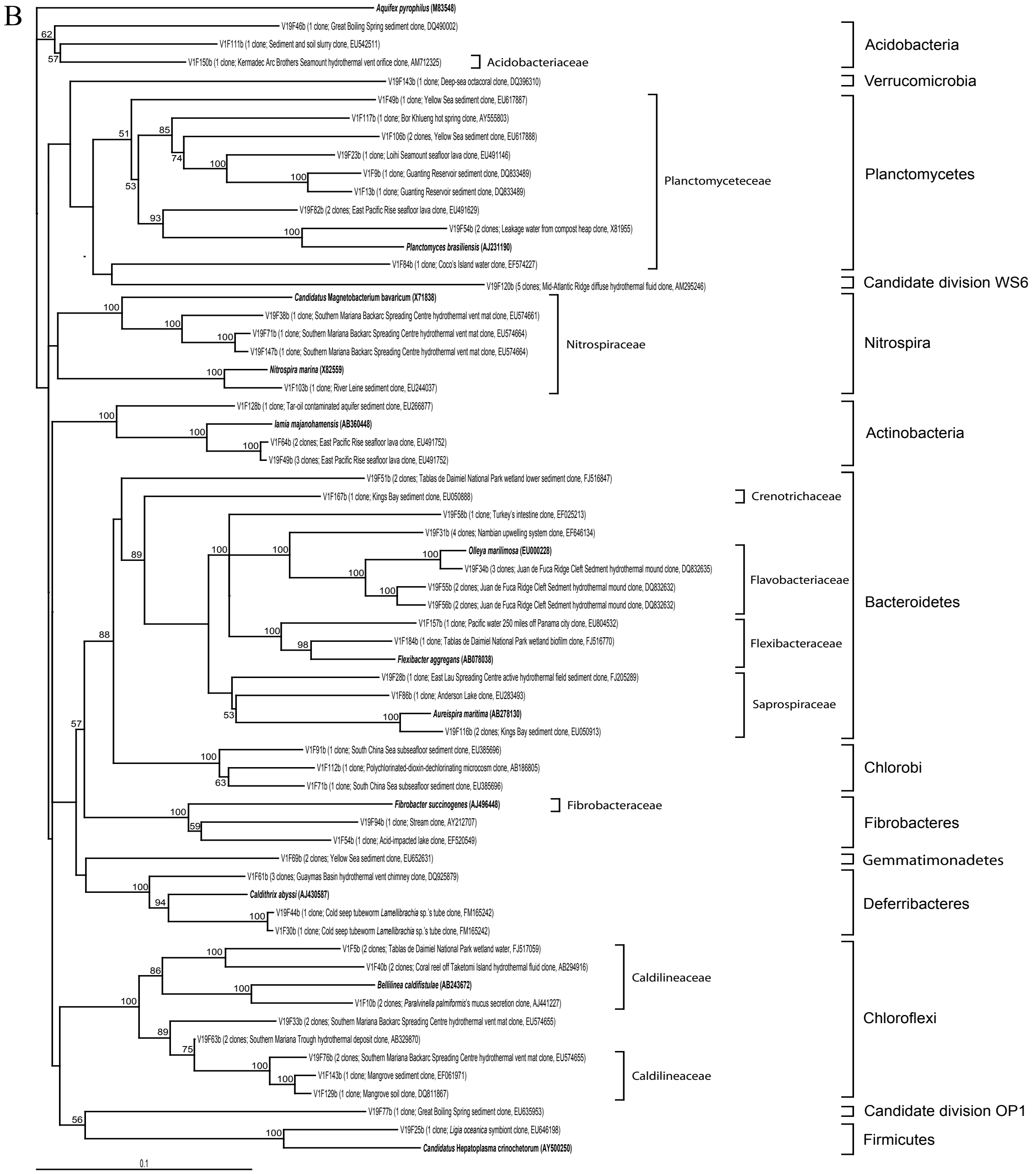


Figure 2.4

Neighbor-joining phylogenetic trees for both samples from Volcanoes 19 and 1. The number of clones representing an OTU, as well as the origin of the closest sequence and its accession number in the GenBank database are in parentheses. The clones were named as V19F for 'Volcano 19 Fe sample' and V1F for 'Volcano 1 Fe sample', followed by the bacterial clone number. Closest named organisms followed by their accession number are indicated in bold. Bootstrap values for nodes with > 50% support, based on 1000 replicates, are displayed as percentages. Scale bar represents the estimated sequence divergence. (A) Affiliation of OTUs belonging to the *Proteobacteria*. (B) Affiliation of the OTUs belonging to the non-*Proteobacteria*.

the *Zeta*- and *Gammaproteobacteria*, the *Actinobacteria* and the *Deferribacteres*, were shared between the two libraries. Comparison of our OTUs against the GenBank database showed that almost half of the clone libraries had ≥ 97%, and fewer than 1% had < 80% similarity to published sequences (Fig. 2.5A). However, only 8.3% of the clone libraries were ≥ 97% similar to cultured organisms, while 20.5% had less than 80% similarity to cultured organisms (Fig. 2.5B). In order to compare the similarity of the two samples, the NPMLE was determined. This index scales from 0.0, indicating samples entirely dissimilar, to 1.0 for samples that are identical. As opposed to traditional similarity indices that only consider the number of species and the proportions of shared species, the NPMLE weighs community overlap by taking into account the community structure, i.e. the abundance of OTU representatives from both the shared and non-shared OTUs (Yue *et al.*, 2005). For our clone libraries, the value of this index was estimated at 0.59. Non-shared OTUs accounted for 95.1% of the total OTUs and most of them had only one representative in either sample, which supports the results of the diversity analysis and suggests that more sequencing would discover a greater number of shared species.

Proteobacteria

A total of 253 clones out of 334, representing 71.5% of the clone library from Volcano 19 and 79.5% of the clone library from Volcano 1, were grouped within five subdivisions of the *Proteobacteria* (Figs 2.4A and 2.6). The largest number of clones belonged to the *Gammaproteobacteria* which account for 32.9% of the Volcano 19 library (52 clones) and 21.6% of the Volcano 1 library (38 clones) (Fig. 2.6). In terms of diversity, the *Gammaproteobacteria* predominated in both samples with 23 and 20 OTUs, respectively, distributed in at least 15 different families (Fig. 2.4A). Most of the OTUs were related to

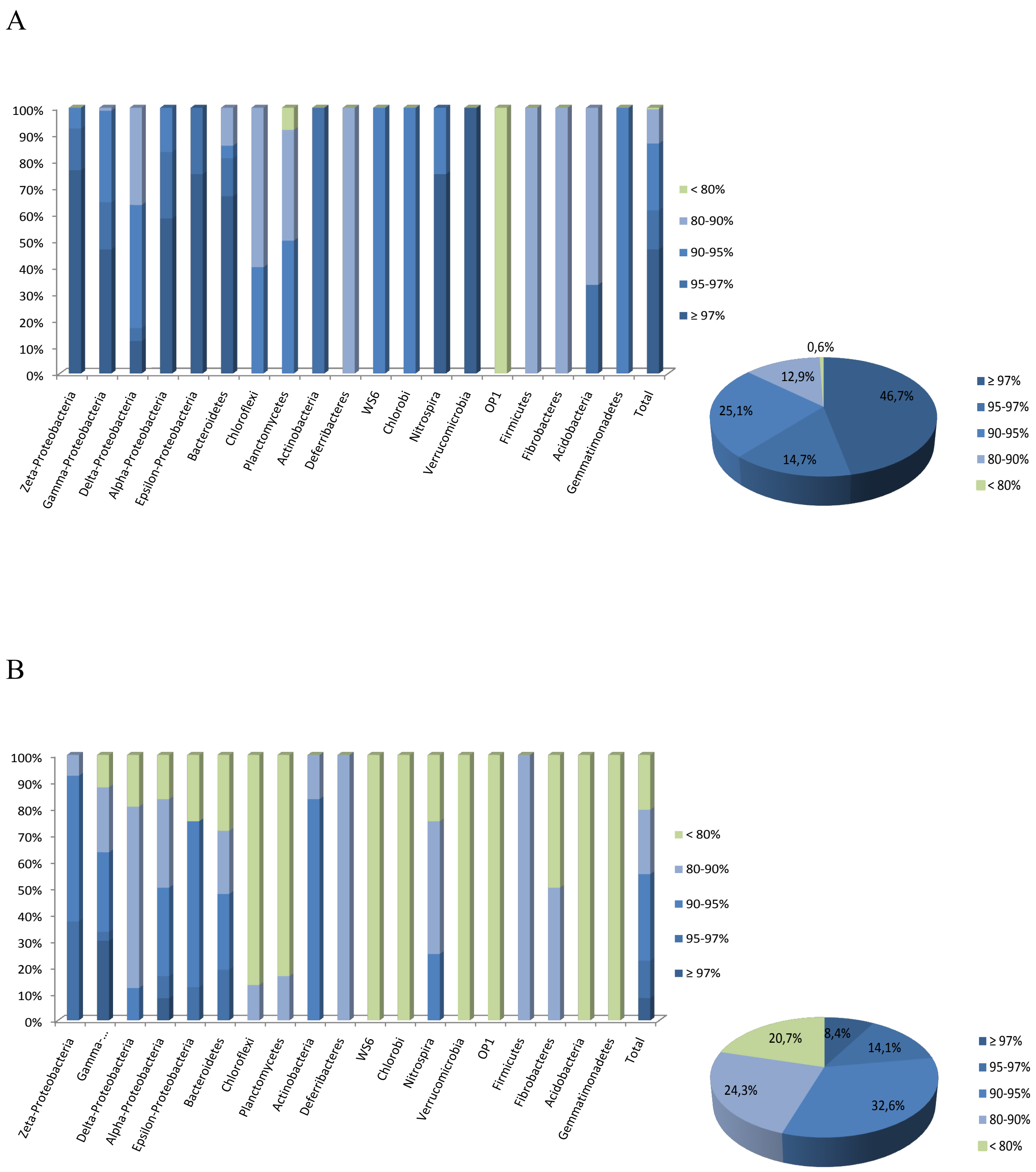


Figure 2.5

Proportion of percent similarity with the closest sequences in the GenBank database based on BLAST search for each taxonomic group detected in the clone libraries. (A) For the closest sequences regardless of its origin. (B) For the closest cultured organism.

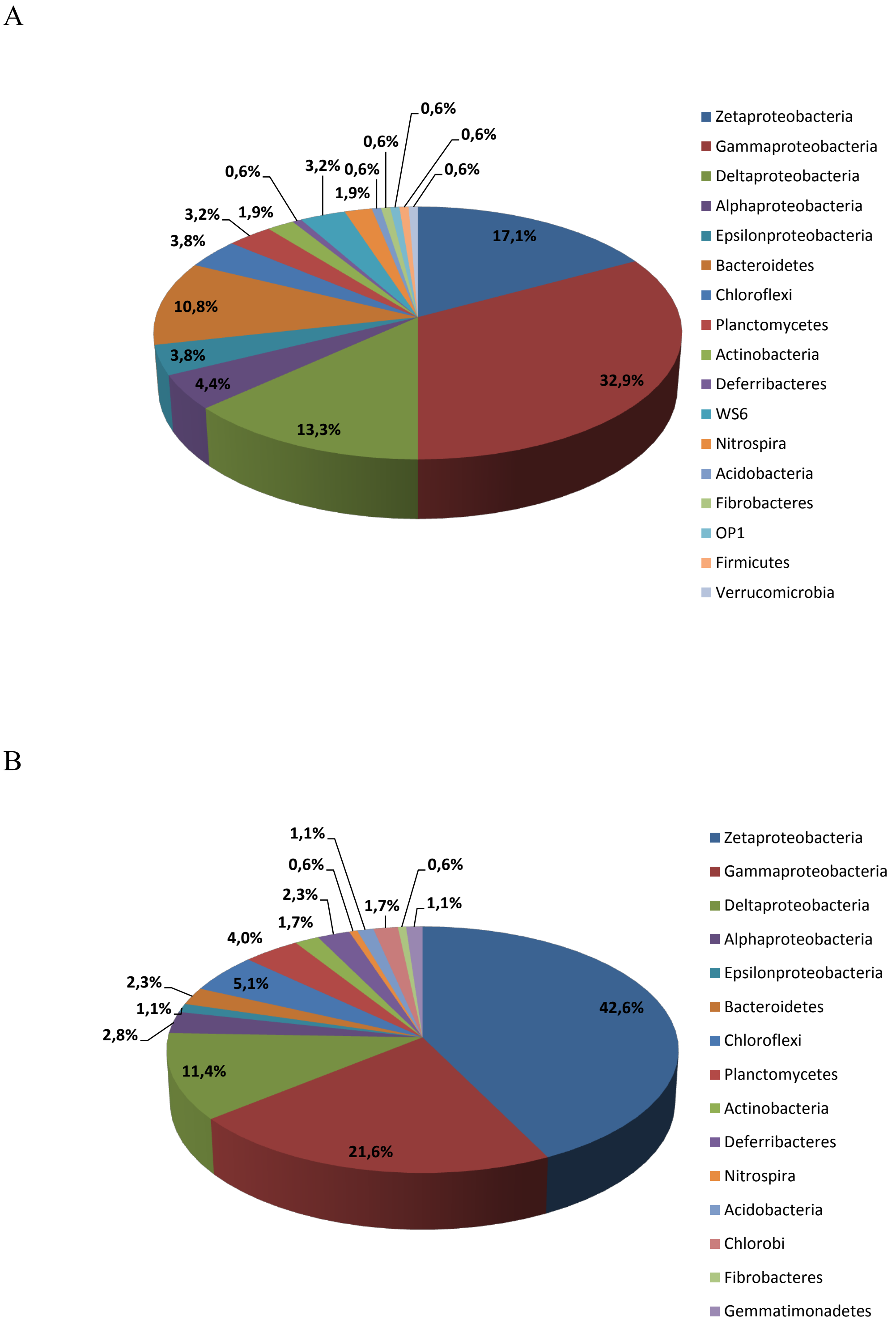


Figure 2.6

Distribution of the SSU rRNA gene sequences into taxonomic groups, and relative abundance of these groups. (A) In sample from Volcano 19. (B) In sample from Volcano 1.

chemoheterotrophic bacteria from different marine environments, with a few CH_4 and S oxidizers. Members belonging to the family *Alteromonadaceae* were dominant, and they all had over 99.5% similarity with *Alteromonas alvinellae*, a chemoheterotrophic organism isolated from the deep-sea hydrothermal vent polychaete *Alvinella pompejana* (GenBank accession no. AF288360). The second most abundant families were the *Methylococcaceae*, in which the OTUs were 92.6-96.0% similar to obligate CH_4-oxidizing *Methylobacter marinus* (Bowman *et al.*, 1993), and the *Legionellaceae*, in which OTUs were 94.1-96.2% similar to the chemoheterotrophic *Legionella buusanensis*, isolated from a cooling tower water (Park *et al.*, 2003). Other families, such as the *Pseudoalteromonadaceae*, in which OTUs were 99.9% similar to the marine chemoheterotrophic bacterium *Pseudoalteromonas atlantica* that was isolated from Japanese coastal waters (Uchida *et al.*, 1993) and the *Chromatiaceae*, composed of phototrophic purple sulphur bacteria isolated from marine and freshwater (Imhoff *et al.*, 1998), were also relatively abundant in the clone libraries (Fig. 2.4A). A few members of the *Gammaproteobacteria* belonged to the obligate marine psychrophilic *Colwelliaceae* (Ivanova *et al.*, 2004), the marine S-oxidizing *Ectothiorhodospiraceae* (Imhoff, 1984), the invertebrate and vertebrate endosymbionts *Coxiellaceae* (Garrity *et al.*, 2005b), the chemoheterotrophic psychrophilic or mesophilic *Moraxellaceae* (Rossau *et al.*, 1991), the chemoheterotrophic and widely distributed *Pseudomonadaceae* (Garrity *et al.*, 2005a) and the mesophilic and moderately halophilic *Alcanivoraceae* (Golyshin *et al.*, 2005). The phylogenetic affiliation of the remaining OTUs was uncertain; many of these OTUs belonged to the order *Chromatiales*, containing purple sulphur and non-phototrophic bacteria (Imhoff, 2005), and most of them were closely related to uncultured organisms from deep-sea hydrothermal vent environments or seafloor lavas (Santelli *et al.*, 2008; GenBank accession no. FJ205280). Two clones were 96.7% similar to the thermotolerant, halophilic and chemoheterotrophic marine bacterium *Marinobacter lutaoensis* (Shieh *et al.*, 2003).

The *Zetaproteobacteria* dominated the clone library from Volcano 1 (42.6%) and was the second most abundant group of the clone library from Volcano 19 (17.1%) (Fig. 2.6A and B). Six OTUs, representing 16.8% of all clones sequenced, had 97.3-98.0% similarity to *M. ferrooxydans*, the only cultured organism belonging to this group so far (Fig. 2.4A). The bacterium was described by Emerson et al. (2007) as an obligate chemolithoautotroph requiring micro-aerobic conditions, marine salts and Fe^{2+} as the energy source. The other nine OTUs, representing 30.5% of the clones sequenced, were 91.1–95.7% related to *M. ferrooxydans*. All the clones were related to uncultured organisms isolated from hydrothermal vent environments, such as active hydrothermal sediments from the East Lau Spreading Centre (GenBank accession nos FJ205309 and FJ205310) and microbial mats from the Southern Mariana Back-Arc Spreading Centre (Davis *et al.*, 2008).

In both libraries, the *Deltaproteobacteria* was the third most abundant group, representing 13.3% of the Volcano 19 library and 11.4% of the Volcano 1 library (Fig. 2.6), and the second most diversified, with 14 and 12 OTUs respectively (Fig. 2.4A). As observed in Fig. 2.4A, most of the clones in this group (56.1%) belonged to the family *Desulfuromonaceae*, a group comprising mesophilic strictly anaerobic chemolitho- or chemoorganotrophs isolated from various aquatic environments, in which some members can utilize S or Fe^{3+} as an electron acceptor (Kuever *et al.*, 2005d). Other clones clustered in families that include organisms associated with S or Fe cycling, such as the *Desulfobulbaceae*, to which belong strictly anaerobic chemoorganotrophs, chemolithoheterotrophs or aerobic chemolithoautotrophs using mostly sulphate as the electron acceptor (Kuever *et al.*, 2005c), the mesophilic or moderately thermophilic SO_4-reducing *Desulfohalobiaceae* (Kuever *et al.*, 2005b) and the Fe-reducing *Geobacteraceae*, inhabiting aquatic sedimentary environments (Kashefi *et al.*, 2003). The mesophilic *Syntrophobacteraceae*, in which many genera use sulphate as an electron acceptor (Kuever *et al.*, 2005a), were also represented in the clone libraries. A few clones belonged to the family *Nitrospinaceae*, characterized by aerobic obligate lithototrophs that require NO_2^- as the energy source (Spieck *et al.*, 2005). The families *Bdellovibrionaceae* and *Sorangineae* were also detected.

The *Alpha*- and *Epsilonproteobacteria* were not very abundant in the clone libraries compared with the other subclasses of *Proteobacteria* (Fig. 2.6). The first group represented 4.4% of the Volcano 19 library and 2.8% of the Volcano 1 library, while the second group represented 3.8% and 1.1%, respectively. These subclasses also had the lowest diversity of the *Proteobacteria* (Fig. 2.4A). In the *Alphaproteobacteria*, the metabolically diverse *Rhodobacteraceae* family was dominant, and the clones were 94.9-97.0% similar to *Roseovarius aestuarii*, a marine mesophilic bacterium isolated from a tidal flat of the Yellow Sea (Yoon *et al.*, 2008). Two clones clustered in the *Kordiimonadaceae* and were closely related to an uncultured organism isolated from the bacterioplankton of the Arctic Ocean (GenBank accession no. EU919796), and one clone clustered in the *Rhodospirillaceae* and was closely related to a bacterium isolated from a hydrothermal vent mat at Loihi Seamount (GenBank accession no. FJ516840). The affiliation of one OTU, which was closely related to an uncultured bacterium recovered from the lower sediments of a semi-arid wetland (GenBank accession no. FJ516840), was too uncertain to be assigned. Within the *Epsilonproteobacteria*, members of the *Helicobacteraceae* family were the most abundant and they were 96.1-97.2% related to *Sulfurovum lithotrophicum*, described as a marine mesophilic facultative anaerobic bacteria growing chemolithoautotrophically with elemental S or $S^2O_3^{2-}$ as an electron donor and with O_2 and NO_3^- as an electron acceptor (Inagaki *et al.*, 2004). The *Nautiliaceae*, a family characterized by marine thermophilic obligately anaerobic or micro-aerobic chemolithoautotrophs and occasionally mixotrophs, using S or NO_3^- as electron acceptors (Miroshnichenko *et al.*, 2004), was represented by one clone. No *Betaproteobacteria* were detected.

Bacteroidetes

Clones belonging to the *Bacteroidetes* represented 10.8% of the Volcano 19 library (17 clones), while only 2.3% of the clones from the Volcano 1 library clustered within this group (four clones) (Figs 2.4B and 2.6). This group was the most diverse of the non-*Proteobacteria* phyla (Fig. 2.4B). The 12 OTUs clustered in four different families, with the *Flavobacteriaceae* being the most abundant. The strictly aerobic marine chemoheterotroph *Olleya marilimosa* (Nichols *et al.*, 2005) was the closest cultured organism (92.3–97.8% similar) belonging to this family. Members of the *Saprospiraceae* had 84.1–96.6% similarity with the mesophilic *Aureispira maritima*, isolated from marine barnacle debris in Thailand (Hosoya *et al.*, 2007b). The marine and strictly aerobic chemoorganotroph *Flexibacter aggregans* (Hosoya *et al.*, 2007a) was 88.5–92.4% similar to the clones within the family *Flexibacteraceae*. Finally, the marine

Crenotrichaceae, known for its extreme halophilic and thermophilic members (Bjornsdottir *et al.*, 2006), had one representative in the Volcano 19 clone library. The family affiliation of three OTUs within the *Bacteroidetes* was uncertain; however, they were closely related to uncultured marine organisms from the deeper sediments of a semi-arid wetland (GenBank accession no. FJ516847) and the Namibian upwelling system (Woebken *et al.*, 2007).

Chloroflexi

The *Chloroflexi* represented 3.8% of the Volcano 19 library and 5.1% of the Volcano 1 library, with three and five OTUs respectively (Figs 2.4B and 2.6). Two OTUs could not be assigned to a family but were closely related to uncultured organism recovered from hydrothermal vent systems in the Southern Mariana Back-Arc Spreading Centre (Davis *et al.*, 2008; GenBank accession no. AB329870). The other members belonged to the thermophilic facultative anaerobic *Caldilineaceae* (Sekiguchi *et al.*, 2003; Yamada *et al.*, 2006). All the clones within the *Chloroflexi* had 80.7–89.6% relatedness to *Bellilinea caldifistulae*, a thermophilic and strictly anaerobic organism isolated from a heated reactor vessel for waste water/waste treatment (Yamada *et al.*, 2007).

Planctomycetes

Approximately 3.2% of the Volcano 19 library and 4.0% of the Volcano 1 library grouped in the *Planctomycetes*. These OTUs all belonged to the same family, i.e. the budding *Planctomyceteceae*, known to be mainly composed of chemoorganotrophic and obligate aerobic members (Schlesner *et al.*, 1986). The closest cultured organism was *Planctomyces brasiliensis*, a halotolerant bacterium isolated from a water sample of a salt pit (Schlesner *et al.*, 1986). However, only one OTU belonging to the *Planctomycetes* had similarity with this organism (between 83.6% and 88.5%); the other eight OTUs had under 80% similarity with any cultured organism.

Others

The other phyla detected in the samples occupied 5.1% of the Volcano 19 library and 4.8% of the Volcano 1 library (Fig. 2.6). Within the Volcano 19 library, representatives of the candidate division WS6 (five clones and one OTU), the *Actinobacteria* (three clones and one OTU) and the *Nitrospira* (three clones and three OTUs) were the most abundant. The candidate division OP1, the *Acidobacteria*, the *Firmicutes*, the *Fibrobacteres*, the *Deferribacteres* and the *Verrucomicrobia* had only one representative each in the library (Fig. 2.4B). In the library from Volcano 1, the rest of the clones belonged to the *Deferribacteres* (four clones and two OTUs), the *Actinobacteria* (three clones and two OTUs), the *Chlorobi* (three clones and three OTUs), the *Acidobacteria* (two clones and two OTUs), the *Gemmatimonadetes* (two clones and one OTU), the *Fibrobacteres* (one clone) and the *Nitrospira* (one clone) (Fig. 2.4B). The closest sequences related to these clones mostly originated from diffuse hydrothermal fluids from the Mid-Atlantic Ridge (Perner *et al.*, 2007), seafloor lava from the East Pacific Rise (Santelli *et al.*, 2008), hydrothermal vent mats from the Southern Mariana Back-Arc Spreading Centre (Davis *et al.*, 2008) and other sediment samples.

Discussion

Bacterial diversity in Fe oxides

Examination of the clone libraries reveals that a phylogenetically highly diverse bacterial community inhabits Fe-rich sediments on these hydrothermally active submarine volcanoes. The majority of the OTUs detected (58.7%) were represented by a single clone. Together with the results of the rarefaction curves and the coverage analysis, this reinforces the conclusion that the actual diversity was only partially surveyed and that more species (or even phyla) would be expected with more sequencing. Within the 334 sequenced clones, we detected 150 OTUs belonging to 19 taxonomic bacterial groups that were widely distributed in the domain *Bacteria*. Although other recent SSU rRNA gene sequence studies from marine environments reported similar diversity, most of the species richness recovered from marine sediments falls within 15 bacterial lineages (e.g., Li *et al.*, 1999; Ravenschlag *et al.*, 1999; López-García *et al.*, 2003; Zeng *et al.*, 2005; Liang *et al.*, 2007; Uthicke *et al.*, 2007; Davis *et al.*, 2008; Dang *et al.*, 2009; Polymenakou *et al.*, 2009; Tian *et al.*, 2009). Teske et al. (2002) recovered at least 16 bacterial groups in hydrothermally active sediments of the Guaymas Basin (Gulf of California), Huber et al. (2003) found 17 bacterial lineages in a diffuse flow hydrothermal vent habitat at Axial Volcano (Juan de Fuca Ridge) and Santelli et al. (2009) recovered 16 bacterial groups in seafloor lavas from the East Pacific Rise. The percentage of similarity between clone libraries and sequences previously published in the GenBank database can be used as an indicator of the uniqueness of our samples. More than half of our clone libraries had less than 97% similarity to previously published sequences, which suggests that these clones could represent novel species that have not been previously detected. Moreover, a large proportion of our clones (36.8%) had less than 95% similarity with other sequences, and 13.5% had less than 90% similarity, suggesting that new genera, and probably new families, were recovered and might be specific to this environment.

Phylogenetic composition of the clone libraries and comparison with other SSU rRNA gene libraries

The morphological and mineralogical description of the samples given in Langley et al. (2008), i.e. filamentous structures encrusted in poorly ordered oxyhydroxides resembling biogenic Fe oxides produced by *Gallionella* and *Leptothrix*, suggested the presence of Fe oxidizers in microbial mats associated with Fe oxide deposits on the seafloor of Volcanoes 1 and 19. The phylogenetic analysis of our SSU rRNA gene clone libraries confirms this hypothesis; the *Zetaproteobacteria* dominated the Volcano 1 clone library and were the second most abundant group of the Volcano 19 clone library. This contrasts with the observations of Hodges and Olson (2009) who found the *Zetaproteobacteria* to be poorly represented in clone libraries from Fe oxide samples from hydrothermally active volcanoes on the Kermadec Arc. In addition to submarine volcano settings, the *Zetaproteobacteria* were also previously detected in Fe-dominated microbial mats collected from a hydrothermal chimney at the Fryer Site on the Southern Mariana Back-Arc Spreading Centre (Davis *et al.*, 2008), and in hydrothermally active sediments at the East Lau Spreading Centre (GenBank accession nos FJ205309 and FJ205310). The only pure culture isolate of this newly described subclass of *Proteobacteria* is *M. ferrooxydans*, an obligately neutrophilic and micro-aerophilic Fe-oxidizing chemoautotroph isolated from Fe-rich microbial mats forming at Loihi Seamount, a hydrothermally active submarine volcano (Emerson *et al.*, 2007). In addition to the *Zetaproteobacteria*, a few of our OTUs clustered within the *Nitrospira* and *Chlorobi*, two other phyla that comprise members capable of oxidizing Fe^{2+} (Rawlings *et al.*, 1999; Bryant *et al.*, 2006). These results suggest that the bacterial communities associated with Fe oxide crusts at Volcano 1 and chimney-like structures at Volcano 19 are dominated by chemolithoautotrophic Fe oxidizers. These bacteria could represent an important source of primary production for heterotrophic bacteria inhabiting this environment. A few putative Fe-reducing bacteria were also detected, primarily from Volcano 1, suggesting the presence of complete Fe cycling. These organisms belonged to the *Deferribacteres*, also reported by (Hirayama *et al.*, 2007) from shallow hydrothermal sediments, and the *Deltaproteobacteria* family *Geobacteraceae*.

The presence of bacterial species from the *Betaproteobacteria* genera *Gallionella* or *Leptothrix* in marine Fe oxides at circumneutral pH is often inferred from morphological descriptions of mineral textures (e.g., Bogdanov *et al.*, 1997; Halbach *et al.*, 2001; Thorseth *et al.*, 2001; Kennedy *et al.*, 2003b, a; Hanert, 2006). To our knowledge, this morphological evidence remains to be complemented by culture studies or molecular analyses, confirming the presence of these genera in Fe oxide sediments. In fact, Emerson and Moyer (2002) have described two bacterial strains, PV-1 and JV-1, which are morphologically similar but not phylogenetically related to *Gallionella* spp., and as mentioned previously, PV-1 was eventually described as *M. ferrooxydans*. This species also produces Fe oxide structures analogous to the Fe-rich stalks produced by *G. ferruginea* (Emerson *et al.*, 2007). In order to confirm their absence in our samples, representative sequences from the genera *Gallionella* and *Leptothrix* were added to the *Proteobacteria* phylogenetic tree. The results showed that none of the clones was related to either genus, and no *Betaproteobacteria* were detected. Although they described similar Fe oxide filaments and sheaths, Hodges & Olson (2009) arrived at the same conclusion.

Representatives of the metabolically diverse *Gammaproteobacteria* were very abundant in our clone libraries and accounted for the largest portion of the sample from Volcano 19. This subclass of *Proteobacteria* has also been found to dominate other marine habitats, such as hydrothermally active sediments and fluids (e.g., Huber *et al.*, 2003; López-García *et al.*, 2003; Brazelton *et al.*, 2006; Hirayama *et al.*, 2007), ocean floor basalts (e.g., Lysnes *et al.*, 2004; Santelli *et al.*, 2008; Santelli *et al.*, 2009), permanently cold sediments (e.g., Li *et al.*, 1999; Zeng *et al.*, 2005; Tian *et al.*, 2009), carbonate sediments of coral reefs (e.g., Uthicke *et al.*, 2007) and mangrove forest sediments (e.g., Liang *et al.*, 2007). The phylogenetic affiliation of many *Gammaproteobacteria* clones from both volcanoes indicated the presence of S oxidizers. These organisms require a source of reducing sulphur species for metabolic energy, which is often rare or absent at low temperature vents. However, while no signs of emerging flow were visible at either site in 2007, Stoffers et al. (2006) described active venting and steam and gas discharge in 2005. Thus, reducing sulphur species could have recently been available for S oxidizers. In addition, the presence of S-reducing bacteria at both sites suggests that even though reducing sulphur species were not available from hydrothermal sources in 2007, they might still be biologically provided, allowing for some growth of S oxidizers, and completing the sulphur cycle. Most of the S-reducers were retrieved from the *Deltaproteobacteria*, a very frequent group in marine sediments (e.g., Ravenschlag *et al.*, 1999; Teske *et al.*, 2002; Zeng *et al.*, 2005; Hirayama *et al.*, 2007; Liang *et al.*, 2007; Uthicke *et al.*, 2007), and the third most abundant and diverse group in the clone libraries.

Some other members of the *Gammaproteobacteria* clustered within the strictly aerobic CH_4-oxidizing *Methylococcaceae* (Bowman *et al.*, 1993) but only from Volcano 1. Methanotrophs have also been recovered from young seafloor basalts (Santelli *et al.*, 2009), microbial mats associated with a shallow submarine hydrothermal system (Hirayama *et al.*, 2007), diffuse hydrothermal vent fluids (Huber *et al.*, 2003), hydrothermal sediments (Teske *et al.*, 2002) and fluids and carbonate material associated with a hydrothermal system (Brazelton *et al.*, 2006). The possible sources of methane in marine sediments are hydrothermal fluids charged with CH_4 from the ocean crust and methanogenesis by strictly anaerobic *Euryarchaeota* (Liu *et al.*, 2008). The other *Gammaproteobacteria* of the clone libraries were mostly ubiquitous heterotrophs.

The *Epsilonproteobacteria* detected in our samples, mostly affiliated with the *Helicobacteriaceae* and *S. lithotrophicum*, indicating an S^- or $S_2O_3^{2-}$-oxidizing type of metabolism (Inagaki *et al.*, 2004), accounted for only a small proportion of our clones. By contrast, this group has been found to be dominant or abundant in other studies from hydrothermal sediments and fluids (e.g., Moyer *et al.*, 1995; Teske *et al.*, 2002; Huber *et al.*, 2003; Brazelton *et al.*, 2006; Hirayama *et al.*, 2007; Hodges *et al.*, 2009). The intensity of the hydrothermal flow, and thus the availability of greater concentrations of reducing sulphur, could be responsible for this difference, as these other studies all sampled sites characterized by active venting. Therefore, like the other S-oxidizing bacteria detected in our libraries, the *Epsilonproteobacteria* from Volcanoes 1 and 19 may have been dependent on reducing sulphur species provided by S-reducing organisms. The only potential S-reducing *Epsilonproteobacteria* in our clone libraries was affiliated to the *Nautiliaceae*.

The *Bacteroidetes*, the *Chloroflexi* and the *Planctomycetes* were the most abundant non-*Proteobacteria* groups of the libraries, and their phylogenetical affiliation suggested that most of them are heterotrophs. These phyla are ubiquitous in marine environments and have been detected in hydrothermal sediments and fluids (e.g., Teske *et al.*, 2002; Huber *et al.*, 2003; López-García *et al.*, 2003; Brazelton *et al.*, 2006; Hirayama *et al.*, 2007; Davis *et al.*, 2008) basaltic seafloor lavas (e.g., Thorseth *et al.*, 2001; Lysnes *et al.*, 2004; Santelli *et al.*, 2008; Santelli *et al.*, 2009), cold marine sediments (e.g., Li *et al.*, 1999; Ravenschlag *et al.*, 1999; Zeng *et al.*, 2005; Dang *et al.*, 2009; Polymenakou *et al.*, 2009; Tian *et al.*, 2009), carbonate sediments of coral reefs (e.g., Uthicke *et al.*, 2007) and mangrove forest sediments (e.g., Liang *et al.*, 2007). Clones affiliated with the *Actinobacteria*, *Fibrobacteres* and *Firmicutes*, suggesting chemo-organotrophic metabolisms, were rarer and they have been observed in the same wide variety of marine environments.

The ecological role of the other bacterial groups detected in our sample is more ambiguous; the members of the phyla *Acidobacteria*, *Verrucomicrobia* and *Gemmatimonadetes* all had lower than 80% similarity with any other cultured organisms, which can be explained by a paucity of characterized species, and the candidate divisions OP1 and WS6 have, to our knowledge, no cultivated representatives so far. These groups were not very abundant in other clone libraries, but they have been observed in marine environments (e.g., Teske *et al.*, 2002; Huber *et al.*, 2003; Polymenakou *et al.*, 2009; Santelli *et al.*, 2009) and most of the closest sequences were isolated from hydrothermal environments (e.g., Perner *et al.*, 2007; Stott *et al.*, 2008, GenBank accession nos DQ490002 and EU635953).

Comparison of Volcanoes 1 and 19

The bacterial taxonomic groups and the OTUs retrieved from Volcanoes 1 and 19 were not identical, but we cannot discuss how different they are based on the clone libraries without considering that the total diversity remains unknown and that more OTUs, and possibly more bacterial groups, would be expected with more extensive sequencing. This shortcoming is addressed in the NPMLE. This index takes into account the non-shared OTUs (Yue *et al.*, 2005); therefore, a low frequency of non-shared OTUs increases the value of the similarity estimation, as it indicates that more shared OTUs would possibly be recovered with additional sequencing.

Most of our OTUs had less than 97% similarity with any cultured organisms, which is the most commonly accepted boundary for defining a species. Sometimes, phylogenetic affiliation with a family can help characterize organisms, but only 47.0% of our samples could reliably be assigned to a family, and some of these families are very diverse metabolically. However, based on family associations, we estimated that at least 48.3% of the clone library from Volcano 1 was composed of autotrophs, while their presence was approximately 31.0% in the clone library from Volcano 19. These more abundant autotrophs were mostly Fe-oxidizing bacteria, which is consistent with the higher Fe concentration measured in sediments at Volcano 1 (390 mg g^{-1}) compared with Volcano 19 (307 mg g^{-1}) (S. Langley, personal communication). Langley et al. (2008) attributed the presence of crystalline Fe oxides, in the Volcano 1 sediments, to a greater intensity of Fe recycling (compared with Volcano 19 samples), which, by extension, suggests a more important presence of Fe-reducing heterotrophic bacteria at Volcano 1. Higher concentrations of photosynthetic pigments and organic matter observed at Volcano 1

(Appendix A, Table A.1) at this shallower site (197 m versus 992 m at Volcano 19) could support a more intensive heterotrophic activity by Fe-reducing organisms. Alternatively, fluctuations of hydrothermal flow through subsurface (or even surface) sediments could create alternating cycles of oxidizing and reducing conditions.

Conclusion

Samples from both Volcanoes 1 and 19 had highly diverse bacterial communities, suggesting that Fe-rich hydrothermal sediments can be exploited by a variety of microorganisms. In contrast to the findings of Hodges and Olson (2009), the clones sequenced in this study were dominated by Fe oxidizers *Zetaproteobacteria*, while only a few representatives of the *Epsilonproteobacteria* were detected. The low similarity of several clones to other published sequences suggested that new species and perhaps new families were recovered from these habitats. All the phyla detected in this study have been found in other marine environments, but this study provides one of the first molecular documentations of a diverse bacterial community dominated by Fe oxidizers.

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Chapter 3 Free-living bacterial communities associated with tubeworm (*Ridgeia piscesae*) aggregations in contrasting diffuse flow hydrothermal vent habitats at the Main Endeavour Field, Juan de Fuca Ridge

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Abstract

We systematically studied free-living bacterial diversity within aggregations of the vestimentiferan tubeworm *Ridgeia piscesae* sampled from two contrasting flow regimes (High Flow and Low Flow) in the Endeavour Hydrothermal Vents Marine Protected Area (MPA) on the Juan de Fuca Ridge (Northeast Pacific). Eight samples of particulate detritus were recovered from paired tubeworm grabs from four vent sites. Most sequences (454 pyrosequencing and Sanger methods) were affiliated with the *Epsilonproteobacteria*, and the sulphur-oxidizing genus *Sulfurovum* was dominant in all samples. *Gammaproteobacteria* were also detected, mainly in Low Flow sequence libraries, and were affiliated with known methanotrophs and decomposers. The co-occurrence of sulphur reducers from the *Deltaproteobacteria* and the

Epsilonproteobacteria suggests internal sulphur cycling within these habitats. Other phyla detected included *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, *Planctomycetes*, *Verrucomicrobia*, and *Deinococcus–Thermus*. Statistically significant relationships between sequence library composition and habitat type suggest a predictable pattern for High Flow and Low Flow environments. Most sequences significantly more represented in High Flow libraries were related to sulphur and hydrogen oxidizers, while mainly heterotrophic groups were more represented in Low Flow libraries. Differences in temperature, available energy for metabolism, and stability between High Flow and Low Flow habitats potentially explain their distinct bacterial communities.

Introduction

Shortly after the discovery of hydrothermal vents and their dense, exotic fauna in 1977 (Lonsdale, 1977), the role of chemoautotrophic bacteria and archaea in the production of new organic matter was recognized as the basis of food webs in these ecosystems (Corliss et al., 1979; Jannasch and Wirsen, 1979; Karl et al., 1980). The first surveys of free-living vent microbial communities, based on morphological observations, revealed the presence of different types of sulphur-oxidizing organisms as well as possible methylotrophic and nitrifying bacteria (Jannasch and Wirsen, 1981). Subsequent culture-based studies of hydrothermal vent microorganisms focused mainly on specific groups, such as sulphur bacteria (Ruby et al., 1981; Felbeck and Somero, 1982; Wirsen et al., 1986; Ruby et al., 1987) and thermophilic organisms (Baross and Deming, 1983; Jones et al., 1983; Belkin et al., 1986; Jannasch et al., 1988). Eventually, the development of molecular techniques overcame the limitations of culture enrichments, and molecular tools are now permitting a more thorough portrayal of free-living microbial diversity at hydrothermal vents in a broad range of geological settings.

Molecular surveys of prokaryotic diversity at vents are still relatively few, but some general patterns are emerging. For example, many studies have found the class *Epsilonproteobacteria* to dominate free-living microbial communities associated with sulphide chimneys (Pagé et al., 2004; Kormas et al., 2006; Zhou et al., 2009), microbial mats (Moyer et al., 1995; Longnecker and Reysenbach, 2001; Moussard et al., 2006), hydrothermal fluids (Huber et al., 2003; Huber et al., 2010), and vent fauna (Polz and Cavanaugh, 1995; Alain et al., 2002; López-García et al., 2002; Alain et al., 2004; Pagé et al., 2004; Petersen et al., 2010). Most studies are also finding that phylogenetic diversity at vents tends to be higher than in other marine environments (Forget et al., 2010) and it is beginning to appear that this diversity can be quite dynamic, varying considerably between vents within the same field (Nakagawa et al., 2005c; Davis and Moyer, 2008; Huber et al., 2010), at individual vents under different environmental conditions (Reysenbach et al., 2000; Byrne et al., 2009), and between different zones of sulphide edifices (Schrenk et al., 2003; Kormas et al., 2006; Pagé et al., 2008). Faunal aggregations at vents provide physical habitat for free-living microorganisms and a few studies have attempted to characterize the free-living microbial communities associated with different faunal communities (Prieur et al., 1990; Polz and Cavanaugh, 1995; Campbell et al., 2001; Zbinden et al., 2008). Compositional changes in macrofaunal communities have been investigated within a framework of identifying successional processes driven by habitat change (Fustec et al., 1987; Hessler et al., 1988; Tunnicliffe and Juniper, 1990; Sarrazin et al., 1997; Tunnicliffe et al., 1997; Sarrazin and Juniper, 1999; Sarrazin et al., 1999; 2002; Cuvelier et al., 2009; 2011), but little is known of how the composition and diversity of associated free-living microbial communities might be related to habitat dynamics and faunal succession. One example is the comparison by Pagé et al. (2004) of the microbial diversity associated with the 'sulphide worm' *Paralvinella sulfincola* with a similar study from Alain et al. (2002) involving the 'palm worm' *P. palmiformis* at Axial volcano, Juan du Fuca Ridge. These two species of alvinellid polychaetes represent the first and second colonizers of newly formed chimney surfaces in Northeast Pacific vents, respectively (Sarrazin et al., 1997). The major taxonomic bacterial groups, dominated by *Epsilonproteobacteria*, were comparable between the two studies, but thermophilic bacterial groups and *Archaea* were also detected in association with the first colonizer *P. sulfincola* (Pagé et al., 2004). However, the lack of replication limited the interpretation of these results.

We present here a systematic study of microbial diversity in two contrasting, previously described habitats of the vestimentiferan tubeworm *Ridgeia piscesae* located in the Endeavour Hydrothermal Vents Marine Protected Area (MPA), Juan de Fuca Ridge. *R. piscesae* represents the major foundation species of the MPA, providing food, colonization surfaces and shelter to smaller species such as gastropods and other polychaetes. The first habitat corresponds to the Assemblage V-High Flow faunal community described in Sarrazin and Juniper (1999) and Sarrazin et al. (1999; 2002), which is dominated by the short-fat morphotype of *R. piscesae* exposed to vigorous fluid flow, hydrogen sulphide concentrations around 40 μM and temperatures up to 41.9°C. High faunal density and biomass, but low species diversity characterize this "High Flow" habitat. Other macrofaunal species present include the alvinellid polychaetes *Paralvinella* *sulfincola* and *P. palmiformis*, as well as a few polynoid polychaete species. The second habitat, which will be referred to as “Low Flow”, is dominated by widespread aggregations of the long-skinny morphotype of *R. piscesae* in areas of weaker hydrothermal discharge. At the tubeworm plume level, the temperature is usually just above that of ambient seawater temperature, around 2°C, and sulphide concentrations are extremely low (< 0.1 μM) (Urcuyo et al., 1998; 2003). This study constitutes the first survey of free-living bacterial diversity associated with this vestimentiferan.

Our comparative study, which combined Sanger sequencing and 454 pyrosequencing, was based on four replicates for each of the two habitats (Table 3.1), allowing for thorough statistical analyses and a better understanding of the distinguishing features of their respective microbial communities.

Methods

Site description and sample collection

The samples used in this study were collected from three different sites in the Main Endeavour vent field, i.e. the Smoke & Mirrors (S&M) edifice, Hotspot 1 and Hotspot 2 on the Grotto edifice; and from a single site in the Clam Bed vent field, during a 2008 research expedition on board the Canadian Coast Guard Ship *John P. Tully.* At each site (Table 3.1 and Fig. 3.1), paired grab samples of the most contrasting morphotypes of the tubeworm *Ridgeia piscesae*, known as the “short-fat” and the “long-skinny” morphotypes, were collected from their respective habitats within a distance of 10 m using the remotely-operated vehicle (ROV) ROPOS and placed in separate, closing bioboxes. Aggregations of the short-fat morphotype were typically found near a black

Table 3.1 Description and location of sampling sites.

Sample	Tubeworm morphotype	Flow regime	Vent Site	Latitude	Longitude	Site description	Depth (m)	Max. temp. at plume (°C)
HF8SMb	Short-fat	High	Smoke & Mirrors	47° 56.879' N	129° 5.912' W	Lots of shimmering, proximity to black smoker	2180.6	10.0
LF8SMb	Long-skinny	Low	Smoke & Mirrors	47° 56.880' N	129° 5.911' W	Little shimmering, no black smoker visible	2180.5	5.0
HF8GH1b	Short-fat	High	Grotto (Hotspot 1)	47° 56.948' N	129° 5.896' W	Black smoke all around	2188.2	30.0
LF8GH1b	Long-skinny	Low	Grotto (Hotspot 1)	47° 56.955' N	129° 5.900' W	No shimmering visible	2187.9	3.6
HG8GH2b	Short-fat	High	Grotto (Hotspot 2)	47° 56.951' N	129° 5.899' W	Black smoke all around	2188.1	30.0
LF8GH2b	Long-skinny	Low	Grotto (Hotspot 2)	47° 56.944' N	129° 5.899' W	Little shimmering, no black smoker visible	2190.4	11.4
HF8CBb	Short-fat	High	Clam Bed	47° 57.778' N	129° 5.490' W	Lots of shimmering, proximity to black smoker	2188.3	27.0
LF8CBb	Long-skinny	Low	Clam Bed	47° 57.770' N	129° 5.490' W	No shimmering visible	2191.3	2.4

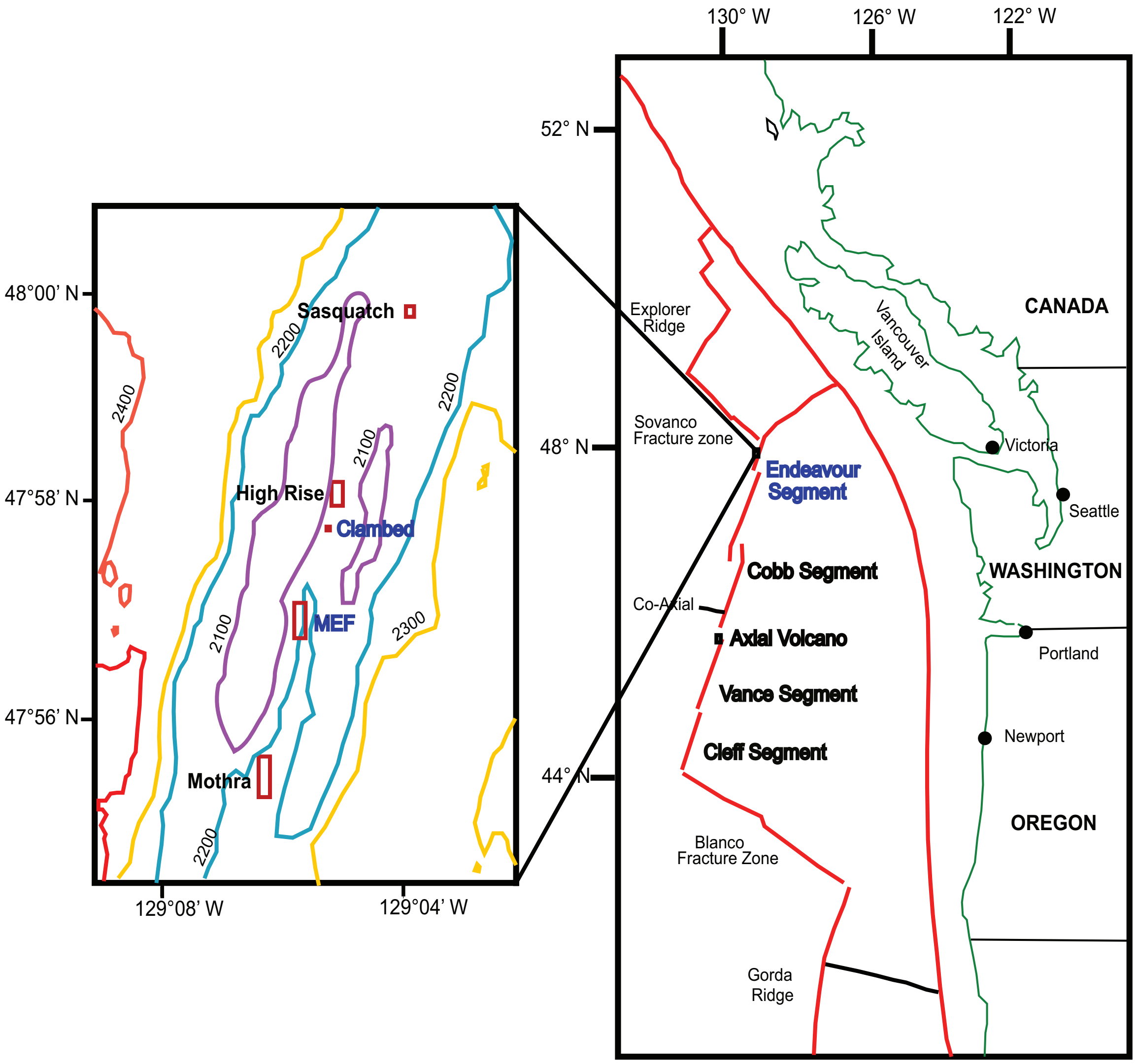

Figure 3.1

Map of the Northeast Pacific (modified from Bourbonnais et al. 2012) showing the location of the Endeavour Segment on the Juan de Fuca Ridge, as well as the major vent fields of this segment. The sample collection sites are in blue. (MEF = Main Endeavour vent field).

smoker in relatively high temperature and vigorous diffuse fluid flow areas (Fig. 3.2A). The long-skinny morphotype, which is more common and widely spread, was collected in regions of low-temperature diffuse hydrothermal fluid away from black smoker activity (Fig. 3.2B). For simplicity, samples collected from short-fat tubeworm habitats are referred as "High Flow samples" and those collected from long-skinny tubeworm habitats as "Low Flow samples". On shipboard, residual particulate detritus from the tubeworm grabs was collected from the bottom of the ROV bioboxes and immediately frozen at -80°C until further processing on shore.

Carbon and nitrogen contents

A few grams of wet particular detritus from the three paired samples collected at S&M and Grotto vents, free of visible faunal organisms, were dried at 60°C for 24 hours. After grinding to a fine powder, 30-80 mg of dry particular detritus were sent to the UC Davis Stable Isotope Facility (Davis, CA; http://stableisotopefacility.ucdavis.edu) for carbon and nitrogen analysis.

DNA extraction and 454 pyrosequencing

The eight samples were prepared for DNA extraction by sorting out the meiofauna using a Leica MZ16 stereomicroscope in order to minimize eukaryotic DNA contamination. DNA was extracted and purified as described in Forget et al. (2010) and quantified using a Nanodrop ND-1000 spectrophotometer. From this purified DNA, 10 µl of each sample, with a concentration of approximately 10 ng/µl of DNA or higher, was sent to the Research and Testing Laboratory (Lubbock, TX) where the primers Gray28F 5'-TTT GAT CNT GGC TCA G-3' and Gray519r 5'-GTN TTA CNG CGG CKG CTG-

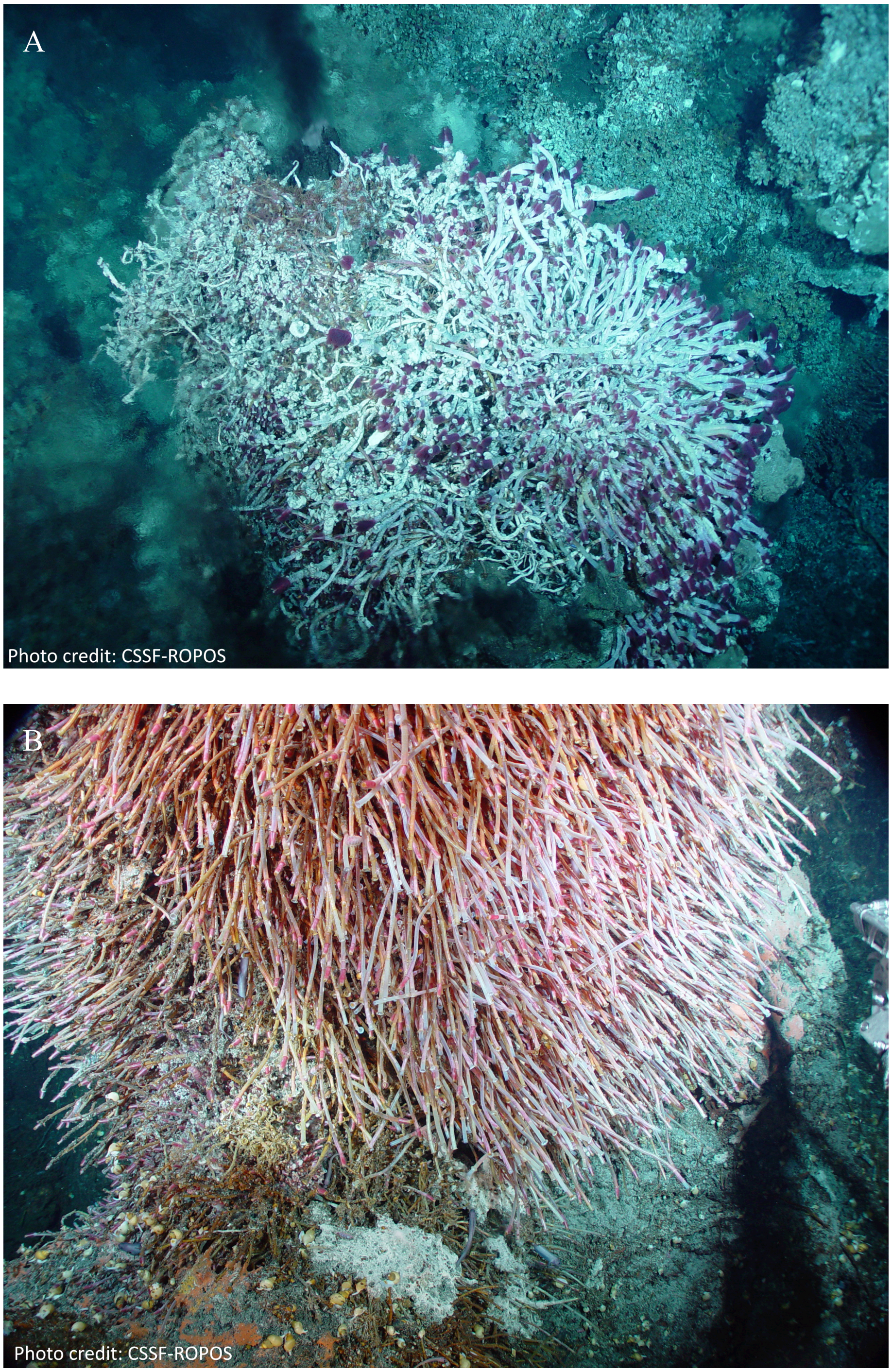

Figure 3.2

Examples of typical sampling sites. (A) High Flow environment inhabited by the short-fat phenotype of *Ridgeia piscesae*. The shimmering indicates the presence of hydrothermal fluid venting through the aggregation of tubeworms. (Note their white tube and bright-red healthy-looking branchial plume.) (B) Low Flow environment inhabited by the long-skinny phenotype of *R. piscesae*. No shimmering is visible. (Note the brown-orange colour of the tube and the reduced branchial plume.)

3’ were used to generate PCR amplicons of the bacterial SSU rRNA gene hypervariable regions V1-V3. Sequencing was carried out with a Roche 454 FLX instrument using the Research and Testing Laboratory protocols (http://www.researchandtesting.com).

Pyrosequencing read analysis

In order to obtain high-quality sequences for statistical analysis, the original reads were passed through quality filters to reduce the error rate (Huse et al., 2007) using the software mothur v.1.22.1 (Schloss et al., 2009). Sequences with an average quality score below 25, containing ambiguous bases (N), shorter than 200 base pairs or not perfectly matching the forward primer or the barcode at the beginning of the read were eliminated. After trimming the primers and barcodes, potential chimeras were identified (and subsequently eliminated) using the program UCHIME (Edgar et al., 2011). The remaining sequences were aligned and classified using a SILVA reference database (Pruesse, 2007) provided by mothur for bacterial sequences. A distance matrix was generated with mothur, and sequences having ≥97% similarity were treated as a same operational taxonomic unit (OTU).

Statistical analysis of pyrosequencing reads

All of our analyses were performed using the software mothur and were based on the distance matrix and OTU definition mentioned above. Bacterial diversity within each of the eight samples was estimated with rarefaction analysis, coverage values, Chao1 richness and Shannon diversity indices. Comparison of the structure and composition of the communities between samples were performed using a hierarchical clustering analysis and a nonmetric multidimensional scaling (NMDS) analysis with the Morisita-

Horn calculator of dissimilarity (Horn, 1966). The significance of the hierarchical clustering was tested using the weighted UniFrac algorithm (Lozupone and Knight, 2005). We used an analysis of molecular variance (AMOVA) to test the significance of the spatial separation between High Flow and Low Flow samples in the NMDS plot, and an analysis of homogeneity of molecular variance (HOMOVA) to test the homogeneity of the bacterial communities between High Flow and Low Flow habitats. The command "metastats", based on the program designed by White et al. (2009), was used to determine which OTUs were (significantly) differentially represented in the High Flow and Low Flow libraries.

Sanger sequencing

Approximately 10 ng/μl of DNA were used to amplify SSU rRNA gene sequences with the universal bacterial primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GTT TAC CTT GTT ACG ACT T-3'). A range of $MgCl_2$ concentrations and annealing temperatures were tested and the most stringent combinations with sufficient yield in the target size range were used. The final reaction mixture used (20 μl) contained 1 μl template DNA, 1.875 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 0.25 μM (each) primer, 1X PCR buffer (Invitrogen, Valencia, CA, USA), and 1 U of *Taq* DNA polymerase (Invitrogen). A first initialization step of 2 min at 94°C was followed by 26-30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 2 min. The final extension step was performed at 72°C for 10 min. The number of cycles was optimized for each sample in order to get a product concentration still in the exponential phase, as visualized on 1.2% (w/v) agarose gels stained with SYBR Safe (Invitrogen). Products of four parallel PCRs were combined, purified and cloned, and white colonies were randomly chosen and screened for inserts by PCR reaction using the vector primers M13F and M13R a as described in Forget et al. (2010). Sequencing of the inserts was completed at the High-Throughput Genomics Unit (Seattle, WA) using the PCR primers 8F and 1492R, and 515F (5'-GTG CCA AGC MGC CGC GGT AA-3'), 519R (5'-GWA TTA CCG CGG CKG CTG-3'), 926F (5'-AAA CTY AAA KGA ATT GAC GG-3') and 907R (5'-CCG TCA ATT CMT TTR AGT TT-3').

Repeated attempts to amplify *Archaea* with universal archaeal SSU primers ARCH-8F (5'-TCC GGT TGA TCC TGC C-3') and ARCH-1492R (5'GGC TAC CTT GTT ACG ACT T-3') yielded no product for any sample.

Sanger sequence analysis

The SSU rRNA gene sequences were assembled and checked manually for errors using Sequencher v4.7 (Gene Codes Corporation). The sequence data set was screened for potential chimeric structures using the Bellerophon server (Huber et al., 2004), available through the Greengenes website (DeSantis et al., 2006). Putative chimeras were further investigated with the program Pintail (Ashelford et al., 2005) by comparing each of them with closely related sequences recovered from the online tool BLAST (available through the National Centre for Biotechnology Information website (NCBI)). The sequences from each library were aligned independently using CLUSTAL W v2 (Larkin et al., 2007) and clones having 97% sequence similarity or higher were treated as the same OTU using DOTUR (Schloss and Handelsman, 2005), based on the distance matrices obtained with the PHYLIP software package v3.68 (Felsenstein, 2005). The phylogenetic affiliation of each sequence was assigned using the Classifier tool available through the Ribosomal Database Project (RDP) (Wang et al., 2007).

Statistical analysis of Sanger sequences

The analyses described previously for the pyrosequencing data were also performed on the Sanger sequences. However, since the amount of information obtained from the first method provided more power to statistical tests, only the results from the phylogenetic affiliation of the sequences and the “metastats” command are discussed in this paper.

Nucleotide sequence accession numbers

The original pyrosequencing reads as well as their quality files were submitted to NCBI’s Sequence Read Archive (SRA) and the project was assigned the accession number SRA056333. The SSU rRNA gene sequences representing unique OTUs have been submitted to the GenBank database and assigned the accession numbers JN662022 to JN662309.

Results

Carbon and nitrogen analysis

Three of the four paired samples were analysed for carbon and nitrogen contents, while no more detritus was available for the two samples from Clam Bed. Total carbon and nitrogen concentrations were consistently higher in the Low Flow sample at each site (Table 3.2). In sample LF8GH2b, these concentrations were an order of magnitude higher than in the other samples, which could suggest the presence of tubeworm or meiofauna residue in the sample. The carbon/nitrogen molar ratios were also consistently higher in Low Flow samples.

Table 3.2 Carbon and nitrogen contents and molar ratio.

Sample ID	%C	%N	C/N molar ratio
HF8SMb	0.28	0.06	5.56
LF8SMb	1.05	0.20	6.30
HF8GH1b	1.64	0.34	5.62
LF8GH1b	2.53	0.45	6.54
HF8GH2b	1.02	0.22	5.41
LF8GH2b	12.29	1.48	9.69

Composition of sequence libraries

For both the Sanger and 454 pyrosequencing approaches, a total of eight bacterial sequence libraries were constructed from the four paired samples. Several attempts to amplify archaeal SSU rRNA gene proved unsuccessful, which was also the case for other studies of microbial communities from diffuse hydrothermal environments (Corre et al., 2001; Alain et al., 2002; Alain et al., 2004; Forget et al., 2010). Similar efforts using samples from more severe hydrothermal conditions successfully amplified *Archaea* (Pagé et al., 2004; Byrne et al., 2009; Opatkiewicz et al., 2009). In this study, measured temperatures at sampling sites did not exceed 30°C.

454 pyrosequencing-based survey

A total of 187 935 sequences covering the V1-V3 region of the bacterial 16S gene were obtained through 454 pyrosequencing of our eight samples, 140 019 of which passed the quality filters described in the Methods section. This conservative approach reduced the size of our data set by approximately 25%, similar to the results obtained by Sogin et al.

(2006). The number of unique reads varied between 927 and 4563 per sample, and the number of OTUs varied between 364 and 1600 per sample (Appendix C, Table C.1). These OTUs were affiliated with 29 different phyla of the bacterial domain, and 15 OTUs with less than 80% similarity to any cultured organism could not be confidently classified. The *Proteobacteria* dominated all libraries, accounting for over 80% of the total number of sequences per library, and up to 98.3% in the case of the High Flow sample HF8GH1b. Within this group, the *Epsilonproteobacteria* were the most abundant, representing from about 50% to 96% of the sequence libraries (Fig. 3.3A). Figure 3.3B shows the breakdown of this class at the genus level. The genus *Sulfurovum* was dominant in all libraries and accounted for 99.4% of the total number of *Epsilonproteobacteria* in the Low Flow sample LF8CBb, while the genus *Sulfurimonas* was more abundant in High Flow samples. The genera *Thioreductor*, *Hydrogenimonas*, *Nitratifractor*, and *Nitratiruptor* were also relatively abundant (Fig. 3.3B). The category "Other and Unclassified" representing less than 1% of the total *Epsilonproteobacteria* in any library, included metabolically diverse genera such as *Sulfurospirillum*, *Arcobacter*, *Wolinella*, *Campylobacter*, *Caminibacter* and *Nautilia*.

The *Gammaproteobacteria*, mostly detected in the Low Flow samples, was the second most abundant class within the *Proteobacteria* (Fig. 3.3A). Sequences within this group was highly diverse, with over 60 different genera detected, most of which represented less than 1% of the total number of sequences (Appendix C, Table C.1). Unclassified sequences accounted for 43% of the *Gammaproteobacteria*, but this number varied between libraries and reached 98.3% in the case of the High Flow sample HF8CBb. The most abundant genera that could be confidently identified were *Leucothrix*,

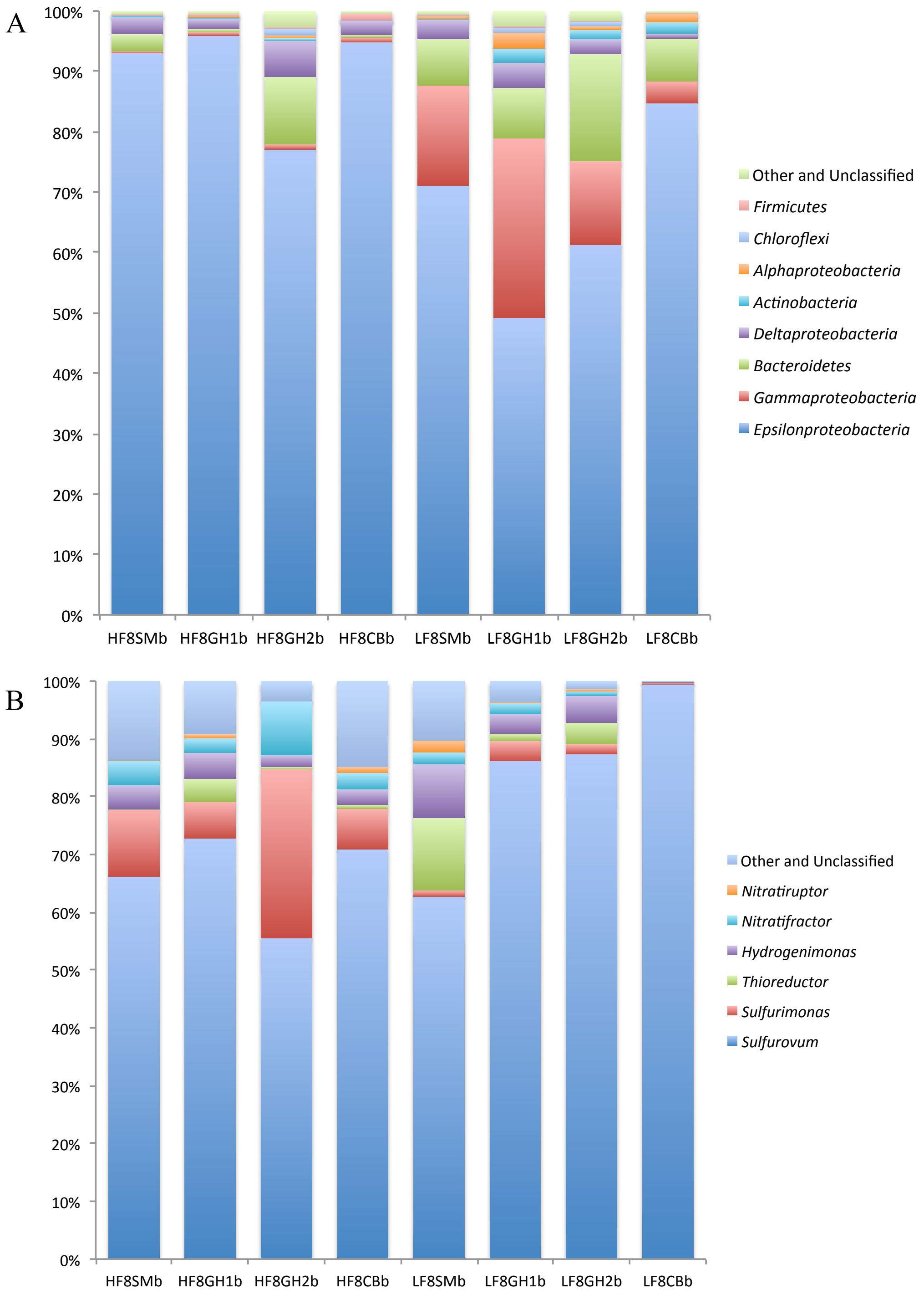

Figure 3.3

Relative abundance of (A) the major taxonomic groups and (B) the genera detected within the class *Epsilonproteobacteria* in the eight clone libraries constructed from 454 pyrosequencing. Genera that did not reach 1% of the relative abundance of at least one library and unidentified OTUs were grouped under the category “Other and Unclassified”.

Endozoicomonas and *Methylosarcina*. *Thiohalophilus* was another relatively abundant genus, followed by *Dasania*, which accounted by itself for more than 50% of the gammaproteobacterial sequences detected in the Low Flow sample LF8CBb, and *Ectothiorhodosinus*, representing 1.4% of the total number of sequences.

The *Deltaproteobacteria*, which represented 2.6% of the total number of sequences, were also very diversified, with over 50 different genera detected (Appendix C, Table C.3). Abundant genera included *Desulfobulbus*, *Desulfocapsa*, *Desulfonema*, *Desulfuromusa*, *Desulforhopalus* and *Desulfoluna.* A few *Alpha*- and *Betaproteobacteria* were also detected (Appendix C, Tables C.4 and C.5, respectively).

The *Bacteroidetes* was the second most abundant phylum, followed by the *Actinobacteria*, the *Chloroflexi*, and the *Firmicutes* (Fig 3.3A). The other phyla, including the *Acidobacteria*, *Lentisphaerae*, *OD1, Cyanobacteria, Deinococcus-Thermus, Chlorobi, Spirochaetes, Synergistetes, Tenericutes, Fusobacteria, WS3, SR1, Chlamydia, TM7, Gemmatimonadetes, Thermodesulfobacteria, Nitrospira, Planctomycetes, Verrucomicrobia,* and *Deferribacteres*, accounted for less than 1% of the relative abundance of any library and were grouped under the category “Other and Unclassified” in Figure 3.3A.

Sanger sequencing-based survey

For the clone libraries constructed from Sanger sequencing, the number of clones sequenced per sample varied between 101 and 175, for a total of 1045 partial sequences. In each library, sequences that had 97% or greater similarity were considered as unique OTUs, and a representative of each OTU was fully sequenced. The number of OTUs detected per library varied between 18 and 51, for a total of 288. These OTUs were affiliated with seven different phyla also detected with 454 pyrosequencing: the *Proteobacteria*, *Bacteroidetes*, *Actinobacteria, Planctomycetes*, *Verrucomicrobia, Deinococcus-Thermus* and *Firmicutes* (Fig. 3.4A). Thirteen OTUs had less than 80% similarity to any cultured organism and could not be classified.

The general patterns of relative abundances of the most common groups were very similar to those observed in the pyrosequencing results (Figs. 3.3A, 3.4A). The *Proteobacteria* largely dominated the clone libraries, representing approximately 95% of the clones sequenced. Within this group, the *Epsilonproteobacteria* were the most abundant group, representing 63 to 97% of the clone libraries (Fig. 3.4A). The *Gammaproteobacteria*, the second most abundant group, were mainly found in the Low Flow samples. The *Bacteroidetes*, *Deltaproteobacteria*, *Actinobacteria* and *Alphaproteobacteria* followed in terms of relative abundance, similar to results obtained from pyrosequencing (Figs. 3.3A, 3.4A). The fully sequenced 16S gene allowed more precision when assigning phylogenetic affiliation; OTUs could be compared with previously published sequences and those with at least 97% similarity to cultured organisms could be identified at the species level. As only OTUs belonging to the *Epsilonproteobacteria* could be assigned to a species, Figure 3.4B shows the relative abundance of the identified species within this class. Between 32 and 42% of the *Epsilonproteobacteria* from the High Flow samples were affiliated with *Sulfurovum lithotrophicum*, while this species represented only a small proportion of the Low Flow libraries and was not detected in LF8CBb. A few OTUs from High Flow and Low Flow samples were affiliated with *Nitratifractor salsuginis*, while *Sulfurimonas paralvinellae* was detected only in High Flow samples (Fig. 3.4B).

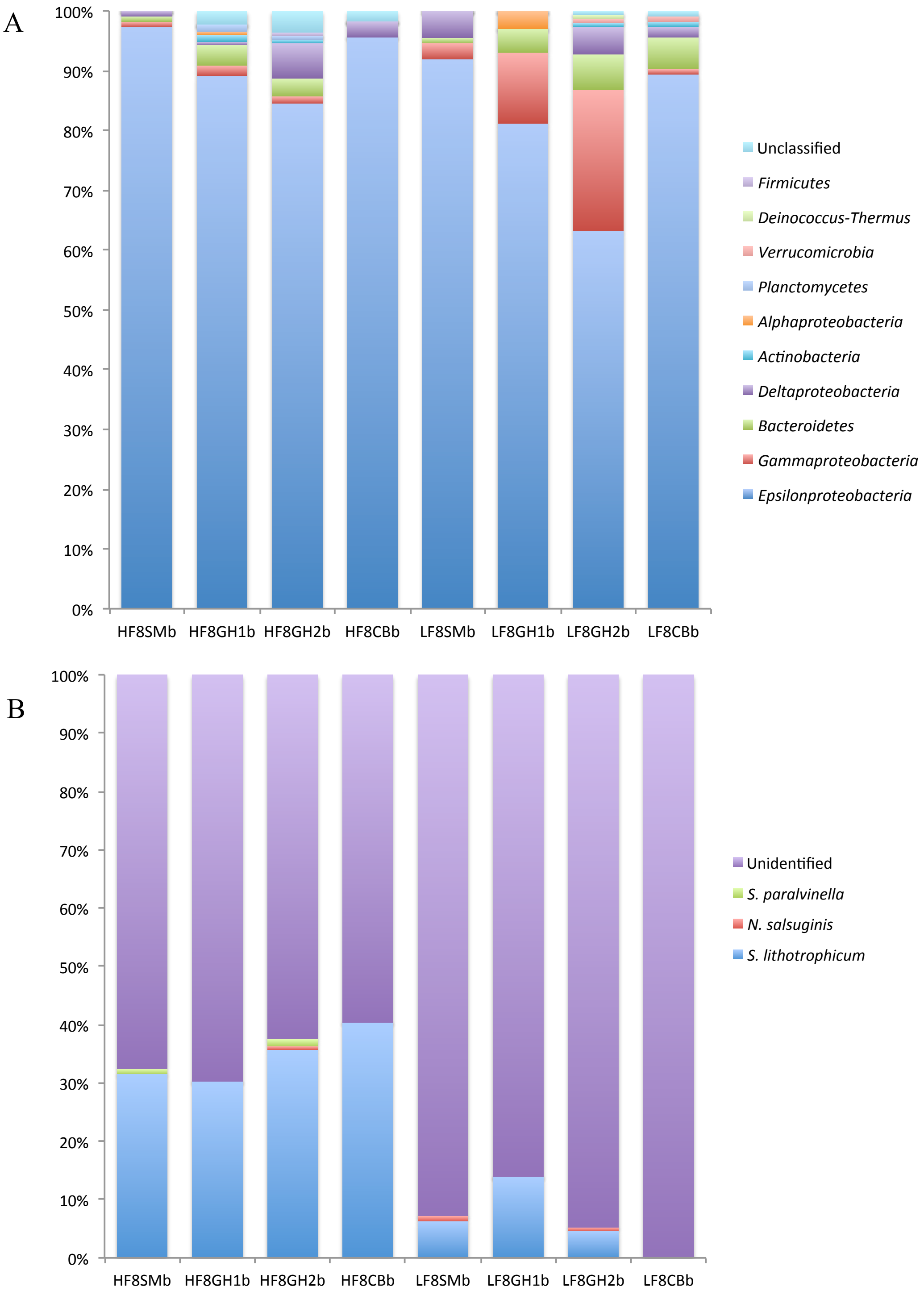

Figure 3.4

Relative abundance of (A) the phyla (the phylum *Proteobacteria*, which dominated all clone libraries, was divided in to the four classes detected) and (B) species confidently identified within the class *Epsilonproteobacteria* in the eight clone libraries constructed from Sanger sequencing.

Most epsilonproteobacterial OTUs could not be classified to the species level, and OTUs with less than 95% similarity to any cultured organism could not be assigned to a genus. Their closest relatives in the GenBank database were environmental clones collected from hydrothermal vent chimneys, biofilms and sediments or uncultured symbionts of hydrothermal fauna such as the polychaetes *Alvinella pompejana*, *Paralvinella palmiformis* and *Riftia pachyptila*, the alvinocarid shrimp *Rimicaris exoculata* and the recently discovered decapod *Kiwa hirsuta*.

A few gammaproteobacterial OTUs could be affiliated to the strictly aerobic genera *Granulosicoccus*, *Leucothrix* and *Methylobacter*. However, most of them could not be confidently assigned a genus, but were closely related to environmental clones collected from hydrothermal vent systems and permanently cold marine sediments. The deltaproteobacterial genera *Desulfobulbus* and *Desulfocapsa* were detected. None of the *Alphaproteobacteria* detected could be confidently assigned a genus (data not shown).

Within the *Bacteroidetes*, the genus *Actibacter* was identified, but most of the phylotypes could not be assigned a genus. However, they were closely related to clones collected from hydrothermal vent environments, cold seeps, and permanently cold marine sediments. The OTUs detected in the *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Deinococcus-Thermus* and *Firmicutes* were not similar enough to any cultured organism to be identified at the genus level, but they were closely related to clones collected from deep-sea sediments, seafloor lavas, and polychaetes mucus and tubes.

Diversity of the bacterial communities

The pyrosequencing data were used for diversity analysis since this method yielded considerably more information than with the Sanger sequencing. First, because the number of read obtained differed among samples, rarefaction analyses, relating the number of OTUs detected to the sequencing effort, were used to compare the richness of the sequence libraries (Fig. 3.5). The samples from the Grotto vent site, except for HF8GHb1, had the highest richness, while the samples from Clam Bed had the lowest. High coverage values indicate that most of the bacterial diversity was encompassed by our sequencing effort (Table 3.3). The samples from Grotto whose rarefaction curves showed the highest richness had the lowest coverage scores. However, coverage, as well as the Chao1 index, is highly dependent on the sequencing effort, which explains the lower values for the paired samples from Hotspot 2 at Grotto vent, HF8GH2b and LF8GH2b (see Appendix C, Table C.1 for total number of reads). The paired samples from Clam Bed, HF8CBb and LF8CBb, also had a lower Chao1 richness index; while the number of reads sequenced for these samples was more than three times greater than the samples from Hotspot 2 at Grotto, and the number of OTUs represented by only one sequence (singletons) was lower, decreasing the Chao1 index value (Table 3.3). The Shannon-Wiener diversity index, which takes into account the richness and the evenness of the community, confirmed the lower diversity of the samples from Clam Bed. HF8GH1b had a high Chao1 index value, but also showed a lower Shannon-Wiener value, suggesting an uneven community.

Comparison of the bacterial communities

Two different visualization tools were used to compare the composition and structure of the bacterial communities between the libraries obtained from 454 pyrosequencing. The tree constructed from the hierarchical clustering analysis showed all High Flow

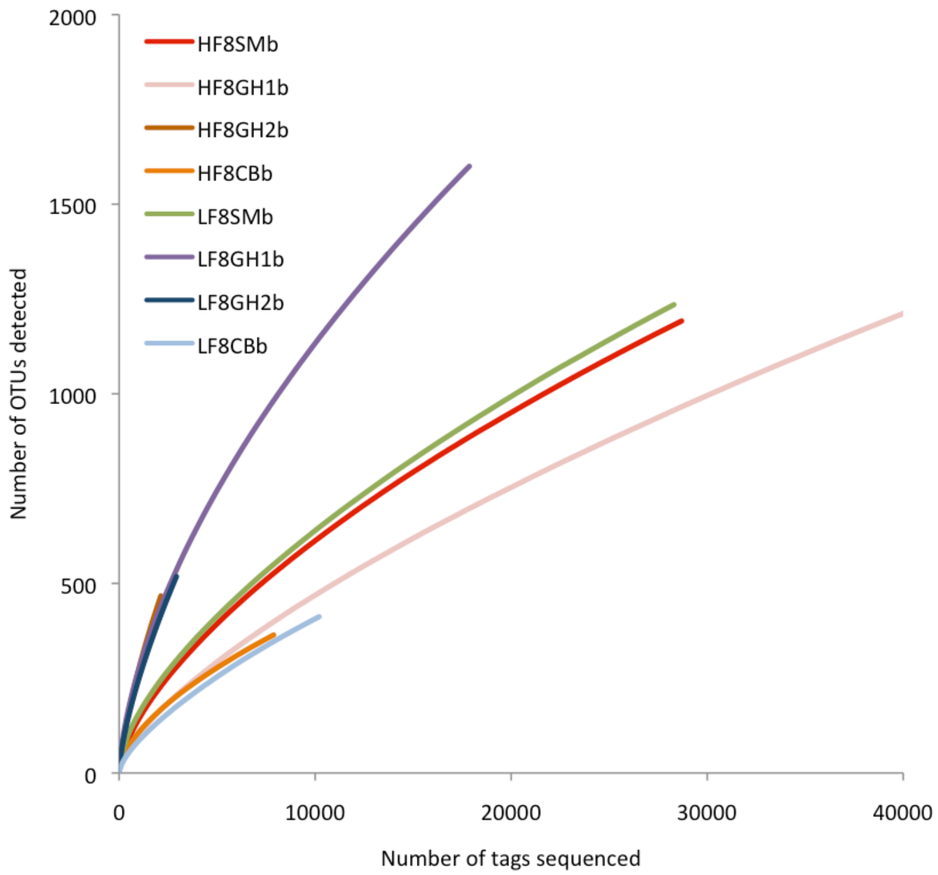

Figure 3.5

Rarefaction analysis of the libraries created from 454 pyrosequencing showing the richness of each clone library at the 97% similarity level.

Table 3.3 Diversity analyses of the clone libraries constructed from 454 pyrosequencing.

Sample ID	Coverage (%)	Chao1 richness index[a]	Number of singletons	Shannon-Wiener diversity index[a]
HF8SMb	97.4	3025 (2664-3474)	742	3.21 (3.18-3.24)
HF8GH1b	98.0	3581 (3140-4125)	850	2.84 (2.82-2.86)
HF8GH2b	85.0	1299 (1056-1618)	320	4.06 (3.96-4.16)
HF8CBb	97.3	869 (703-1115)	216	2.86 (2.81-2.91)
LF8SMb	97.3	3119 (2755-3568)	772	4.06 (4.04-4.08)
LF8GH1b	94.7	3725 (3357-4171)	943	4.97 (4.94-5.00)
LF8GH2b	87.8	1554 (1264-1956)	356	4.40 (4.32-4.47)
LF8CBb	97.3	1031 (847-1292)	273	2.19 (2.15-2.23)

[a]Numbers in parenthesis indicate the 95% confidence intervals

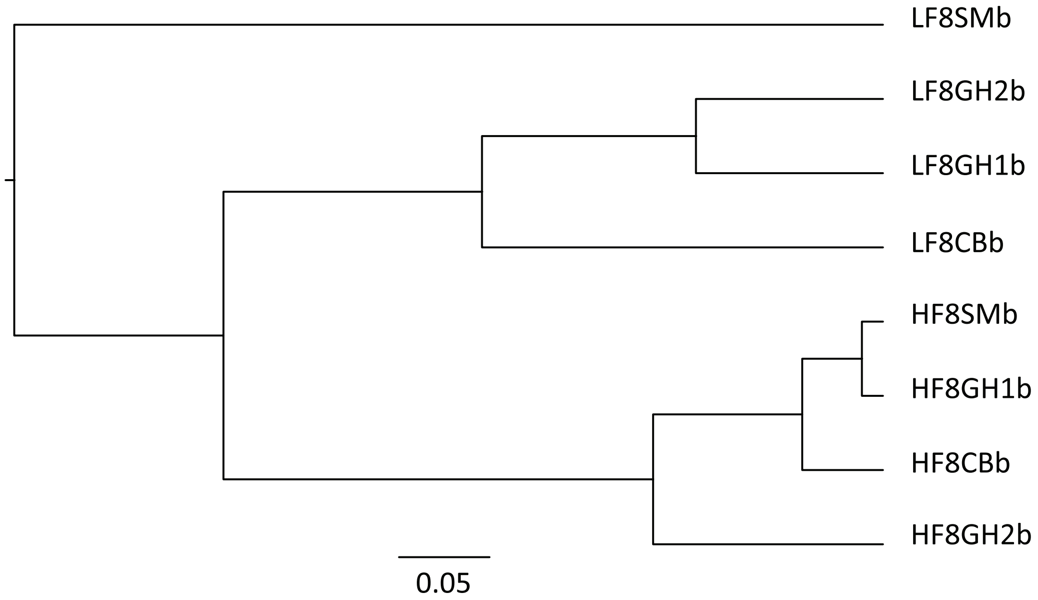

Figure 3.6

Hierarchical clustering analysis showing the similarity of the libraries constructed from 454 pyrosequencing to each other using the Morisita-Horn calculator of dissimilarity. The scale bar represents the estimated divergence between the libraries.

samples to be more similar to each other than to any other sample (Fig. 3.6). Three samples from the Low Flow habitats also grouped together, but sample LF8CBb from the Clam Bed Low Flow habitat did not cluster with any other sample, suggesting a unique bacterial community. A weighted UniFrac analysis, which tests the probability that the communities have the same structure by chance, showed that the separation of High Flow and Low Flow samples in the tree was highly significant ($p < 0.001$). The NMDS ordination plot corroborated the pattern observed with the hierarchical clustering analysis, showing all High Flow samples and three of the Low Flow samples grouped together (Fig. 3.7). The location of sample LF8CBb on the plot indicates that the bacterial community detected in this sample is distinct from the others. The AMOVA confirmed the significance of the spatial separation between High Flow and Low Flow samples with a p value < 0.001. The plot also revealed shorter distances between the High Flow samples compared to the Low Flow samples, suggesting a greater diversity among the latter. The HOMOVA indicated that the diversity of the bacterial communities within each habitat was not homogeneous ($p < 0.001$). However, when sample LF8CBb was excluded from the HOMOVA analysis, no significant difference was observed between High Flow and Low Flow samples.

The “metastats” function in mothur allows the identification of sequences that are differently represented between populations ($p < 0.05$). We found a total of 142 OTUs belonging to at least 53 genera differently represented among the High Flow samples, compared to 487 differently represented OTUs belonging to at least 167 genera in the Low Flow samples, respectively accounting for 4.3% and 12.9% of the total number of

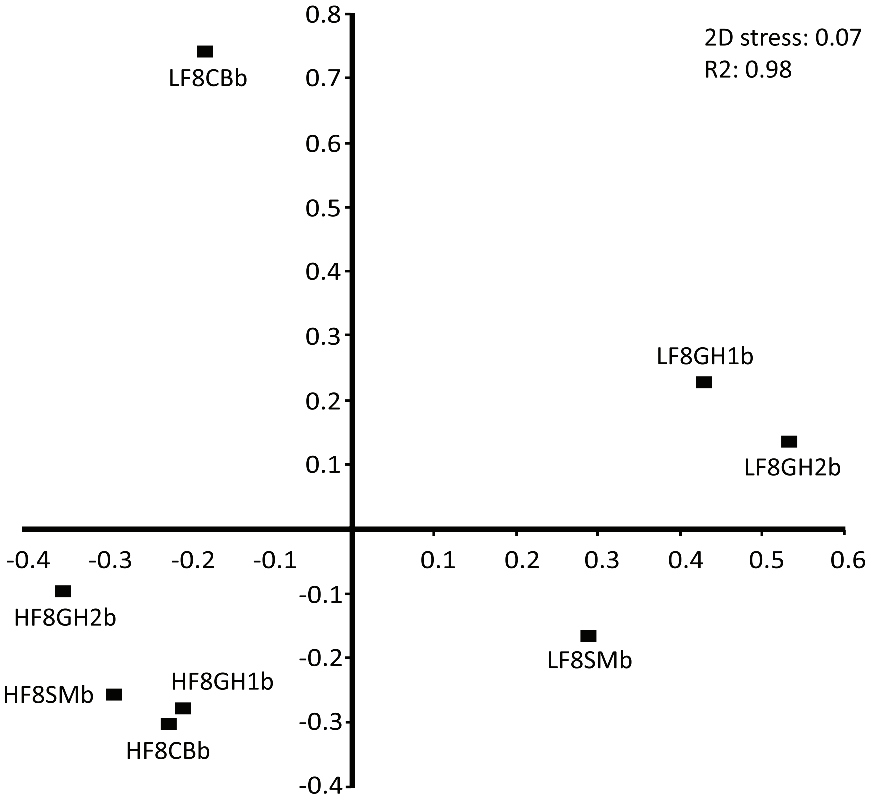

Figure 3.7

Nonmetric multidimensional scaling (NMDS) 2D similarity plot showing the distance between the libraries constructed from 454 pyrosequencing based on the Morisita-Horn calculator of dissimilarity.

OTUs in each habitat. A higher number of phyla were detected in Low Flow samples, but in both habitats, most differently represented OTUs belonged to the *Proteobacteria* and the *Bacteroidetes* (Fig. 3.8A and B). Within the *Proteobacteria*, differently represented OTUs in High Flow samples were mostly affiliated with the *Epsilonproteobacteria*, followed by a few *Deltaproteobacteria*, five *Gammaproteobacteria* and one *Alphaproteobacteria* (Fig. 3.8C), while in Low Flow samples, most differently represented OTUs were affiliated with the *Gammaproteobacteria*, followed by the *Epsilonproteobacteria*, the *Deltaproteobacteria*,

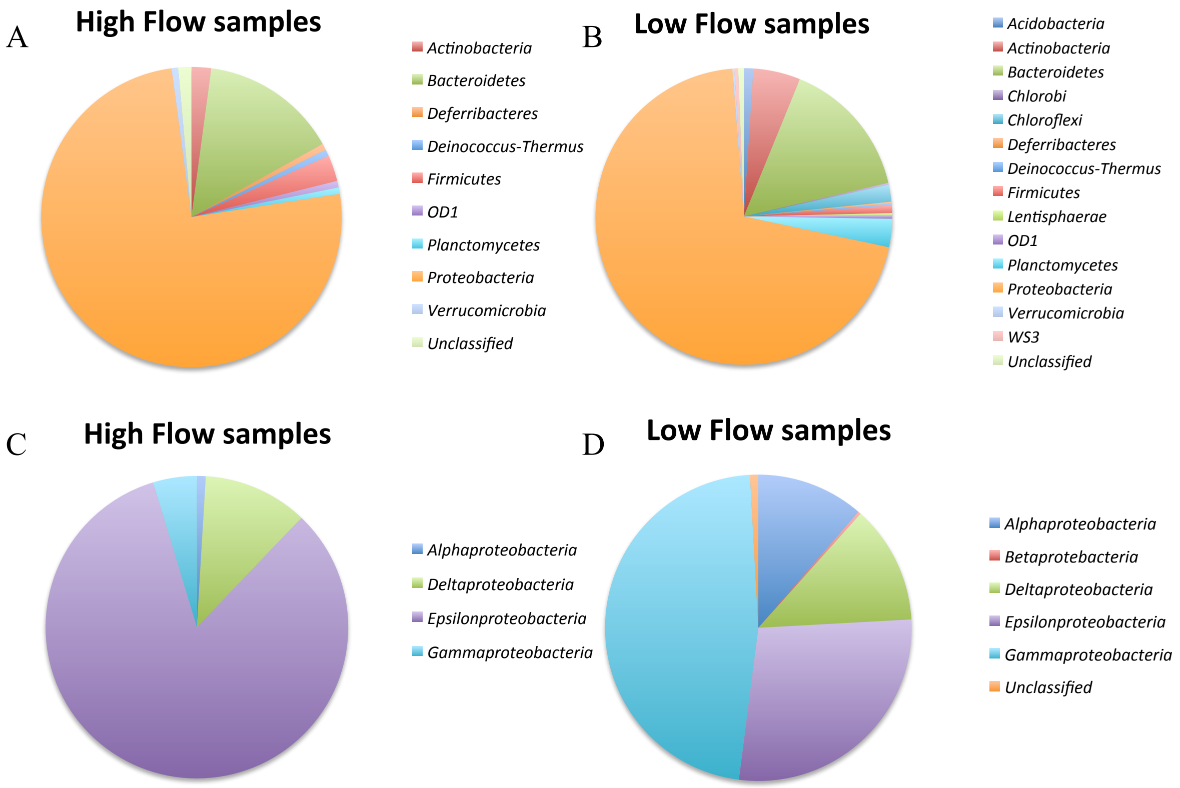

Figure 3.8

Relative abundance of the significantly differently represented OTUs ($P < 0.05$) in (A) High Flow phyla, (B) Low Flow phyla, (C) High Flow *Proteobacteria* and (D) Low Flow *Proteobacteria*.

the *Alphaproteobacteria*, one *Betaproteobacteria* and three unclassified *Proteobacteria* (Fig. 3.8D). A closer look at the genus level revealed that High Flow and Low Flow samples were colonized by different species that belong mostly to the *Sulfurovum*, *Sulfurimonas*, *Hydrogenimonas* and *Nitratifractor* within the *Epsilonproteobacteria*, and to the genus *Desulfobulbus* within the *Deltaproteobacteria*. Within the *Gammaproteobacteria*, the most abundant genera detected in the Low Flow libraries were *Leucothrix* and *Endozoicomonas*. Other relatively abundant phyla differently represented between habitats included the *Actinobacteria*, the *Planctomycetes* and the *Chloroflexi,* mostly detected in Low Flow libraries. The same analysis performed on the clone libraries constructed from Sanger sequencing showed *S. lithotrophicum* to be more represented in High Flow samples ($p < 0.001$), while the other OTUs with significantly differently represented between habitats had < 97% sequence similarity to any cultured organism and could not be classified to the species level.

Discussion

Our survey combined conventional Sanger sequencing with 454 pyrosequencing; the first approach provided more precise information on the taxonomy of the sequences, while the second allowed rapid generation of large numbers of sequences without the bias introduced by the cloning step, thereby enabling a deeper survey and better coverage of bacterial diversity and the detection of rare species. Furthermore, while only semi-quantitative, 454 pyrosequencing provides a more accurate estimate of the relative abundance of OTUs in the microbial communities (Sogin et al., 2006).

Neither of the two molecular approaches used in this study yielded any archaeal sequences. Alain et al. (2004) pointed out that while mesophilic *Archaea* are known to be ubiquitous, only thermophilic and hyperthermophilic strains have been detected in hydrothermal vent environments. Furthermore, these strains were mostly anaerobic or microaerophilic organisms, while diffuse flow habitats are zones of mixing between hydrothermal fluids and well-oxygenated background seawater (Alain et al., 2004). Roussel et al. (2011) used nested PCR to improve yield of 16S rRNA gene PCR product from samples from three hydrothermal vent sites on the Mid-Atlantic Ridge. Even with this approach results were mixed with not all samples yielding sufficient PCR product for cloning.

The use of 454 pyrosequencing technology allowed the detection of 29 different bacterial phyla, more than four times the number of phyla detected by Sanger sequencing. The substantially greater information yield from the 454 pyrosequencing-based survey increased the power of the statistical analyses, thereby reducing the probability of type II errors, the failure to reject a false null hypothesis, that could have prevented detection of patterns related to habitat type. Yet, the relative abundance of the major taxonomic groups detected in each sample was comparable with the Sanger sequencing survey. Unsurprisingly, the *Epsilonproteobacteria*, which play a major role in the cycling of nitrogen and sulphur, were dominant in all libraries. This has been observed in numerous microbial diversity surveys of hydrothermal habitats, including venting fluids (Huber et al., 2010), mats covering surfaces in the vicinity of vents (Zhou et al., 2009; Flores et al., 2011; Lanzen et al., 2011) or in association with vent fauna (Alain et al., 2002; López-García et al., 2002; Alain et al., 2004). Both of our surveys found the facultative anaerobic mesophilic sulphur-oxidizing genus *Sulfurovum* to be dominant in all samples (Inagaki et al., 2004). The genus *Sulfurimonas*, whose members are also mesophilic chemolithoautotrophs using similar electron donors but in aerobic conditions (Inagaki et al., 2003), was mostly represented in High Flow samples. Other relatively abundant epsilonproteobacterial genera included the mesophilic to thermophilic and strictly chemolithoautotrophic *Thioreductor*, *Hydrogenimonas*, *Nitratifractor*, and *Nitratiruptor*, all using molecular hydrogen as electron source in anaerobic to microaerophilic habitats (Takai et al., 2004; Nakagawa et al., 2005b; Nakagawa et al., 2005a). These genera have in common metabolisms well adapted to vent conditions and are therefore frequently detected in hydrothermal environments (Campbell et al., 2006).

Both surveys also agreed on the high relative abundance of the class *Gammaproteobacteria* in Low Flow libraries, but a large proportion of the OTUs could not be classified to the genus level, and the majority of the identified genera accounted for < 1% of the sequences. However, the Sanger survey showed that most of these OTUs were closely related to uncultured bacteria collected from vent chimneys or living in association with vent fauna that were also sampled from the Endeavour Segment of the Juan de Fuca Ridge, and described, respectively, as methanotrophs and decomposers involve in the degradation of organic debris (Alain et al., 2002; Wang et al., 2009). These descriptions are consistent with the most abundant genera that were confidently identified in this study, such as *Leucothrix*, *Endozoicomonas, Methylosarcina* and *Dasania* from the pyrosequencing survey and *Granulosicoccus* and *Methylobacter* from the Sanger sequencing survey (Harold and Stanier, 1955; Bowman et al., 1993; Wise et al., 2001; Kurahashi and Yokota, 2007; Lee et al., 2007a; Lee et al., 2007b). The occurrence of methanotrophs in our samples suggests the presence of CH_4 in hydrothermal fluids emitted at these sites, confirmed by the elevated concentrations measured by de Angelis et al. (1993). Free-living methanotrophs have been reported from many hydrothermal sites (Takai et al., 2006) and are also part of the epibiotic fauna found on the Mid-Atlantic shrimp *Rimicaris exoculata* (Zbinden et al., 2008; Guri et al., 2012). Interestingly, only two relatively abundant *Gammaproteobactera* genera found in our samples, *Ectothiorhodosinus* and *Thiohalophilus,* have been described as sulphur oxidizers (Gorlenko et al., 2004; Sorokin et al., 2007). The dominance of *Epsilonproteobacteria* at all sites would suggest that metabolisms using sulphur compounds as electron donors should also be common among the metabolically diverse

Gammaproteobacteria. It is possible that diffuse flow conditions at these sites provided sulphur-metabolizing *Epsilonproteobacteria* with a competitive advantage over their *Gammaproteobacteria* counterparts.

The most abundant genera detected within the class *Deltaproteobacteria* included strictly anaerobic and heterotrophic genera such as *Desulfobulbus*, *Desulfocapsa*, and *Desulforhopalus* detected by both methods, and *Desulfonema*, *Desulfuromusa*, and *Desulfoluna* detected only by 454 pyrosequencing (Widdel and Pfennig, 1982; Widdel et al., 1983; Liesack and Finster, 1994; Isaksen and Teske, 1996; Janssen et al., 1996; Suzuki et al., 2008). Members of these taxonomic groups are sulphur reducers, and their presence along with sulphur oxidizers from the *Epsilonproteobacteria* and the *Gammaproteobacteria* classes might suggest internal sulphur cycling within these habitats, as proposed by Forget et al. (2010) and Lanzen et al. (2011) in studies of other low temperature hydrothermal environments.

The OTUs that belonged to the other detected major phyla, including the *Bacteroidetes*, *Actinobacteria*, *Chloroflexi* and *Firmicutes*, were all members of heterotroph genera, and most likely involved in the decomposition of organic matter. The minor groups were metabolically diverse, and a relatively large proportion could not be classified to the genus level, but identified genera were also heterotrophs.

Diversity indices and community composition

Our results showed a significant relationship between microbial community composition and habitat type, suggesting a predictable pattern in High Flow and Low Flow environments. In the tree constructed from the cluster analysis, all libraries sampled from a same habitat at different vent sites, except one, grouped together, and the spatial separation of the High Flow and Low Flow libraries in the NMDS ordination plot was highly significant. The Low Flow library from Clam Bed did not cluster with any other habitat, indicating that the microbial communities from this Low Flow site were distinct from all other communities investigated. Furthermore, the spatial distribution of the Low Flow libraries on the NMDS plot, more widely spread along both axes, suggested that the overall diversity within this habitat, when considering all samples, was higher than that of the High Flow habitat. The distinct composition of the Low Flow sample from Clam Bed was primarily responsible for the apparently higher diversity in the Low Flow habitat. Removing the Clam Bed Low Flow library from the NMDS analysis resulted in there being no significant difference between High Flow and Low Flow libraries in terms of overall diversity. The High Flow library collected from the same site did not diverge from the other libraries in terms of composition. However, both samples from Clam Bed had significantly lower Chao1 richness and Shannon-Wiener diversity indices compared to most other samples, and high coverage values, suggesting that the microbial communities at this vent site were less complex than the other sites.

This result could be explained by a difference in sources of energy available between sites, especially between High Flow habitats, possibly limiting the diversity of the bacterial taxonomic groups adapted to the environmental conditions at Clam Bed. The observation of chemical gradients in the composition of hydrothermal fluids observed along the Endeavour Segment (Butterfield et al., 1994), together with the location of the sites (while the three other sites sampled are all within 150 m of each other, Clam Bed is approximately 1750 m away from Grotto and Smoke & Mirrors) supports this explanation. This is also coherent with the results obtained by Flores et al. (2011)

showing that the composition of the microbial communities to be more similar within vent fields than between them, suggesting that the effect of distinct environmental conditions between vent fields is more important than any other variables within a vent field. However, the chemical components of fluids in Low Flow habitats are very diluted. Therefore, the difference in the composition of the fluids between sites is unlikely to explain the lower diversity in the Low Flow sample from Clam Bed. Also, the composition of the microbial communities showed no statistically significant difference within or between vent field relationships (data not shown). This was also the case in a recent study of the patterns of distribution of *Epsilonproteobacteria* in hydrothermal fluids along the Mariana Arc, where the communities from individual vents seemed to be unique (Huber et al., 2010). These authors suggested that the high diversity of chemical conditions in hydrothermal environments might explain the distinctness of the epsilonproteobacterial populations.

Another fact to take into account is the temporal stability of the sampled habitats. Except for the Low Flow sample from Clam Bed, all samples were collected directly on active sulphide edifices, with the High Flow samples typically found adjacent to vigorous venting and the Low Flow samples further away, on the same edifice, where little or no shimmering flow was visible. These structures are known to be very unstable environments that rapidly evolve at small spatial scales, affecting the distribution and structure of the macrofaunal communities (Sarrazin et al., 1997; Sarrazin et al., 2002). In contrast, the Low Flow sample from Clam Bed was collected from a basaltic substratum in an area where diffuse flow has supported communities of *R. piscesae* since at least 1991 (Reyes et al., 1995; Tivey et al., 1999; Durand et al., 2006). The stability of this site compared with the others may explain the development of a distinct microbial community.

In order to understand other features distinguishing the High Flow and Low Flow habitats, we explored the taxonomic affiliation of the OTUs that were differently represented between habitats. OTUs that were significantly more represented in our Low Flow libraries were over three times more abundant than in our High Flow libraries. This observation suggests that bacterial groups representing only a small proportion of High Flow communities might play a more important ecological role in Low Flow communities. Also, several species were significantly more abundant in the High Flow libraries than in the Low Flow libraries. These include mesophilic to thermophilic microaerophilic sulphur and hydrogen oxidizers from the class *Epsilonproteobacteria,* with one organism classified to the species level, *Sulfurovum lithotrophicum*, as well as a few heterotrophs from the phylum *Bacteroidetes* and sulphur reducers from the *Deltaproteobacteria*. Different species from the same genera having similar metabolisms also proved to be more represented in Low Flow samples, but most of the OTUs significantly more abundant in this habitat belonged to heterotrophic groups, including methanotrophs of the class *Gammaproteobacteria*. Other relatively abundant heterotrophic genera from the phyla *Actinobacteria*, *Planctomycetes* and *Chloroflexi* were also more represented in Low Flow libraries.

Differences in temperature, available energy for metabolism and stability between High Flow and Low Flow habitats potentially explain their distinctive bacterial communities. Even though sulphur and hydrogen oxidizers dominate both types of habitat, the fact that some species were significantly more abundant in High Flow or Low Flow samples, suggests that environmental conditions that control tubeworm morphotypes and the composition of their associated macrofauna, can also shape the composition of free-living bacterial communities. Environmental shaping of hydrothermal vent microbial communities has also been observed in other studies (Schrenk et al., 2003; Byrne et al., 2009; Kato et al., 2010; Flores et al., 2011), although it is not usually considered in the broader ecological context of factors controlling the composition of vent faunal assemblages. The abundance of heterotrophic organisms in Low Flow libraries (versus High Flow) indicates that detrital organic matter may be a more important source of energy and carbon for the microbial communities in this habitat. Perner et al. (2007) also pointed out the potential importance of heterotrophic metabolism in microbial assemblages at low-temperature vents, although their study did not include analysis of available organic matter. Our carbon and nitrogen analyses consistently showed High Flow sites to have fresher, less degraded detritus (lower C/N ratios), which should normally provide more energy for heterotrophic growth than the more refractory organic material (higher C/N ratios) sampled from the Low Flow sites. This is consistent with findings by Venkitachalam (2005) that both autotrophic and heterotrophic microbial enzyme activity were more intensive in detrital samples from High Flow tubeworm colonies than in samples from Low Flow sites. Despite the fact that Low Flow habitats represent lower energy environments (compared to High Flow sites), where the availability of reducing sulphur species is limited (Urcuyo et al., 2003) and fresh organic matter is scarce, there were no major differences in overall bacterial diversity between the two habitats. All conditions being equal, one would expect the higher energy habitat to support a greater level of bacterial diversity. One important difference between the High

Flow and Low Flow habitats may be related to their relative stability and longevity. High Flow habitats have been observed to be short-lived (Sarrazin et al., 1997; Tunnicliffe et al., 1997) while at least one of the Low Flow sites sampled here (Clam Bed) is known to have been active for at least two decades. The greater stability of the Low Flow sites may have therefore favoured a diversification of both autotrophic and heterotrophic bacteria, despite the less favourable growth conditions.

Hydrothermal vents are extremely dynamic environments where physical and chemical conditions vary rapidly both in time and space, and this is known to affect the composition of the macrofaunal communities (Sarrazin et al., 1999; Sarrazin et al., 2002). To date, logistic considerations have limited the use of replicate sampling in studies of the dynamics of vent faunal communities, thus limiting the scope of any interpretation of observed differences between samples. For similar practical reasons, replication and statistical comparisons have been even less common in comparative studies of hydrothermal vent microbial communities. As the field progresses from general descriptive studies to probing relationships between microbial diversity and habitat dynamics, replicate sampling and statistical comparisons will become essential to identifying trends and testing hypotheses. In this study, we have provided some examples of the insights that can be derived from a combination of systematic, replicated field collections and molecular analysis of microbial diversity. Even if the small number of cultured microorganisms known from hydrothermal vent habitats, combined with the limitation of taxonomic assignment by 454 pyrosequencing data prevented us from investigating which species defined the bacterial communities of High Flow and Low Flow habitats, our survey revealed interesting general characteristics about these communities, such as their high overall diversity and a probable relationship of composition with habitat type. Furthermore, results underline the ubiquity of *Epsilonproteobacteria* in hydrothermal vent environments, which were mainly related to sulphur- and hydrogen-oxidizing genera. Other approaches, such as metagenomic and proteomic analysis, could help to define more specifically the ecological role of the microorganisms detected by providing information on genes and enzymes specific to each habitat.

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Chapter 4 Molecular study of bacterial diversity within the trophosome of the siboglinid tubeworm *Ridgeia piscesae*

Abstract

A large proportion of the faunal biomass in hydrothermal vent ecosystems relies on symbiotic relationships with bacteria as a source of nutrition. Multiple symbionts within single faunal hosts have been described in many vent and cold seep invertebrate families, but it has been thought that the siboglinid tubeworms harbour a single symbiont phylotype. Two previous studies suggested the presence of more than one symbiont within the trophosome of the siboglinid tubeworm *Ridgeia piscesae*, and this study aimed to confirm these findings and explore the diversity of trophosome bacteria using Sanger sequencing, pyrosequencing and catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH). In addition, we compared symbiotic assemblages between tubeworms from different sites as well as from High Flow and Low Flow vent habitats, contrasted by discharge intensity, temperature and the availability of reducing sulphur compounds. Results from both sequencing methods showed that *R. piscesae*'s symbiotic assemblages are dominated by *Gammaproteobacteria*, and more specifically by one OTU previously identified as *R. piscesae*'s sulphur-oxidizing endosymbiont, followed by *Epsilonproteobacteria*. While only *Proteobacteria* were detected with Sanger sequencing, many other phyla were revealed by pyrosequencing, among which *Bacteroidetes* were relatively abundant. Statistical analysis indicated that membership and structure of the libraries were significantly different between sampling sites, while only membership differed between High Flow and Low Flow habitats. CARD-FISH

confirmed the presence of *Gammaproteobacteria* and *Epsilonproteobacteria* in trophosome tissue.

Introduction

Hydrothermal vent environments host highly productive faunal communities relying on primary production by chemosynthetic microorganisms (Corliss et al., 1979; Jannasch and Wirsen, 1979; Karl et al., 1980). While free-living microbial communities represent an important source of organic carbon for suspension- and deposit-feeders, the bulk of the faunal biomass at most vent sites is supported by associations with symbiotic chemolithoautotrophic bacteria (Cavanaugh, 1994). At Eastern Pacific vents, symbioses are dominated by large populations of siboglinid tubeworms. These gutless polychaetes host symbionts in an organ known as the trophosome (Cavanaugh et al., 1981; Felbeck, 1981). Most studies have detected a single, specific endosymbiont that is common to this group of worms (Edwards and Nelson, 1991; Feldman et al., 1997). In contrast, other symbiont-bearing invertebrates known from deep-sea reducing habitats (vents, cold seeps and whale and wood falls), such as mytilid mussels (Distel, 1995; Fiala-Medioni et al., 2002), alvinocarid shrimp (Petersen et al., 2010) and provannid snails (Suzuki et al., 2005; Urakawa et al., 2005), host phylogenetically and metabolically diverse chemosynthetic partners. Investigation of the phylogenetic position of siboglinid symbionts has revealed two clusters corresponding to either cold seep or vent hosted organisms (Di Meo et al., 2000), between which small subunit (SSU) ribosomal RNA (rRNA) gene sequence divergence was 4.3% on average (Vrijenhoek, 2010).

One exception to the siboglinid single symbiont trend appears to be the northeast Pacific tubeworm *Ridgeia piscesae*, for which there is some evidence of a more diverse trophosomal symbiotic assemblage. *R. piscesae* is found in variable hydrothermal conditions and exhibits a remarkable phenotypic plasticity, ranging from the "short-fat" to the "long-skinny" morphotypes. Short-fat tubeworms typically colonize diffuse High Flow environments in the vicinity of black smokers and are exposed to hydrogen sulphide concentrations of 40 μM or more and temperatures up to 41.9°C (Sarrazin et al., 2002), whereas the "long-skinny" morphotype is found in Low Flow environments, where dissolved hydrogen sulphide is often undetectable and temperatures are close to those of ambient seawater, around 2° C (Urcuyo et al., 2003). Ultrastructural studies of *R. piscesae* trophosomes suggested that similar symbionts are found across worm morphotypes, but that two morphologically distinct bacteria could occur within a single host (de Burgh et al., 1989). Using terminal-restriction fragment length polymorphism (t-RFLP), Chao et al. (2007) provided further evidence of multiple symbioses within *R. piscesae*, detecting the expected gammaproteobacterial phylotype plus two novel phylotypes from the same class, together with one *Alphaproteobacteria*, and one *Bacteroidetes*.

The goal of the present study was to confirm these preliminary findings and to explore the diversity of the bacteria within *R. piscesae*'s trophosome collected from different sampling sites and in contrasting habitats, referred hereafter as High Flow and Low Flow habitats combining multiple approaches. SSU rRNA gene Sanger sequencing allowed a first screening of trophosome bacterial diversity and detailed characterization of the phylogenetic affiliation of detected operational taxonomic units (OTUs). 454 pyrosequencing, performed on a total of 40 individuals, permitted a deeper probing of potential symbiont phylotypes. This technique has not previously been used for screening endosymbiont diversity in vestimentiferans and is therefore considered exploratory. The number of replicates allowed the comparison of trophosome assemblages between sampling sites and as well as between High Flow and Low Flow habitats. Finally, catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) confirmed the presence of at least two bacterial phylotypes within the trophosome of individual tubeworms. In order to control for possible contamination of trophosome tissue by bacteria from external tubeworm surfaces during experimental manipulations, we compared pyrosequencing data from free-living bacteria from the same habitats with results from trophosome tissue.

Methods

Sample collection

Samples of *R. piscesae* were collected during three separate research expeditions in July 2008, 2010, and 2011 onboard the R/V Atlantis, using the submersible *Alvin* (2008 and 2010) and onboard the R/V Thomas G. Thompson, using the remotely-operated vehicle ROPOS (2011). Grabs of the "short-fat" and "long-skinny" morphotypes of the tubeworm were collected in High Flow and Low Flow habitats, respectively (Fig. 4.1), from Axial Volcano, Mothra, Clam Bed and Main Endeavour vent fields on the Juan de Fuca Ridge (Table 4.1). Samples were transported to the surface in sealed, separated bioboxes to prevent contamination between samples and from ambient seawater. Once on board, samples intended for Sanger sequencing were packed individually and immediately frozen at -80°C. Samples collected for pyrosequencing were pre-processed in a 5˚C cold room: the bodies of the worms were carefully removed from their tubes and

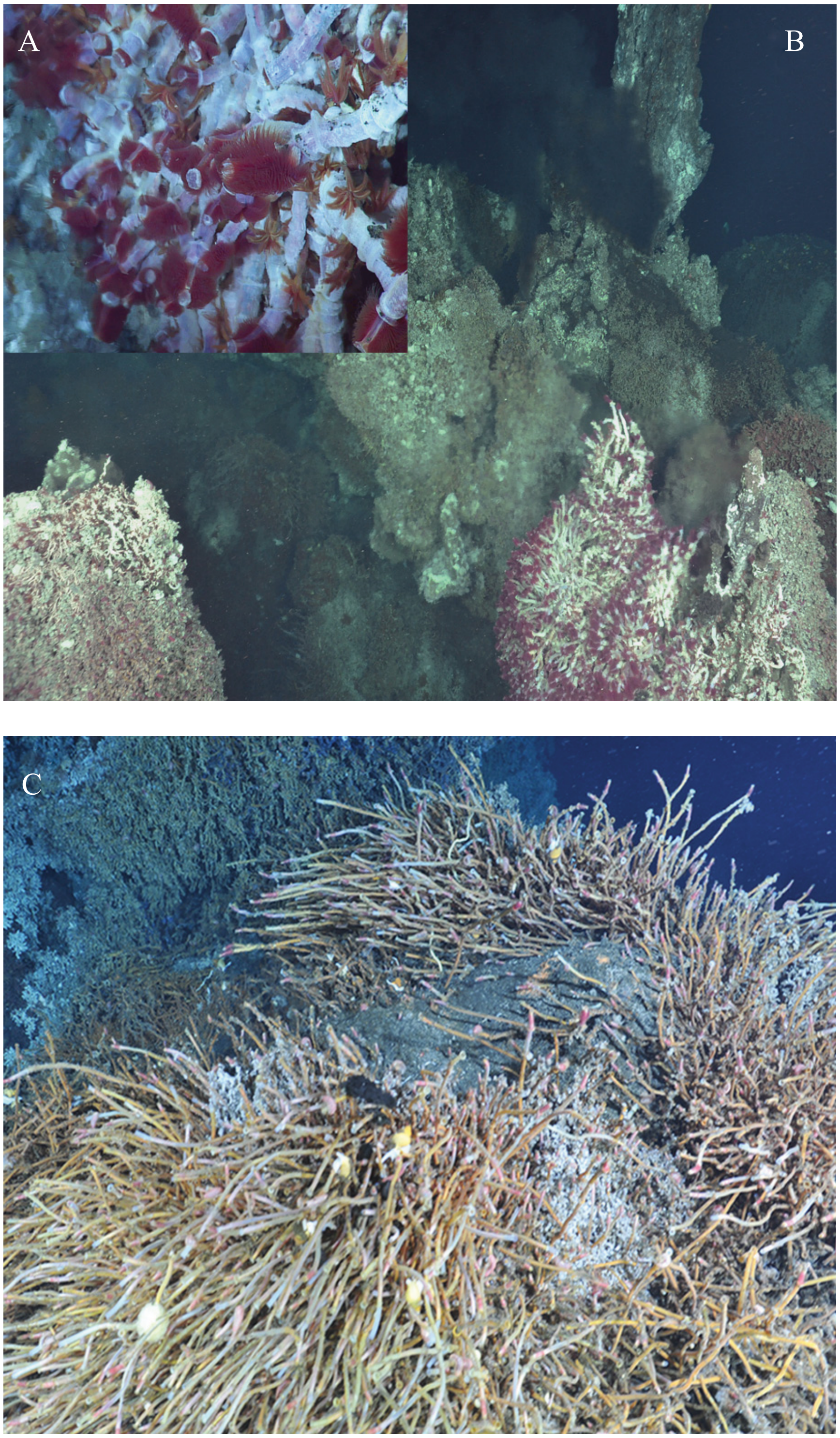


Figure 4.1

Examples of typical sampling sites. (A) Aggregation of the “short-fat” morphotype of *R. piscesae.* (B) Zoom out showing a High Flow environment with a black smoker in the surrounding area. (C) Low Flow environment inhabited by the ‘”long-skinny” morphotype of *R. piscesae*. Here, no shimmering is visible.

Table 4.1 Description and location of sampling sites.

Sampling Site ID	Tubeworm Morphotype	Habitat Type[b]	Vent Site	Latitude	Longitude	Depth (m)	Max. temp (°C) at plume	Collection Date	Analysis Technique(s)	Nb. of ind. analyzed
HF8Mb	Short-fat	HF	Mothra	47° 55.432' N	129° 6.533' W	2271	21.0	July-08	Sanger sequencing	3
LF8Mb	Long-Skinny	LF	Mothra	47° 55.435' N	129°6.520' W	2274	20.0	July-08	Sanger sequencing	1
LF8CMb	Long-Skinny	LF	Clam Bed	47° 57.792' N	129° 5.479' W	2189	13.0	July-08	Sanger sequencing	2
LF10AV1b	Long-Skinny	LF	Axial Volcano (Hollywood Flats 1)	45° 56.147' N	129° 58.888' W	1518	na	July-10	Pyrosequencing	5
HF10AV2b	Short-fat	HF	Axial Volcano (Hollywood Flats 2)	45° 56.155' N	129° 58.893' W	1517	4.1	July-10	Pyrosequencing & CARD-FISH	5 & 3[c]
LF10AV2b	Long-Skinny	LF	Axial Volcano (Hollywood Flats 2)	45° 56.156' N	129° 58.890' W	1517	2.0	July-10	Pyrosequencing & CARD-FISH	5 & 3[c]
LF10TPb	Long-Skinny	LF	Main Endeavour (TP)	47° 56.971' N	129° 5.854' W	2197	2.4	July-10	Pyrosequencing	5
HF10HUb	Short-fat	HF	Main Endeavour (Hulk)	47° 57.007' N	129° 5.824' W	2190	14.0	July-10	Pyrosequencing & CARD-FISH	5 & 3[c]
LF10HUb	Long-Skinny	LF	Main Endeavour (Hulk)	47° 57.007' N	129° 5.825' W	2191	2.5	July-10	Pyrosequencing & CARD-FISH	5 & 3[c]
HF11GRb	Short-fat	HF	Main Endeavour (Grotto)	47° 56.953' N	129° 5.903' W	2188	21.6	July-11	Pyrosequencing	5
HF11LBb	Short-fat	HF	Main Endeavour (Lobo)	47° 56.965' N	129° 5.900' W	2191	12.2	July-11	Pyrosequencing	5

[a] The letter(s) at the beginning of the number indicate(s) the submersible used for the sampling ("AD" = "Alvin Dive"; "R" = "ROPOS").

[b] HF = High Flow; LF = Low Flow

[c] For pyrosequencing and CARD-FISH, respectively.

cleaned with 70% ethanol, individually packed and frozen at -80°C. For CARD-FISH, subsamples of tubeworm trophosome were fixed as described by Dubilier et al. (1995).

SSU rRNA gene clone library construction

In the laboratory, a total of six tubeworms, three from High Flow and three from Low Flow habitats, were used for Sanger sequencing analysis. The individuals were dissected and trophosomes were aseptically removed (Table 4.1). DNA was extracted from approximately 25 mg of tissue using the DNeasy Blood and Tissue Kit (Qiagen, Carlsbad, CA, USA), following the manufacturer's instructions. SSU rRNA gene was amplified by PCR using approximately 100 ng/µl of gDNA with the universal bacterial primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GTT TAC CTT GTT ACG ACT T-3') (Turner et al., 1999). $MgCl_2$ concentrations and annealing temperatures were optimized in order to get sufficient yield in the target size range with the most stringent combinations. The final reaction mixture had a final volume of 20 µl containing 1 µl template DNA, 1.875 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, 0.25 µM (each) primer, 1X PCR buffer (Invitrogen, Valencia, CA, USA), 1 U of *Taq* DNA polymerase (Invitrogen), and sterile MiliQ H_2O to up to 20 µl. A first initialization step of 2 min at 94°C was followed by 27-30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 2 min. A final extension step was performed at 72°C for 10 min. The number of cycles was optimized for each sample in order to get a product concentration still in the exponential phase, as visualized on 1.2% (w/v) agarose gels stained with SYBR Safe (Invitrogen). Products of four parallel PCRs per sample were combined, purified with the Qiagen MinElute PCR purification kit (Qiagen) and cloned with Invitrogen's TOPO-TA cloning kit (Invitrogen).

White colonies were randomly chosen and screened for inserts by PCR reaction using the vector primers M13F and M13R as described in Forget et al. (2010). Sequencing of the inserts was completed at the High Throughput Genomics Centre (Seattle, WA). A first screening of bacterial diversity was performed using the PCR primer 8F. The resulting partial SSU rRNA gene sequences were used to construct a distance matrix with the PHYLIP software package, v.3.69 (Felsenstein, 2005). Sequences displaying ≥ 97% similarity were grouped as operational taxonomic units (OTUs) using the DOTUR software average neighbor algorithm (Schloss and Handelsman, 2005). One representative of each OTU detected was near-completely sequenced using the PCR primers 1492R, 515F (5’-GTG CCA AGC MGC CGC GGT AA-3’) (Turner et al., 1999) and 907R (5’-CCG TCA ATT CMT TTR AGT TT-3’) (Lane, 1991). This combination of primers allowed a complete overlap of the near-complete SSU rRNA gene sequence.

Phylogenetic analysis

Obtained sequences were inspected, assembled, screened for chimeras, and aligned as described in Forget and Juniper (2013). The software program mothur (v.1.30.0) (Schloss et al., 2009) was used to assign phylogenetic affiliation with the SILVA template database and 1000 bootstrap iterations. Closely related sequences were obtained using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) and combined with our sequences, aligned with alignment gap characters conserved using the Greengenes Align tool (DeSantis et al., 2006), imported and edited using BioEdit v7.1.3 (Hall, 1999). A neighbour-joining tree was constructed using PHYLIP with 1000 bootstrap replicates.

454 pyrosequence library construction

DNA was extracted as described above from a total of 40 tubeworm trophosomes: 20 from High Flow and 20 from Low Flow habitats (Table 4.1). DNA was purified and concentrated using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, and 20 µl of DNA with a concentration of 20 ng/µl or higher was sent to the *Plateforme d'Analyses Génomiques* (Institute of Integrative and Systems Biology, Laval University, Quebec City, QC, Canada). The hypervariable region V1-V3 of the bacterial SSU rRNA gene was amplified by PCR using Takara Ex *Taq* premix (Fisher Scientific, Toronto, ON, Canada). PCR reactions were performed in a final volume of 50 µl containing 25 µl of Premix *Taq*, 1 µM of each primer and sterile MiliQ H_2O to up to 50 µl (a list of the primers is available in Appendix D). PCR conditions were as follows: after a denaturing step of 30 s at 98°C, samples were processed through 30 cycles consisting of 10 s at 98°C, 30 s at 55°C and 30 s at 72°C. A final extension step was performed at 72°C for 4 min 30 s. Following amplification, samples were purified using magnetic AMPure Beads (Beckman Coulter Genomics) to recover PCR amplicons, separating them from contaminants. Samples were adjusted to 100 µl with EB buffer (Qiagen), to which 63 µl of beads were added. Samples were mixed and incubated for 5 min at room temperature (RT.) Using a Magnetic Particle Concentrator (MPC), the beads were pelleted against the wall of the tube and supernatant was removed. The beads were washed twice with 500 µl of 70% ethanol and incubated for 30 s each time. Supernatant were removed and beads were allowed to air dry for 5 min. Tubes were removed from the MPC and 15 µl of EB buffer were added. Samples were vortexed to resuspend the beads. Finally, using the MPC, the beads were pelleted against the wall once more and supernatant were transferred to a new clean tube. DNA concentrations in sample were quantified by Nanodrop and mixed in equal amounts. Pyrosequencing was performed using a 454 GS-FLX DNA Sequencer with the Titanium Chemistry (Roche) according to the procedure described by the manufacturer.

Pyrosequencing read preprocessing

All data processing and analyses were performed using the software program mothur. Raw pyrosequences were checked for different quality criteria. Reads with an average quality score below 27 (Kunin et al., 2010), containing an error in the forward primer sequence at the beginning of the read (Sogin et al., 2006), containing one or more ambiguous bases (Ns) (Sogin et al., 2006; Huse et al., 2007), or shorter than 250 base pairs (De Leon et al., 2012), were eliminated. A set of unique reads was created and aligned against the SILVA-based bacterial reference alignment (Pruesse, 2007) provided by mothur using the Needleman-Wunsch pairwise alignment algorithm (Needleman and Wunsch, 1970). A pre-clustering step was applied to group sequences differing by less than 2% (corresponding to 5 mismatches for a 250-base-pair sequence) (Huse et al., 2010). Potential chimeras were identified using the program UCHIME (Edgar et al., 2011) and removed from the data set. Because there was a large range between the minimum and maximum number of reads found in our samples, the three samples with the lowest numbers of reads (one sample from HF10AV2 with 809 reads, one sample from HF10HUb with 2269 reads, and one sample from LF10AV1b with 1568 reads) were eliminated from further analyses. Then, the number of reads across samples was standardized by subsampling based on the lowest number of sequences (3551) found in any of the remaining 37 samples. The remaining sequences were classified using the SILVA template database with 1000 bootstrap iterations.

Statistical analysis

A distance matrix was generated based on uncorrected pairwise distances between the sequences, following which OTUs were assigned using the average neighbor algorithm at 4% distance level, as suggested by Kim et al. (2011) for bacterial analysis of the V1-V3 region of the SSU rRNA gene. Singleton OTUs (*i.e.* OTUs represented by a single sequence) were excluded from subsequent analyses in order to eliminate artefacts, as recommended by Tedersoo et al. and Zhou et al. (2010; 2011). Furthermore, since OTU inflation is a function of the sampling effort, spurious OTUs can represent a high proportion of the total number of OTUs detected in low diversity samples such as symbiotic assemblages (Huse et al., 2010). Analyses were performed on a shared file, which described the number of times each OTU was observed in all samples. First, the coverage, Chao1 and ACE richness indices, Shannon-Wiener diversity index, and Shannon's evenness index (SHEI) were calculated for each sample. Then, both membership and structure (*i.e.* the relative abundance of the members) of the trophosome assemblages were evaluated and compared between sampling sites and between High Flow and Low Flow habitats. Dendrograms illustrating the distance between the samples based on the Jaccard and the Yue and Clayton indices (Yue and Clayton, 2005), describing the membership and the structure of the trophosome assemblages, respectively, were generated using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The significance of the clustering was evaluated with a parsimony test. Distance matrices were calculated based on the shared file using the same similarity indices. Based on these matrices, analyses of molecular variance (AMOVAs) tested whether the variation in the membership and structure of the trophosome assemblages within each pair of sampling sites was significantly different from that of both sites pooled together. The same analyses were performed to compare High Flow and Low Flow habitats. Analyses of homogeneity of molecular variance (HOMOVAs) tests compared variability within each sampling site as well as within each habitat. When the tests were statistically significant, distance matrices were visualized using a nonmetric multidimensional scaling (NMDS) algorithm. Finally, the command “metastats” (White et al., 2009) indicated which OTUs were differentially represented between sampling sites and between habitats.

Comparison of OTUs revealed by Sanger sequencing and 454 pyrosequencing

Using mothur, the OTUs detected by Sanger sequencing as well as the sequences published by Chao et al. (2007) were aligned to our pyrosequence reads and cut to overlap the same region of the SSU rRNA gene. A distance matrix was generated and sequences were clustered at the 4% distance level. We used a shared file in order to verify if the OTUs obtained by Sanger sequencing corresponded to any pyrosequencing OTUs.

Comparison with 454 pyrosequencing data from free-living bacterial communities

Using mother, a shared file describing the number of times each OTU obtained from pyrosequencing was observed in our libraries and in free-living bacterial community libraries was created. Data from free-living communities were obtained from eight samples collected in High Flow and Low Flow habitats in the Clam Bed and Main Endeavour vent fields, Juan de Fuca Ridge (Forget and Juniper, 2013).

Nucleotide sequence accession numbers

The near-complete SSU rRNA gene sequences representing unique OTUs were submitted to the GenBank database and assigned accession numbers JX570594-JX570609. The pyrosequence reads were deposited in NCBI's Sequence Read Archive (SRA) under the accession number SRA058565.

Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH)

Fixed tissues were embedded in paraffin and sectioned (5 μm thickness) onto glass slides. The pre-hybridization treatments were performed as previously described (Dubilier et al., 1995) with the following modifications: sections were deparaffinised in CitriSolv (Fisher Scientific), a less toxic alternative to xylene, and the post-fixation step in 3.7% formaldehyde was omitted. CARD-FISH was carried out as described by Blazejak et al. (2005) with the following horseradish peroxidase (HRP)-labelled oligonucleotide probes: EPSY549, specific to the *Epsilonproteobacteria*, GAM42a with an unlabelled competitor BET42a, covering most *Gammaproteobacteria*, EUB338, targeting the domain *Bacteria* as a positive control, and NON338, a complementary negative control (Table 4.2). Sequences for these general probes were obtained through the probeBase website (Loy et al., 2007). For each probe, formamide concentration was optimized using a range of different concentrations in order to get the best signal with the highest formamide concentration (most stringent conditions possible) (Table 4.2). The fluorescently labelled tyramides were prepared as described by Pernthaler et al. (2004) with the Alexa Fluor 488, 555 or 546 dye (Molecular Probes - Invitrogen). A few sections were hybridized without a probe to control for background fluorescence. For multiple hybridizations, the CARD-FISH protocol was repeated with the same sections

Table 4.2 Oligonucleotide probe description.

Probe	Specificity	Sequence (5’-3’)	Position[a]	[Formamide] (%, v/v)[c]	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338-355	50-**55**-60	Amann et al. 1990
EPSY549	*Epsilonproteobacteria*	CAGTGATTCCGAGTAACG	549-566	50-**55**-60	Lin et al. 2006
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	1027-1043[b]	50-**55**-60	Manz et al. 1992
BET42a[d]	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	1027-1043[b]	25-30-**35**-40	Manz et al. 1992
NON338	Negative control	ACTCCTACGGGAGGCAGC	338-355	25-**30**-35-40	Widdel and Bak 1992; Wallner et al. 1993

[a] Position in the 16S rRNA gene of *Escherichia coli* unless indicated otherwise.

[b] Position in the 23SrRNA gene of *E. coli*.

[c] In hybridization buffer. Numbers in bold indicate the concentration used in this study.

[d] Non labeled probe

with different probes and dyes as described in Blazejak et al. (2005). Slides were imaged under epifluorescence and confocal illumination, using a Nikon C1 Plus confocal microscope (Nikon, Mississauga, ON, Canada).

Results

Sanger sequence library

A total of six clone libraries were constructed from the trophosome of three "short-fat" and three "long-skinny" tubeworms collected from High Flow and Low Flow habitats, respectively. The number of clones varied from 77-80 in High Flow samples, and from 15-34 in Low Flow samples, for a total of 303 sequenced clones. Even after vector-to-insert ratio and ligation step duration were optimized, Low Flow samples had a lower yield of transformed cells (with plasmid containing an insert). The clones represented 3-7 OTUs per library (Table 4.3), for a total of 16 different OTUs at the 3% distance level. All OTUs belonged to the phylum *Proteobacteria*. The six clone libraries were dominated by *Gammaproteobacteria*, which represented > 93% of the High Flow clone libraries and between 66.7-86.7% of the Low Flow clone libraries (Fig. 4.2). Members of the *Epsilonproteobacteria* were also detected in all clone libraries, representing 2.5-6.3% of the High Flow samples, and 11.8-27.8% of the Low Flow samples. *Deltaproteobacteria* were found in three clone libraries from both habitat types, and members of the *Alpha*- and *Betaproteobacteria* were detected in one clone library from a Low Flow sample (Fig. 4.2).

The most common OTU detected, SNG (for Sanger sequencing) OTU 1, belonged to the *Gammaproteobacteria* and had 99 to 100% sequence similarity with previously published sequences isolated from trophosomes of *Riftia pachyptila* or *R. piscesae* (Chao

Table 4.3 Affiliation and occurrence of each Sanger OTU detected within the six clone libraries (all members of *Proteobacteria*).

SNG OTU ID	Nb of clones	Affiliation[a]	HF8Mb1	HF8Mb2	HF8Mb3	LF8Mb1	LF8CMb1	LF8CMb2	Pyrosequence match
1	270	*Gammaproteobacteria - Sedimenticola*	+	+	+	+	+	+	PYRO OTU 1
2	1	*Gammaproteobacteria*	-	-	-	-	+	-	none found
3	1	*Gammaproteobacteria - Oceanospirillales*	-	-	-	-	+	-	PYRO OTU 13
4	6	*Epsilonproteobacteria - Campylobacterales*	-	-	-	+	+	+	rare OTU
5	4	*Epsilonproteobacteria - Arcobacter*	+	+	-	-	+	-	PYRO OTU 11
6	3	*Epsilonproteobacteria - Arcobacter*	-	-	+	+	-	-	rare OTU
7	4	*Epsilonproteobacteria - Sulfurovum*	+	-	-	-	+	+	PYRO OTU 12
8	1	*Epsilonproteobacteria - Arcobacter*	-	+	-	-	-	-	rare OTU
9	1	*Epsilonproteobacteria - Sulfurovum*	+	-	-	-	-	-	PYRO OTU 10
10	1	*Epsilonproteobacteria - Sulfurovum*	-	+	-	-	-	-	PYRO OTU 14
11	1	*Epsilonproteobacteria - Sulfurovum*	+	-	-	-	-	-	rare OTU
12	1	*Epsilonproteobacteria - Sulfurovum*	-	+	-	-	-	-	rare OTU
13	5	*Deltaproteobacteria*	-	-	+	-	+	+	PYRO OTU 5
14	2	*Alphaproteobacteria*	-	-	-	-	-	+	rare OTU
15	1	*Alphaproteobacteria - Phyllobacteriaceae*	-	-	-	-	-	+	rare OTU
16	1	*Betaproteobacteria - Ralstonia*	-	-	-	-	-	+	rare OTU

[a] Proteobacterial class and lowest confidently assigned level

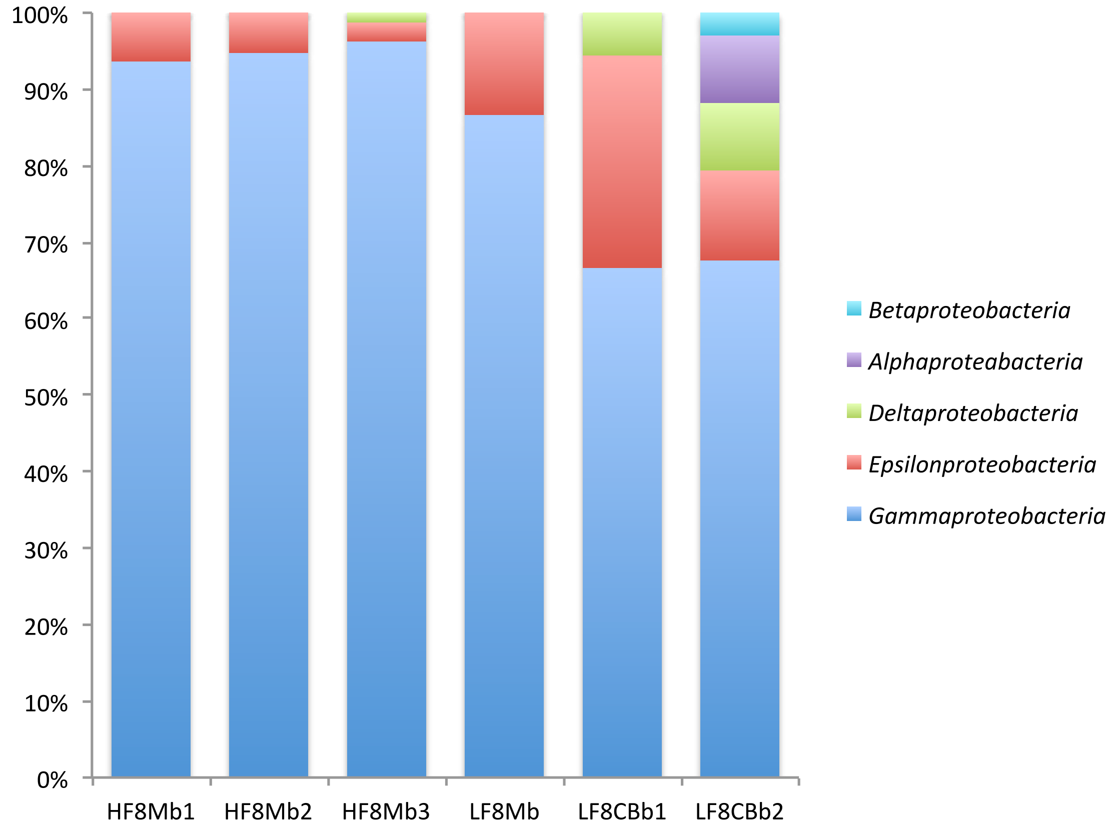


Figure 4.2

Relative abundance of the classes detected with Sanger sequencing for individual tubworms. The three first bars represent the High Flow samples, and the three last bars, the Low Flow samples.

et al., 2007; Vrijenhoek et al., 2007). This OTU, found in all clone libraries, was affiliated to the genus *Sedimenticola* (Table 4.3). Two other OTUs, SNG OTUs 2 and 3, were also classified as *Gammaproteobacteria*, but were both singletons, detected only in one Low Flow clone library. SNG OTU 2 had 98% sequence similarity to an epibiont of the galatheid crab *Shinkaia crosnieri* (Watsuji et al., 2010), while SNG OTU 3 was closely related to a clone isolated from free-living bacterial communities associated with *R. piscesae* (Forget and Juniper, 2013). Neither could be classified to the genus level (Table 4.3).

The most diverse class was the *Epsilonproteobacteria*, in which 9 different OTUs were detected. SNG OTU 4, a member of the order *Campylobacterales* detected in all Low Flow libraries (Table 4.3), was the second most abundant OTU and was closely related to an endosymbiont of the gastropod *Pectinodonta* sp. found on sunken wood (Zbinden et al., 2010). SNG OTU 5, found in both High Flow and Low Flow samples (Table 4.3), was a member of the genus *Arcobacter* closely related to a free-living bacterium associated with *R. piscesae* (Forget and Juniper, 2013). The closest GenBank database matches for SNG OTU 6, also affiliated to the genus *Arcobacter*, and SNG OTU 7, member of the genus *Sulfurovum* were, respectively, an epibiont of the tubeworm *R. pachyptila* (López-García et al., 2002) and a free-living bacterium isolated from hydrothermal fluids (Bourbonnais et al., 2012). Both OTUs were found in at least one High Flow and Low Flow library (Table 4.3; Fig. 4.3). Several OTUs occurred in only one of two High Flow libraries (HF8Mb1 or HF8Mb2; Table 4.3). These include SNG OTU 8, another member of the genus *Arcobacter,* closely related to a free-living bacterium associated with *R. piscesae* (Forget and Juniper, 2013); and SNG OTUs 9 through 12, classified as *Sulfurovum* species (Table 4.3). These latter OTUs were closely related to free-living bacteria isolated from hydrothermal fluids (Bourbonnais et al., 2012; Sylvan et al., 2012b) and associated with *R. piscesae* (Forget and Juniper, 2013).

The third most abundant OTU of the clone libraries, SNG OTU 13, belonged to the *Deltaproteobacteria* and was detected in both High Flow and Low Flow samples (Table 4.3). This OTU could not be classified to the genus level, but was closely related to a sequence isolated from seafloor lavas from Loi'hi Seamount (Santelli et al., 2008). SNG OTU 14 and 15, two members of the *Alphaproteobacteria* closely related to a bacterium

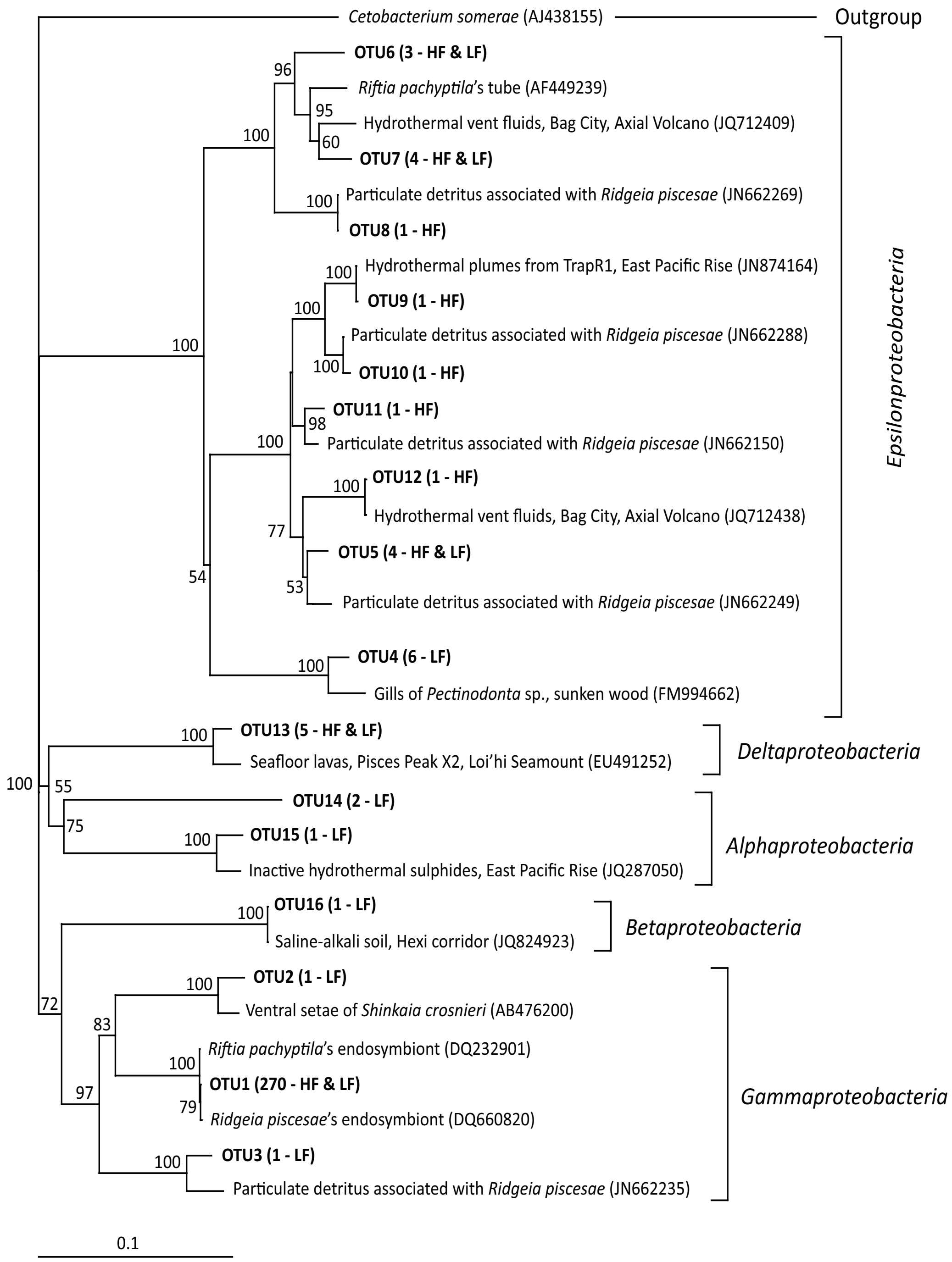


Figure 4.3

Neighbor-joining tree built with the representative sequences of each OTUs detected by Sanger sequencing along with their closest relative in the GenBank database. In parentheses, following the OTU's name, are indicated the number of clones represented by the OTU and the habitat in which these clones were detected (HF=High Flow habitat; LF=Low Flow habitat). Bootstrap values for nodes with > 50% support, based on 1000 replicates, are displayed as percentages. Scale bar represents the estimated sequence divergence.

isolated from inactive hydrothermal sulphides (Sylvan et al., 2012a) and SNG OTU 16, a *Betaproteobacteria* closely related to a bacterium isolated saline-alkali soil (unpublished), were only found in only one Low Flow clone library (Fig. 4.3; Table 4.3). SNG OTU 15 was affiliated to the family *Phyllobacteriaceae* and SNG OTU 16 to the genus *Ralstonia* (Table 4.3).

454 pyrosequence library

For the 40 tubeworm trophosomes sampled, a total of 645 009 reads were obtained through pyrosequencing. After quality filtering and removing the three less abundant samples, 508 399 high-quality pyrosequences remained, representing 2489 unique sequences and 1087 OTUs at the 96% similarity level. Removing singletons eliminated 462 sequences, leaving 625 OTUs. This number dropped to 499 after subsampling 3551 sequences per sample (for a total of 131 387 remaining reads), which was the lowest number of sequences found in our 37 libraries. Subsequent analyses were performed on this final number of reads. On average, each sample had 40 ± 29 OTUs, ranging from 2-131 (Table 4.4). The average coverage was 99.6 ± 0.3%. The Chao1 and ACE richness indices estimated the average number of OTUs to 57 ± 42 and 71 ± 51, respectively. The Shannon-Wiener diversity index ranged from 0.03-2.56, with an average of 0.91 ± 0.77, and the SHEI, which scales from 0, indicating an extremely uneven distribution dominated by one type, to 1, indicating a perfectly even distribution, ranged from 0.02-0.53, with an average of 0.23 ± 0.17 per sample (Table 4.4). The evenness of the trophosome assemblages is also illustrated by the rank abundance curves (Fig. 4.4). Most OTUs (95.2%) had a relative abundance under 0.1%, and 71.9% of the OTUs accounted for less than 0.01% of the library (data not shown). The 10 most abundant OTUs detected

Table 4.4 Diversity estimates from the pyrosequencing data for the whole sequence library.

	Average per sample	Standard deviation	Range
Number of OTUs	40	29	2-131
Coverage	99.6%	0.3%	98.8-100%
Chao1	57	42	2-163
ACE	71	51	0-193
Shannon-Wiener	0.91	0.77	0.03-2.56
SHEI	0.23	0.17	0.02-0.53

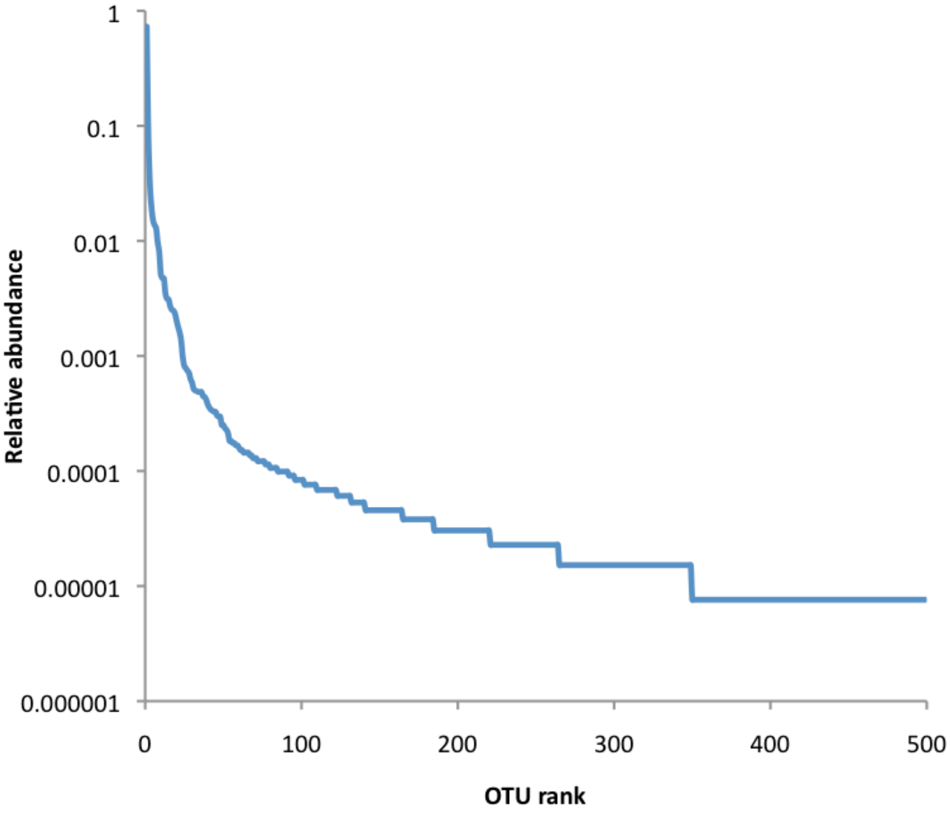


Figure 4.4

Rank abundance curve for pyrosequencing read for the whole sequence library.

through pyrosequencing represented 93.5% of the sequence library, while the 25 most abundant represented 97.3%, and the 50 most abundant, 98.4%. Of the 499 OTUs, 54.5% were found only in one sample, 72.3% were found in ≤ two samples, and 80.1% were found in ≤ three samples. Only 12 OTUs were detected in more than half of the samples, but they accounted for 90.1% of the sequences. Two OTUs were detected in all 37 samples (Table 4.5).

Thirteen different phyla were detected, but only two represented more than 1% of the sequence library: the *Proteobacteria* and *Bacteroidetes*. Within the *Proteobacteria*, which represented 97.0% of the sequences, *Gammaproteobacteria* were clearly the most abundant class, followed by *Epsilonproteobacteria, Deltaproteobacteria*, *Alphaproteobacteria*, and *Betaproteobacteria* (Fig. 4.5). The other phyla detected, accounting for 0.3% of the sequence library, included *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Spirochaetes*, *Chlorobi*, *Acidobacteria*, *Cyanobacteria*, *Lentisphaerae*, and the candidate divisions BD1-5, TM6 and TM7. (For an exhaustive list of the genera detected, see Appendix E, Table E.1).

Table 4.5 describes the phylogenetic affiliation and occurrence of the OTUs representing ≥ 0.1% of the sequence library, which correspond to the 25 most abundant OTUs. The number at the end of the OTU ID reflects its abundance rank. The three most abundant OTUs detected in the sequence library, i.e. PYRO (for pyrosequencing) OTUs 1, 2 and 3, belonged to the gammaproteobacterial genera *Methylomicrobium*, *Halomonas*, and *Shewanella* (Table 4.5). These OTUs represented 72.2, 9.0, and 3.2% of the sequence library, respectively. PYRO OTU 1 was one of the OTUs detected in all samples. Five other OTUs, members of the genera *Methylomicrobium*, *Nitrosococcus*, *Pseudomonas*,

Table 4.5 Affiliation and occurrence of the OTUs representing ≥ 0.1% of the pyrosequence library. The p-value indicates whether each OTU is differentially represented between High Flow (HF) and Low Flow (LF) habitats.

PYRO OTU ID	Affiliation[a]	Total	Number of samples	Average in HF	Average in LF	p-value	Total in free-living communities[c]
1	*Gammaproteobacteria - Methylomicrobium*	72.9%	37	78.3 ± 6.6%	67.9 ± 6.8%	ns[b]	0.0%
2	*Gammaproteobacteria - Halomonas*	9.0%	31	7.9 ± 2.5%	10.0 ± 2.6%	ns	0.0%
3	*Gammaproteobacteria - Shewanella*	3.2%	34	3.2 ± 1.0%	3.2 ± 0.8%	ns	0.0%
4	*Bacteroidetes - Gaetbulibacter*	2.0%	16	< 0.1 ± < 0.1%	3.9 ± 1.9%	0.001	0.0%
5	*Deltaproteobacteria - Desulfuromonadales*	1.5%	27	0.3 ± 0.1%	2.7 ± 1.3%	ns	0.0%
6	*Epsilonproteobacteria - Sulfurovum*	1.4%	10	< 0.1 ± < 0.1%	2.7 ± 2.5%	0.009	1.2%
7	*Epsilonproteobacteria - Arcobacter*	1.3%	24	2.4 ± 1.8%	0.3 ± 0.1%	ns	0.0%
8	*Gammaproteobacteria - Nitrosococcus*	1.0%	8	2.0 ± 1.4%	< 0.1 ± < 0.1%	0.012	0.0%
9	*Gammaproteobacteria - Methylomicrobium*	0.8%	37	0.9 ± 0.1%	0.7 ± 0.1%	ns	0.0%
10	*Epsilonproteobacteria - Sulfurovum*	0.5%	19	0.2 ± 0.1%	0.8 ± 0.4%	ns	4.2%
11	*Epsilonproteobacteria - Arcobacter*	0.5%	21	0.3 ± 0.2%	0.6 ± 0.3%	ns	< 0.1%
12	*Epsilonproteobacteria - Sulfurovum*	0.5%	16	0.9 ± 0.8%	0.1 ± < 0.1%	ns	2.8%
13	*Gammaproteobacteria - Oceanospirillales*	0.3%	15	< 0.1 ± < 0.1%	0.6 ± 0.4%	0.034	< 0.1%
14	*Epsilonproteobacteria - Sulfurovum*	0.3%	13	0.1 ± < 0.1%	0.6 ± 0.4%	ns	6.1%
15	*Alphaproteobacteria - Rickettsiales*	0.3%	18	< 0.1 ± < 0.1%	0.6 ± 0.2%	0.001	< 0.1%
16	*Alphaproteobacteria - Novosphingobium*	0.3%	26	0.2 ± 0.1%	0.3 ± 0.1%	ns	0.0%
17	*Bacteroidetes - Flavobacterium*	0.3%	26	0.2 ± 0.1%	0.3 ± 0.1%	ns	0.0%

18	*Epsilonproteobacteria - Sulfurovum*	0.2%	18	0.5 ± 0.2%	0.1 ± < 0.1%	0.023	2.5%
19	*Gammaproteobacteria - Pseudomonas*	0.2%	27	0.2 ± 0.1%	0.2 ± 0.1%	ns	0.0%
20	*Deltaproteobacteria - Desulfuromonadales*	0.2%	8	0.3 ± 0.3%	0.1 ± 0.1%	ns	0.0%
21	*Epsilonproteobacteria - Sulfurovum*	0.2%	8	< 0.1 ± < 0.1%	0.3 ± 0.3%	ns	2.6%
22	*Gammaproteobacteria - Pseudoalteromonas*	0.2%	4	0.0%	0.3 ± 0.2%	ns	0.0%
23	*Deltaproteobacteria - Desulfobulbus*	0.1%	7	< 0.1 ± < 0.1%	0.3 ± 0.2%	0.030	< 0.1%
24	*Actinobacteria - Propionibacterium*	0.1%	19	0.1 ± < 0.1%	0.1 ± 0.1%	ns	< 0.1%
25	*Bacteroidetes - Polaribacter*	0.1%	7	< 0.1 ± < 0.1%	0.2 ± 0.1%	ns	0.0%

[a] Phylum (or proteobacterial class) and lowest confidently assigned level.

[b] ns = not significant

[c] From Forget et al, 2013

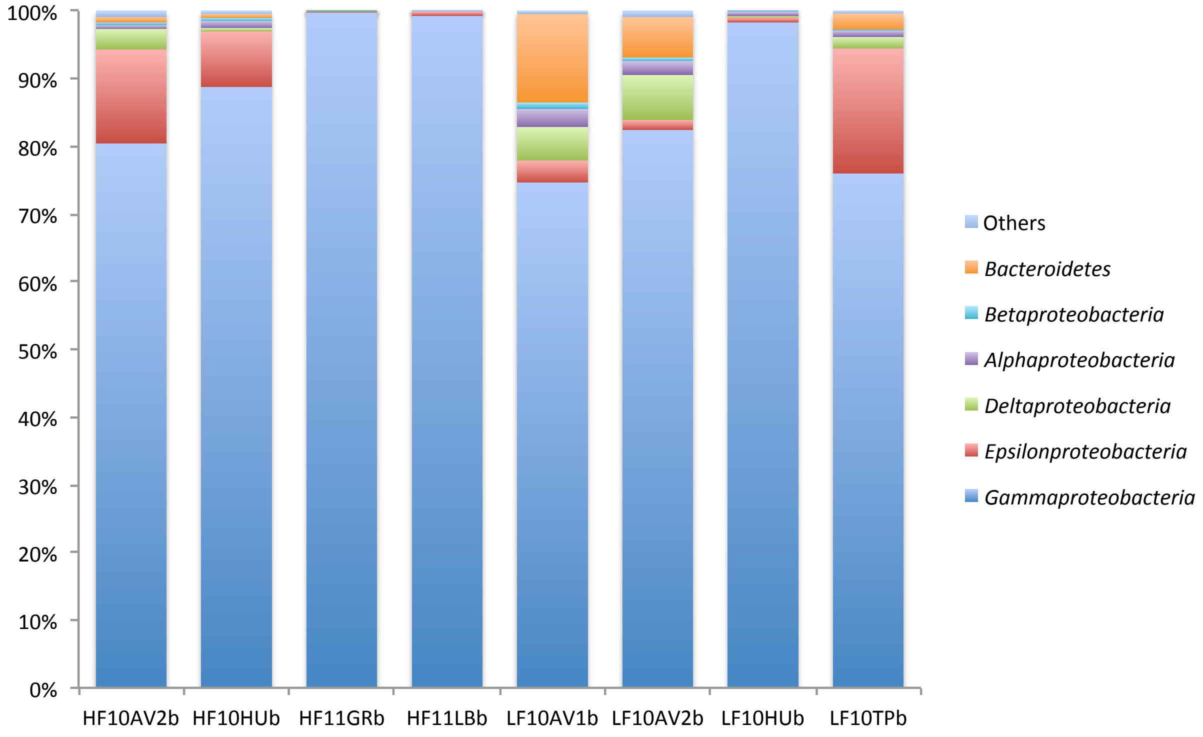


Figure 4.5

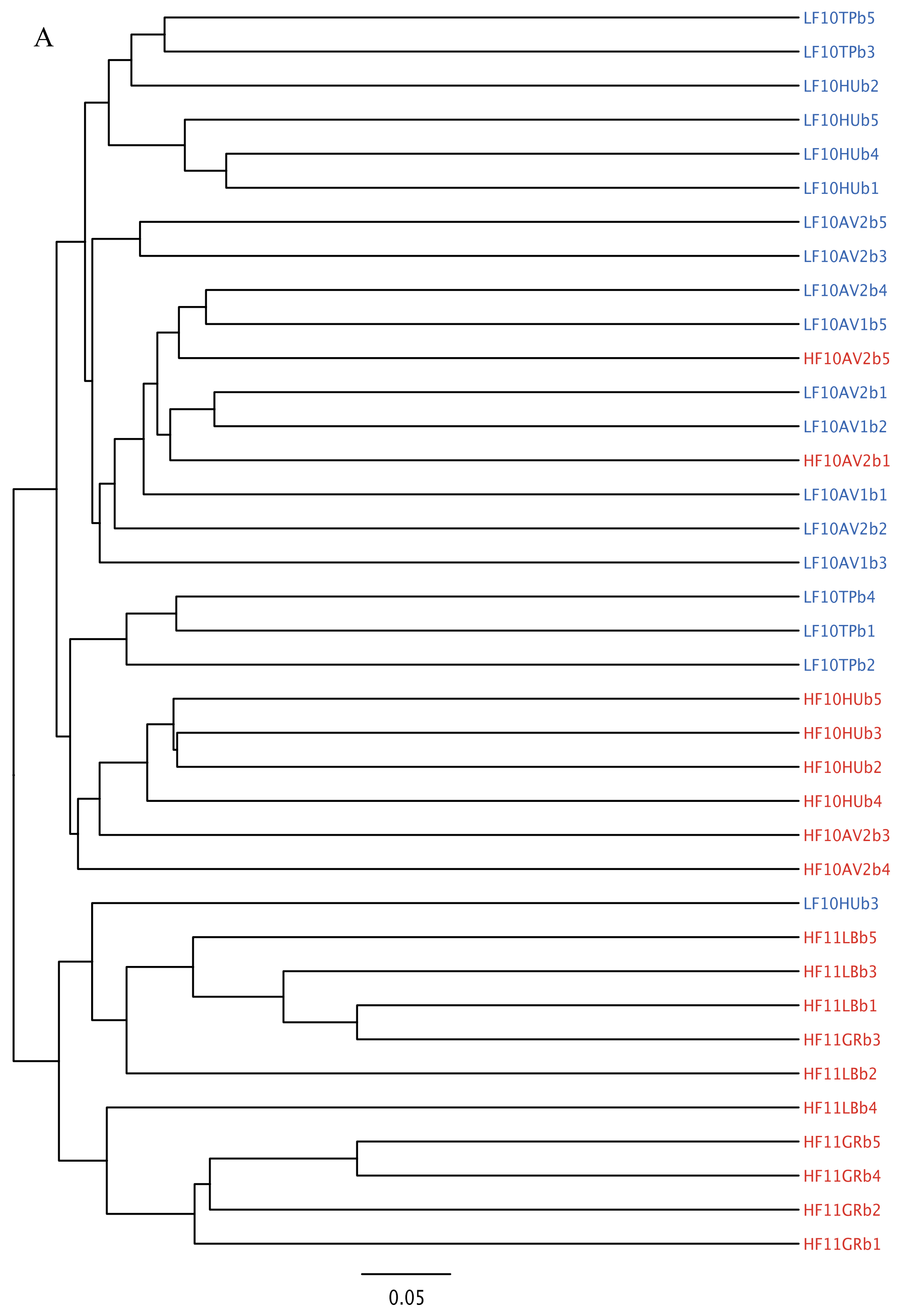
Relative abundance of the phyla detected by 454 pyrosequencing for each sampling location. The four first bars represent High Flow sampling locations, and the four last bars, Low Flow sampling locations. Because *Proteobacteria* dominated the pyrosequence library, the phylum is divided in the five classes detected. The category "Others" includes the phyla that represented < 1% of the pyrosequence library.

Pseudoalteromonas, and an unclassified member of the order *Oceanospirillales*, belonged to the *Gammaproteobacteria*. One of these OTUs, PYRO OTU 5, was the only other OTU detected in all samples (Table 4.5). PYRO OTU 4, a *Bacteroidetes* from the genus *Gaetbulibacter*, accounted for 2.0% of the library. PYRO OTU 17 and 25 were also members of the phylum *Bacteroidetes*, affiliated to the genera *Flavobacterium* and *Polaribacter*, respectively. Two OTUs, PYRO OTU 5 and 20, were members of the deltaproteobacterial order *Desulfuromonadales*, and one of these belonged to the genus *Desulfobulbus*. A total of eight OTUs were members of two genera from the

Epsilonproteobacteria: *Sulfurovum* and *Arcobacter*. Two OTUs from the *Alphaproteobacteria* were also detected: PYRO OTU 15 was a member of the order *Rickettsiales* and PYRO OTU 16, a member of the genus *Novosphingobium*. Finally, one *Actinobacteria* from the genus *Polaribacter* was also part of the 25 most abundant OTUs detected in our pyrosequence library (Table 4.5).

Comparison between sampling sites and High Flow and Low Flow habitats

Since 454 pyrosequencing data analyzed in this study included 37 samples and yielded considerably more information than Sanger sequencing data, which were limited to only six analyzed individuals and a low number of sequences, comparisons between sampling sites and habitats were based on the former method. The number of OTUs, richness, and diversity indices for two High Flow sampling sites, HF111GRb and HF11LBb, were significantly lower than for most other sites, but overall these metrics did not differ significantly between High Flow and Low Flow samples (Appendix E, Table E.2). The dendrogram illustrating the similarity of the samples based on membership of their trophosome assemblages suggested that samples collected from one site or one habitat were more similar to each other than to samples collected from different sites and habitats (Fig. 4.6A). This was confirmed by the parsimony test, which indicated that the clustering of the samples by sampling sites and habitats was significant ($p < 0.001$). Pairwise comparisons were also performed to explore which sampling sites had significantly different trophosome assemblage memberships (Appendix E, Table E.3). The false discovery rate, *i.e.* the proportion of null hypotheses incorrectly rejected, was controlled by applying the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). The clustering of the samples by sampling site in the dendrogram based on the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HF10AV2b5
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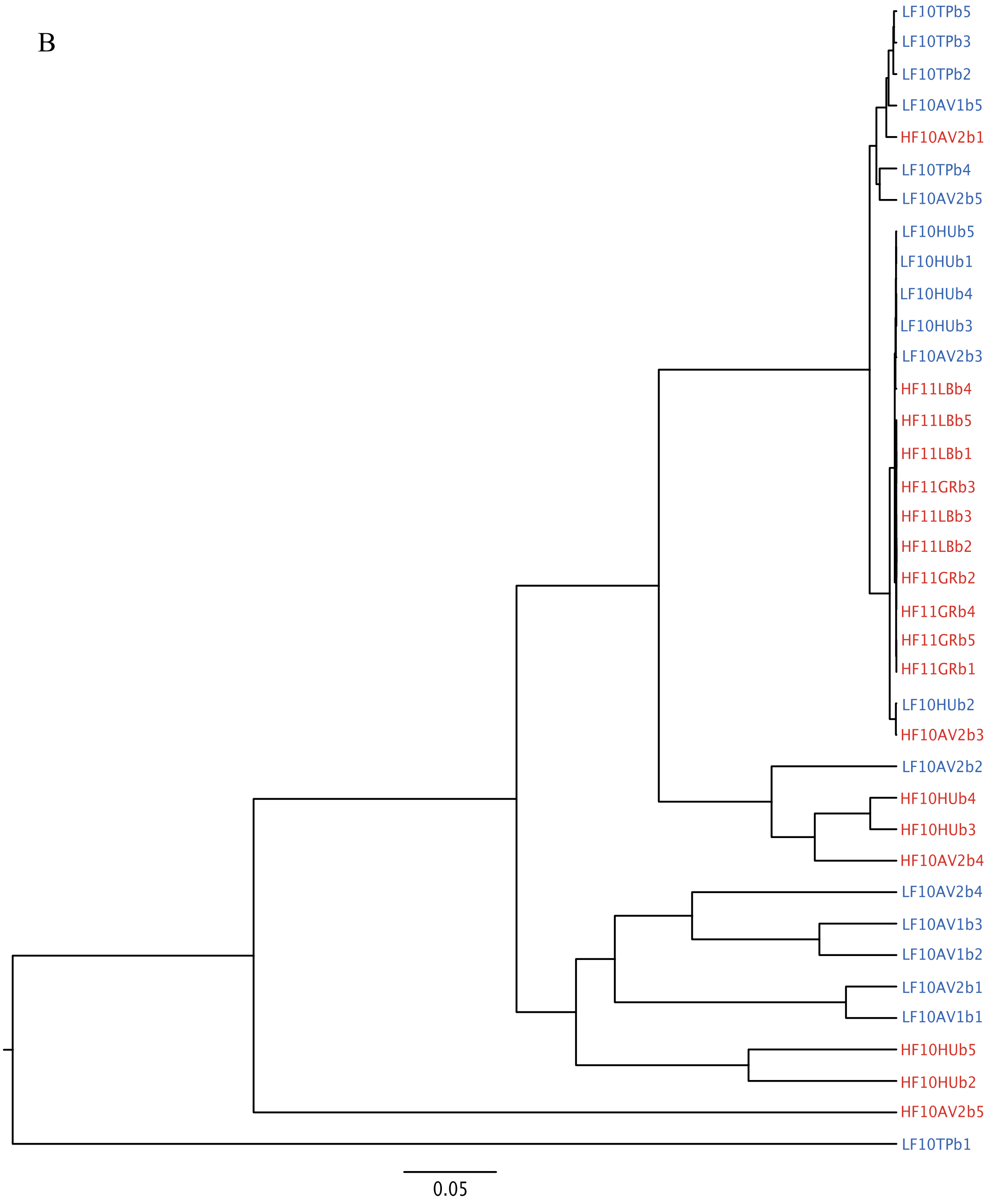


Figure 4.6

UPGMA dendrograms constructed from pyrosequencing data describing the dissimilarity of the samples based on (A) the membership of their trophosome assemblages using the Jaccard index and (B) the structure of their trophosome assemblages based on the Yue and Clayton index. Samples in red were collected from High Flow habitats, and samples in blue, from Low Flow habitats.

structure of the trophosome assemblages was also highly significant ($p < 0.001$; Fig. 4.6B). Pairwise comparison results are found in Appendix E, Table E.3. However, the clustering of High Flow and Low Flow samples in this dendrogram was not significant. The AMOVA test on the distance matrix based on the membership of the trophosome assemblages was significant ($p < 0.001$), indicating that the variation within sampling sites was different from the average variation of two sites pooled together (Appendix E, Table E.3). Similarly, the variation of the trophosome assemblage membership of each habitat was different from the average variation of High Flow and Low Flow samples pooled together ($p < 0.001$). These results can be visualized in a NMDS ordination plot (Fig. 4.7A). The same test performed on the structure of the trophosome assemblages was statistically significant when comparing sampling sites ($p = 0.003$), but not habitat type (Fig. 4.7B). None of the HOMOVA tests on the membership of trophosome assemblages were significant, but the test on trophosome assemblage structure indicated significant variation between sampling sites ($p < 0.001$; Appendix E, Table E.3). Several OTUs were differentially represented between sampling sites and between habitats, but most of them were found in only one of the 37 samples and accounted for < 0.1% of the pyrosequence library. Within the 25 most abundant OTUs detected, 24 were differentially represented in at least two sampling sites (Appendix E, Table E.4) and seven were differentially represented between habitats (Table 4.5).

Pyrosequencing analysis on SNG OTUs

The most abundant OTU detected by Sanger sequencing, SNG OTU 1, as well as the most common OTU revealed by Chao et al. (2007)'s investigation, RAE OTU 1, corresponded to PYRO OTU 1 (Table 4.3). However, while the near-complete SSU

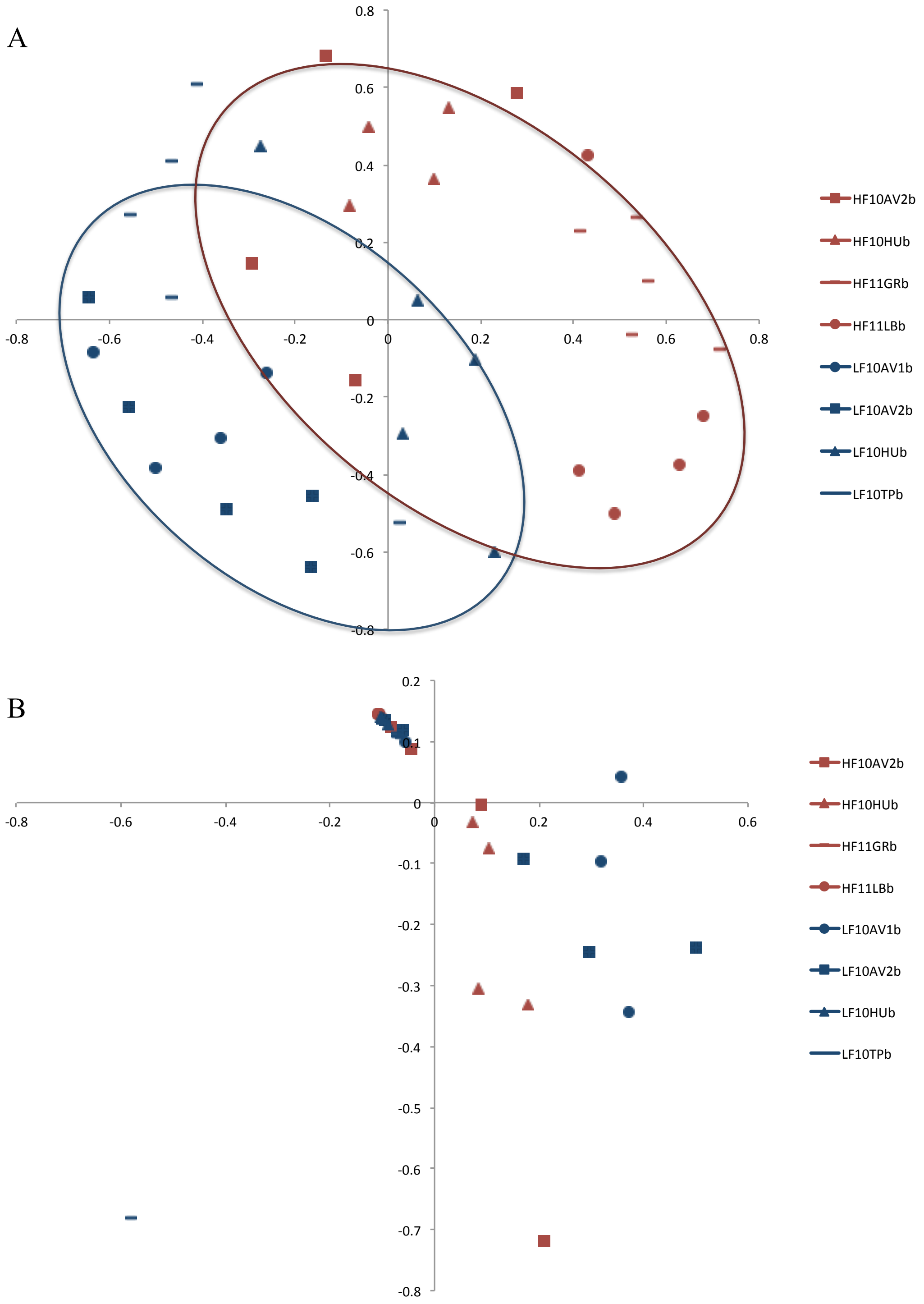


Figure 4.7

NMDS ordination plot constructed from pyrosequencing data describing the dissimilarity of the samples based on (A) the membership of their trophosome assemblages using the Jaccard index (stress value = 0.37; $R^2 = 0.49$) and (B) the structure of their trophosome assemblages based on the Yue and Clayton index. Samples in red were collected from High Flow habitats, and samples in blue, from Low Flow habitats.

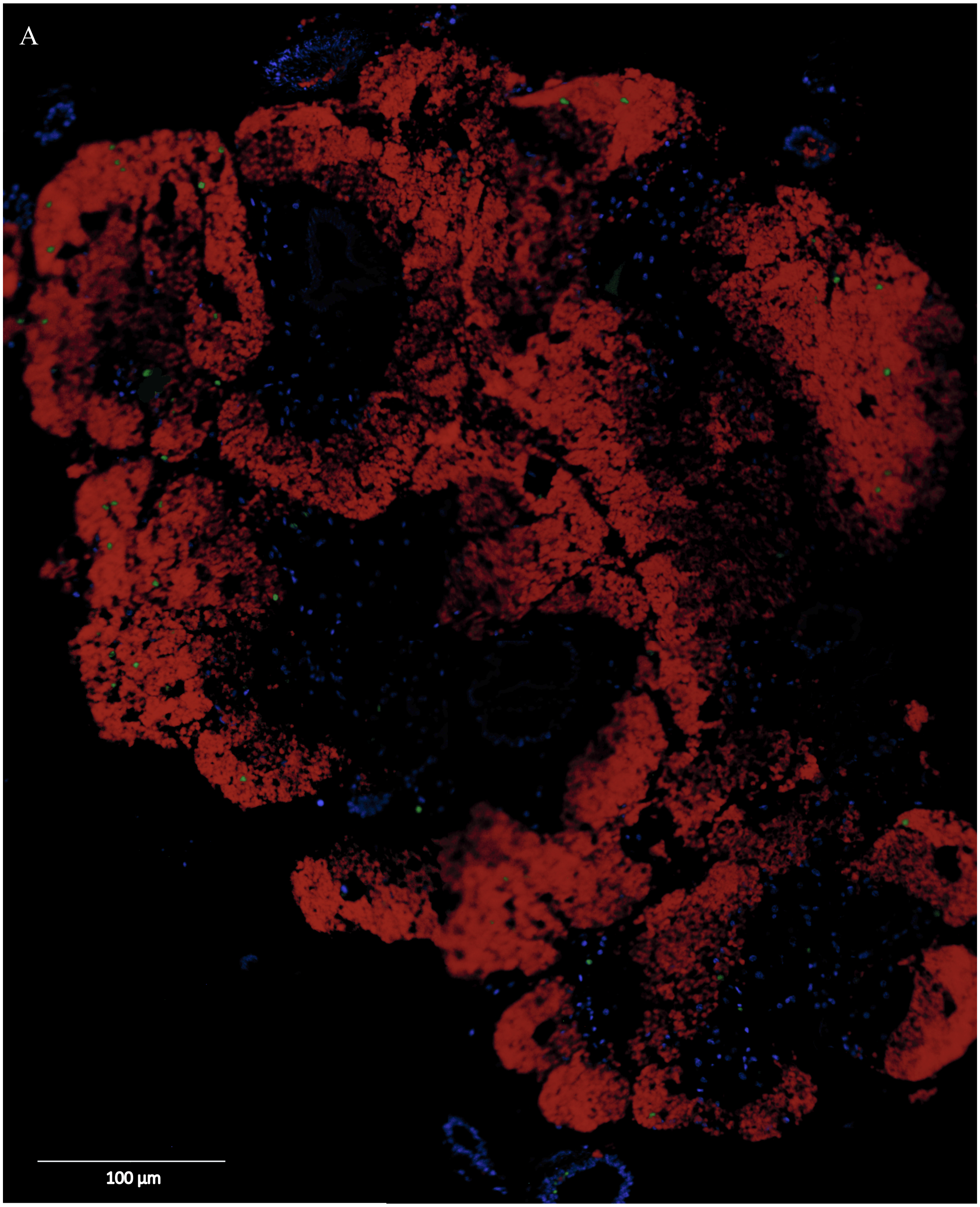
rRNA gene sequence was affiliated to the genus *Sedimenticola*, the V1-V3 region was classified as *Methylomicrobium* (Table 4.5). SNG OTUs 3, 5, 7, 9, 10, and 13 all matched one of the 25 most abundant OTUs detected by pyrosequencing (Table 4.3). The other SNG OTUs corresponded to rare (< 0.1% of the library) PYRO OTUs, except SNG OTU 2, which did not match any of our pyrosequences (Table 4.3). None of the other OTUs detected by Chao et al. (2007) clustered with any of our pyrosequences (data not shown).

Comparison between trophosome and free-living bacterial populations

We compared our pyrosequencing reads to data obtained from free-living bacterial communities from High Flow and Low Flow habitats. Four out of eight free-living community libraries were collected at the same vent site, Grotto, as one of our trophosome libraries (Table 4.5). The five most abundant PYRO OTUs were not detected within the free-living community libraries, and the four most abundant free-living OTUs, accounting for 39.8% of the sequences, represented rare OTUs (< 0.01%) in the trophosome library (data not shown). A total of 11 PYRO OTUs were also found in the free-living communities, but five of them accounted for less than 0.1% of the total number of sequences. The six other OTUs represented the fifth, sixth, eighth, ninth, tenth, and fourteenth most abundant OTUs detected in the free-living community libraries (data not shown).

Symbiont detection in *R. piscesae* trophosome

Multiple hybridizations with EPSY549 and GAM42a combined with BET42a confirmed the presence of members of *Epsilonproteobacteria* together with *Gammaproteobacteria* within the trophosome of single *R. piscesae* in all individuals analyzed (Fig. 4.8). The relative abundance of *Gammaproteobacteria* was clearly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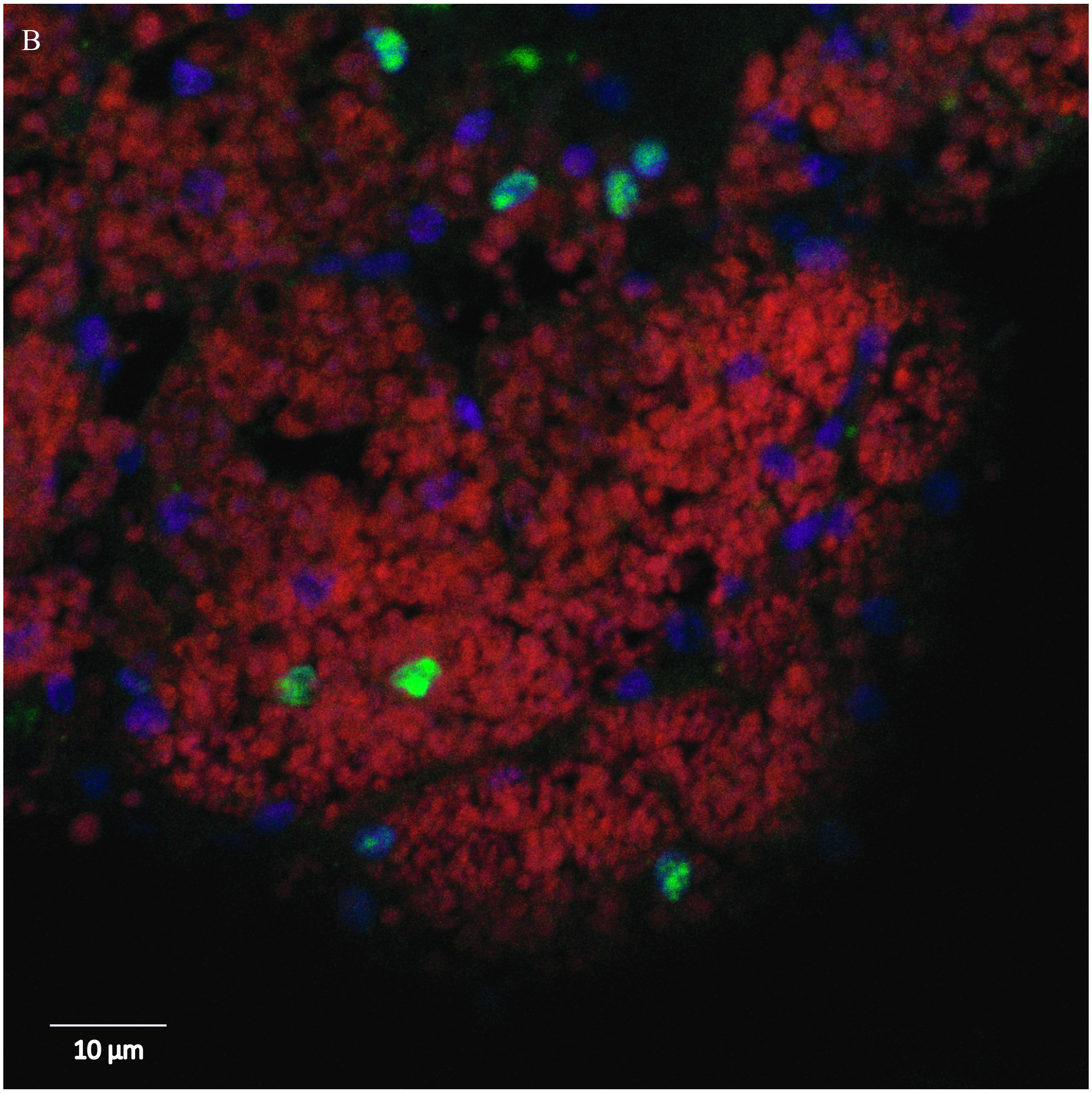

Figure 4.8

In situ localization of bacterial symbionts in *R. piscesae* trophosome. The sections underwent a double hybridization with the GAM42a and BET42a probes, targeting *Gammaproteobacteria* (red) and the EPSY549 probe, targeting *Epsilonproteobacteria* (green). DAPI was used as a general DNA stain (blue). Since all samples provided similar results, we are showing only one trophosome sample from the sampling location HF10AV2b. (A) Epifluorescence image showing an overall view of trophosome section. (B) Confocal image showing a close up on a trophosome section.

superior to that of *Epsilonproteobacteria* in all samples, while comparison of High Flow and Low Flow samples did not reveal any obvious differences between habitats in terms of relative abundance of the two groups.

Discussion

Symbiotic associations between bacteria and eukaryotes are widespread in the biosphere and dominate faunal biomass in reducing habitats such as hydrothermal vents, cold seeps, and whale and wood falls, where the host bridges oxic and anoxic zones, facilitating access to oxidants and reductants for the chemoautotrophic symbionts (Cavanaugh et al., 2006). While the association of vent organisms from many different families with multiple bacterial phylotypes is well documented (Distel, 1995; Fiala-Medioni et al., 2002; Suzuki et al., 2005; Urakawa et al., 2005; Petersen et al., 2010), vent tubeworms have been thought to host only one symbiont phylotype shared across the entire group (Edwards and Nelson, 1991; Feldman et al., 1997; Markert et al., 2007; Robidart et al., 2008). Two previous studies provided preliminary evidence of multiple endosymbionts in the *R. piscesae* trophosome (de Burgh et al., 1989; Chao et al., 2007), but these findings were not definitive. The de Burgh et al. (1989)'s study was based entirely on morphological examination of bacterial cells within trophosome tissue, without molecular evidence that the different morphologies corresponded to phylogenetically distinct bacteria. Chao et al. (2007) provided molecular evidence, but the authors recognized that their conclusion, based solely on T-RFLP data, could have been influenced by contamination from bacteria associated with the worm's tube or the environment where they were collected (Chao et al., 2007). In this study, we began our investigation of *R. piscesae*'s trophosome assemblages by screening bacterial diversity in six tubeworm samples using near-complete SSU rRNA gene sequences, which provided detailed information about the affiliation of the OTUs detected. These findings were reinforced by an extension of the investigation to a larger number of worms (37) and the application of 454 pyrosequencing, which yielded considerably more sequences ($>10^5$). Finally, CARD-FISH results provided direct observational evidence of the presence of at least two distinct bacterial taxonomic groups within the trophosome tissue of *R. piscesae*. We used general probes designed to target *Gamma-* and *Epsilonproteobacteria*, the two most abundant groups detected by both sequencing methods, since visualization of fluorescence signals in FISH is limited by the number of rRNA gene copies present (Hoshino et al., 2008).

The pyrosequencing approach has rarely been used to investigate endosymbiont diversity, and since the rare biosphere detected by this technique is more likely to contain artefacts (Huse et al., 2010; Kunin et al., 2010; Tedersoo et al., 2010), we applied very strict quality filtering to reduce background contamination and a high number of replicates to improve data comparability (Zhou et al., 2011). In addition, we compared our results to data from free-living bacterial communities to control for environmental contamination, and focused our interpretation of the results on the most abundant OTUs detected ($\geq 0.1\%$ of the pyrosequence library). Nevertheless, the results of the analyses performed on pyrosequencing data should be considered exploratory and the detection of rarer bacterial lineages, i.e. other than *Gamma-* and *Epsilonproteobacteria,* remains to be confirmed by PCR-independent methods such as CARD-FISH.

Diversity analyses performed on pyrosequencing data suggested that our sequencing effort covered the bulk of the diversity. As expected, our results also revealed very uneven bacterial assemblages in *R. piscesae* trophosome tissue, with a few OTUs representing the majority of the sequences detected and many OTUs accounting for minor proportions of the sequence library. All proteobacterial classes detected by Sanger sequencing were detected by 454 pyrosequencing. In addition, the relative abundance of each class observed the same general pattern, with *Gammaproteobacteria* clearly dominating both libraries, followed by *Epsilon-*, *Delta-*, *Alpha-,* and *Betaproteobacteria* (Figs. 4.2 and 4.5). The presence and proportional representation of *Gamma-* and *Epsilonproteobacteria* were confirmed by CARD-FISH, validating the results obtained by both sequencing methods. The dominance of the first class is not surprising since the previously known siboglinid endosymbionts are members of the *Gammaproteobacteria*. Pyrosequence analysis performed on SNG OTUs revealed that the most common OTU detected by both methods and retrieved in all samples was the same phylotype, which also matched the most common OTU found by Chao et al. (2007), *R. piscesae*'s previously proposed sulphur-oxidizing endosymbiont. As opposed to Harmer et al. (2008), this OTU was not detected in the free-living populations. When cut to match the V1-V3 region of the SSU rRNA gene, the affiliation of SNG OTU 1 to the family *Sedimenticola* changed to the genus *Methylomicrobium* from the family *Methylococcaceae*. Based on the EzTaxon-e server (Kim et al., 2012), *R. pachyptila*'s endosymbiont, *Candidatus Endoriftia persefone* (Robidart et al., 2008), which is shared among all vent siboglinids (Di Meo et al., 2000), is affiliated to the family *Sedimenticola*, reinforcing the phylogenetic affiliation obtained from the near-complete gene sequence. Okubo et al. (2012) assessed phylogenetic drifts of pyrosequence read classification and suggested that assignment at the genus level is affected by read length. Such classification errors can lead to incorrect conclusions about the ecological role of the community investigated. Thus, while 454 pyrosequencing provides massive data sets and allows semi-quantitative interpretation of the results when carefully handled, Sanger sequencing may be needed as a complement to accurately identify sequences.

Within the other gammaproteobacterial OTUs detected, PYRO OTUs 2 and 3 were found in 31 and 34 libraries and represented on average 9.0 and 3.2% of the reads, respectively. These OTUs, as well as PYRO OTU 9, which was the only other OTU detected in all 37 samples, were absent from the free-living libraries, providing further evidence that they were not environmental contaminants. In addition, SNG OTU 3 corresponded to PYRO OTU 13, which was significantly more abundant in Low Flow libraries and was not detected within the free-living communities. This result suggests that the presence of this gammaproteobacterial member within our libraries is not accidental and might be favoured by Low Flow habitat conditions.

Members of the *Epsilonproteobacteria* were found within the epibiotic community of the alvinocaridid shrimp *Rimicaris exoculata* (Petersen et al., 2010; Zbinden et al., 2010; Guri et al., 2012), the alvinellid polychaetes *Paralvinella sulfincola*, *P. palmiformis* and *Alvinella pompejana* (Haddad et al., 1995; Campbell et al., 2001; Alain et al., 2002; Pagé et al., 2004), as well as the siboglinid polychaetes *R. pachyptila* and *R. piscesae* (López-García et al., 2002; Kalanetra and Nelson, 2010). Epsilonproteobacterial endosymbionts have been previously detected in provannid gastropods from the genus *Alviniconcha* (Urakawa et al., 2005), and in pectinodontid gastropods from the genus *Pectinodonta* (Zbinden et al., 2010), but this is the first report of their presence in vent siboglinids. All epsilonproteobacterial SNG OTUs were also detected in our pyrosequence library, and four of them matched one of the 25 most abundant OTUs. However, all epsilonproteobacterial PYRO OTUs detected within the most abundant OTUs, except PYRO OTU 7, were also detected in the free-living libraries. While this doesn't necessarily imply that they are contaminants, our sequence data are not sufficient to confirm that these OTUs represent real *R. piscesae* symbionts. PYRO OTU 7, affiliated to the genus *Arcobacter*, was not detected in the free-living bacterial communities and was found in more than half of the individuals investigated. Members of this genus were found to dominate vent free-living microbial communities (Moussard et al., 2006; Huber et al., 2007; Lanzen et al., 2011; Forget and Juniper, 2013).

The third most common group identified in our pyrosequence library was the *Bacteroidetes*. This phylum, accounting for 2.7% of the pyrosequences, was not detected through Sanger sequencing, but was revealed by Chao et al. (2007)'s investigation, although their *Bacteroidetes* did not match any of our pyrosequences. Furthermore, none of the three PYRO OTUs detected within the most abundant OTUs and belonging to this phylum were found in the free-living communities. While symbiotic associations with *Bacteroidetes* are common within arthropods such as ants (Eilmus and Heil, 2009), giant scale insects (Matsuura et al., 2009), spiders (Martin and Goodacre, 2009), and termites (Fisher et al., 2007), the only other example of a *Bacteroidetes* member associated with an organism from chemosynthetic-based environments comes from a cold seep mytilid mussel from the genus *Idas* (Duperron et al., 2008). That phylotype was related to the genus *Polaribacter*, which corresponds to the affiliation of PYRO OTU 25. All cultured members of this phylum are heterotrophs and many are pathogenic. Duperron et al. (2008) suggested that their presence in *Idas* spp., which also occurs on sunken wood, could reflect a specific adaptation to their environment, but could also be indicative of an infection. Chao et al. (2007) also discussed the possibility of an infection, since non-gammaproteobacterial OTUs such as *Bacteroidetes* and *Alphaproteobacteria* were only detected in apparently unhealthy tubeworms. Interestingly, the most abundant member of *Bacteroidetes*, as well as the most abundant alphaproteobacterial OTU detected in our pyrosequence library, were significantly more abundant in Low Flow libraries constructed from the trophosomes of the long-skinny morphotype of *R. piscesae* experiencing very diffuse vent flow and sulphide concentrations (Urcuyo et al., 2003). These marginal growth conditions for tubeworms could be more favourable to the proliferation of minor taxa (including infections) in the trophosome.

Sanger sequencing detected only one deltaproteobacterial OTU, but this OTU matched the fifth most abundant pyrosequence OTU, which was found in almost 80% of the tubeworms investigated, but was absent from the free-living libraries. Symbiotic members of *Deltaproteobacteria* were found in association with the oligochaete gutless worm *Olavius algarvensis*, which also hosts gammaproteobacterial symbionts (Dubilier et al., 2001). A metagenomic analysis of these co-occurring bacterial partners by Woyke et al. (2006) confirmed that the gammaproteobacterial symbionts are sulphur oxidizers and that the deltaproteobacterial symbionts are sulphate reducers, suggesting syntrophic cycling of sulphur species between the two groups (Woyke et al., 2006). In addition, these same authors found that all *O. algarvensis* symbionts were capable of carbon fixation, providing the host with nutrition through different metabolic pathways. Having multiple energy sources for carbon dioxide fixation could be advantageous to *R. piscesae* in environments where the availability of hydrogen sulphide is low and subject to interruption.

The other OTUs detected either by Sanger sequencing or within the 25 most abundant pyrosequencing OTUs were putative heterotroph members of *Alphaproteobacteria*, *Betaproteobacteria*, and *Actinobacteria*. Other investigative approaches such as CARD-FISH and metagenomic analysis are needed to confirm and interpret their presence inside the trophosome of *R. piscesae*.

Statistical analyses of our pyrosequence data suggested the presence of unique trophosome assemblages at each sampling site, both in terms of membership and structure. This is supported by the fact that all except one of the 25 most abundant OTUs detected in the pyrosequence library were differentially represented at least in one sampling site. Similar results have been observed in free-living hydrothermal vent bacterial communities (Huber et al., 2010; Forget and Juniper, 2013). Physical and chemical conditions can vary widely in hydrothermal vent environments in both time and space, and it has been suggested that specific conditions at individual vent sites could explain the distinctiveness of bacterial communities (Huber et al., 2010). By extension, where host animals occupy a broad physico-chemical niche, similar site-specific differences might be expected in symbiotic assemblages. Spatial structuring of the symbiont populations was observed in the vent shrimp *R. exoculata* and the vent mussels *Bathymodiolus azoricus* and *B. puteoserpentis* and potentially explained by horizontal transmission of site-specific bacteria and vent chemistry, respectively (Duperron et al., 2006; Petersen et al., 2010). High Flow and Low Flow trophosome assemblages had different members, but no overall difference in terms of structure. This result may be explained by a very uneven distribution of the OTUs detected: while many OTUs were differentially represented between habitats, no significant difference in abundance was detected within the three most abundant OTUs, which represented 85.1% of the pyrosequence library. Within the 25 most abundant OTUs, seven were significantly more abundant in either High Flow or Low Flow samples, but they accounted for only ≤ 2.0% of the pyrosequence library, which was not enough for statistical demonstration of structural differences. If, as discussed above, sampling site conditions account for differences in trophosome assemblages, it is not surprising that between-habitat differences were difficult to detect. The significant differences observed in the membership of the trophosome assemblages between sampling sites as well as between habitats can be explained by the detection of several minor OTUs detected in only one or two tubeworm individuals. Since the rare biosphere revealed through pyrosequencing tends to contain many artefacts (Tedersoo et al., 2010; Zhou et al., 2011), results of analyses based on presence/absence of members must be interpreted carefully, especially in the case of assumed symbiotic assemblage investigations in which diversity is expected to be low.

Conclusion

As previously suggested by de Burgh et al. (1989) and Chao et al. (2007), both sequencing approaches revealed the presence of multiple bacterial lineages within our libraries, and CARD-FISH confirmed the co-occurrence of *Gamma*- and *Epsilonproteobacteria* within *R. piscesae*'s trophosome tissue. Furthermore, the five most abundant PYRO OTUs were not detected within the free-living bacterial community libraries from these habitats, providing further evidence that these OTUs were not environmental contaminants. All OTUs detected by Sanger sequencing, except one, corresponded to an OTU revealed by pyrosequencing. However, except for the expected vent siboglinid symbiont phylotype, none of the OTUs detected by Chao et al. (2007) were found within our pyrosequence library. This may be explained by the site-specific characteristics of the trophosome assemblages discussed here, with a single OTU dominating trophosome assemblages together with different OTUs unique to a single site or relatively more abundant at one site than another. In light of this variability within trophosome assemblages it is not possible to infer which phylotypes were observed in the ultrastructural study by de Burgh et al. (1989).

Our study also provided further evidence of the presence of *Bacteroidetes* in trophosome tissue, mostly detected in the long-skinny morphotype of *R. piscesae* living in Low Flow habitats, which is consistent with Chao et al. (2007)'s findings. Furthermore, members of the *Delta*- and *Alphaproteobacteria* were detected by Sanger sequencing as well as within the most abundant pyrosequencing OTUs. The presence of multiple symbiont phylotypes could be advantageous to the host by providing alternative metabolic pathways for CO_2 fixation. The detection of potential heterotrophs could be related to an infection of the trophosome. Because of the lack of information about closely related sequences and the limits to phylogenetic classification, metagenomic analysis will be needed to understand the interactions of the symbionts and their host. The surprisingly high diversity of taxonomic groups revealed by pyrosequencing must be handled carefully: the majority of the OTUs detected had very low frequency. In order to improve pyrosequence data sets, many studies have recommended eliminating minor OTUs (He et al., 2010; Zhou et al., 2011). This study is the first to provide direct observational evidence (CARD-FISH) of the presence of *Epsilonproteobacteria* within a siboglinid tubeworm and to explore potential symbiotic diversity through a combination of sequencing approaches. Molecular methods based on rRNA genes can be sensitive to the relative abundance of organisms and biased toward those with higher gene copy numbers (Farrelly et al., 1995; Crosby and Criddle, 2003; Hoshino et al., 2008). The preponderance of *Gammaproteobacteria* within tubeworm trophosomes, and of one particular phylotype, could have limited the detection of less abundant groups/phylotypes in other studies.

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Chapter 5 Trophosome bacterial diversity in *Ridgeia piscesae* juveniles

Abstract

Based on recent evidence of multiple bacterial partners in the vestimentiferan tubeworm *R. piscesae*, this study examined trophosome assemblages associated with juveniles scraped directly from adult tubeworms. We constructed seven pyrosequence libraries representing seven different sampling sites. Pyrosequencing data obtained from adult tubeworms collected from the same sites were combined with our analyses in order to compare the membership and structure of trophosome assemblages between juveniles and adults, as well as between sites, and High Flow and Low Flow habitats. Our results indicated that juvenile and adult libraries shared similar members, but with different relative abundances, suggesting a selection process during tubeworm maturation or growth. The membership of the libraries appeared site- and habitat-specific, which could be explained by horizontal transmission of bacteria adapted to local conditions. However, no differences in trophosome assemblage structure were detected between sites and habitats. The extensive variation between juvenile and adult libraries at the site level could be masking potential differences between sites and habitats.

Introduction

Symbiotic associations with chemosynthetic prokaryotes dominate the fauna of deep-sea hydrothermal vent ecosystems, allowing a high biomass of invertebrates to thrive where very little photosynthetically-produced organic matter is available (Jannasch and Mottl, 1985). Hosts and symbiotic partners are very diverse, and their relationships vary from ecto- to endosymbiosis, with a single specific partner or many co-occurring phylotypes (Cavanaugh et al., 2006; Dubilier et al., 2008). Northeast Pacific hydrothermal vent assemblages are often dominated by the siboglinid tubeworm *Ridgeia piscesae* (Tunnicliffe and Juniper, 1990; Southward et al., 1995; Sarrazin and Juniper, 1999). Like other vestimentiferans, this polychaete lacks a digestive track and harbours, instead, chemoautotrophic bacteria inside an organ called the trophosome. All vent vestimentiferans have been thought to share a single endosymbiont phylotype (Edwards and Nelson, 1991; Feldman et al., 1997; Di Meo et al., 2000; Nelson and Fisher, 2000; McMullin et al., 2003; Vrijenhoek, 2010). However, in the case of *R. piscesae*, deBurgh et al. (1989) and Chao et al (2007) provided evidence of multiple symbiont phylotypes in single tubeworm individuals, which has been recently confirmed by a multi-faceted approach study combining Sanger sequencing, pyrosequencing and CARD-FISH (Chapter 4).

One of the most crucial steps for symbioses to be maintained through generations is symbiont transmission. Two distinct strategies of transmission are observed: vertical and horizontal. Symbionts transmitted vertically are inherited from parents, generally through the female germ line, whereas symbionts acquired horizontally come from the surrounding environment during an aposymbiotic life cycle phase (Bright and Bulgheresi, 2010). This strategy was suggested for vestimentiferans, whose early aposymbiotic development stages exhibit a functional digestive system (Jones and Gardiner, 1988; Southward, 1988). Since tubeworms most likely depend on their pelagic trochophore larvae for dispersion between vent sites (Jones and Gardiner, 1989), horizontal transmission is also coherent with recent findings suggesting site-specific trophosome assemblage composition in *R. piscesae* (Chapter 4). Nussbaumer et al. (2006) used specific probes for the common vestimentiferan symbiont phylotype and other groups of bacteria to investigate their location in larvae and very small juveniles of *Riftia pachyptila*, *Tevnia jerichonana* and *Oasisia alvinae*. They concluded that symbionts are acquired through the mucus-coated epidermis of larvae where they undergo a selection process and migrate to the mesodermal tissue which eventually differentiate into the trophosome. A single symbiont phylotype was observed throughout all life stages; other bacterial groups were only detected on the surface of tubes (Nussbaumer et al., 2006).

To our knowledge, symbiont diversity has never been investigated in younger stages of *R. piscesae*. Other cases of horizontally acquired co-occurring symbiont phylotypes have shown that the relative proportion of symbiotic partners can vary during the host life history. For example, Guri et al. (2012) observed a switch between *Gamma*- and *Epsilonproteobacteria* in the composition of *Rimicaris exoculata* epibionts from egg and hatched egg to adult stages. This was also observed in *Acropora tenuis*, a scleractinian coral hosting two strains of endosymbiotic zooxanthellae differentially represented between juveniles and adults (Little et al., 2004), However, in mytilid mussels from the genus *Bathymodiolus*, the relative proportions of chemoautotrophic and methanotrophic symbionts remained stable throughout post-larvae, juvenile and adult stages, but varied between vent and cold seep environments (Salerno et al., 2005). The study reported here explored the composition of trophosome assemblages in *R. piscesae* juveniles found on adult tubes using 454 pyrosequencing. We compared our results to a pyrosequencing dataset obtained from the trophosome of adult individuals collected from the same sites (Chapter 4). Based on the theory that vestimentiferans use the horizontal mode of symbiont transmission as well as on our previous results from adult trophosome assemblages, we hypothesized that adults and juveniles from a same site would have similar bacterial partners.

Methods

Sample collection

Specimens of *R. piscesae* were collected in July 2010 and 2011 from Axial Volcano and the Main Endeavour hydrothermal vent field during two separate research cruises using the submersible *Alvin* onboard the RV Atlantis and the remotely operated vehicle (ROV) ROPOS onboard the RV Thomas G. Thompson, respectively. The most extreme morphotypes of *R. piscesae* were collected from contrasted habitats: short-fat tubeworms were taken from active chimneys, where they were exposed to relatively high temperature and diffuse vigorous vent flow. Long-skinny tubeworms were found in low-temperature diffuse vent flow environments, where no shimmering was visible. These habitats will hereafter be referred as High Flow and Low Flow, respectively. Samples were carried to the surface in sealed bioboxes to prevent contamination from each other and from ambient seawater. Once onboard, for each sampling location, juveniles were scraped from adult tubes, packed together, and immediately frozen at -80°C (Table 5.1). Adult trophosome assemblages were investigated in Chapter 4.

DNA extraction and 454 pyrosequencing

Once in the laboratory, the trophosomes of five juveniles per sample location measuring 2-5 cm were aseptically dissected and pooled together in order to get enough

Table 5.1 Description and location of sampling sites.

Sampling Location ID	Tubeworm Morphotype	Habitat Type[a]	Vent Site	Latitude	Longitude	Depth (m)	Max. T°C at plume	Collection Date
LF10AV1b	Long-Skinny	LF	Axial Volcano (Hollywood Flats 1)	45° 56.147' N	129° 58.888' W	1518	na	July-10
HF10AV2b	Short-fat	HF	Axial Volcano (Hollywood Flats 2)	45° 56.155' N	129° 58.893' W	1517	4.1	July-10
LF10AV2b	Long-Skinny	LF	Axial Volcano (Hollywood Flats 2)	45° 56.156' N	129° 58.890' W	1517	2.0	July-10
LF10TPb	Long-Skinny	LF	Main Endeavour (TP)	47° 56.971' N	129° 5.854' W	2197	2.4	July-10
HF10HUb	Short-fat	HF	Main Endeavour (Hulk)	47° 57.007' N	129° 5.824' W	2190	14.0	July-10
LF10HUb	Long-Skinny	LF	Main Endeavour (Hulk)	47° 57.007' N	129° 5.825' W	2191	2.5	July-10
HF11LBb	Short-fat	HF	Main Endeavour (Lobo)	47° 56.965' N	129° 5.900' W	2191	12.2	July-11

[a] "HF" = "High Flow"; "LF" = "Low Flow".

tissue material for DNA extraction (≈ 25 mg). DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Toronto, Canada), purified and concentrated using the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. A volume of 20 µl of DNA with a concentration ≥ 20 ng/µl was sent to the *Plateforme d'Analyses Génomiques* (Institute of Integrative and Systems Biology, Laval University, Quebec City, QC, Canada). PCR amplicons of the bacterial SSU rRNA gene hypervariable region V1-V3 were obtained using a 454 GS-FLX DNA sequencer with the Titanium Chemistry (Roche) according to the manufacturer's instructions, as previously described (Chapter 4).

Pyrosequence data quality filtering

All data processing and analysis was performed using the software mothur v.1.29.0 (Schloss et al., 2009). Juvenile data were combined with the pyrosequencing data set obtained from 40 adult *R. piscesae* samples collected from the same sampling sites and described in Chapter 4. Since we only had one juvenile library per sampling site resulting from the combination of extracted DNA from five tubeworms, we pooled the data obtained from five adult samples per site, for a total of 15 pyrosequence libraries. Preprocessing to improve data set quality and eliminate background noise was carried out as described in Chapter 4. Sequences displaying ≥ 96% similarity were assigned to operational taxonomic units (OTUs) based on the distance matrix generated on uncorrected pairwise distances. This threshold was chosen as suggested by Kim et al. (2011) for phylogenetic analysis of the V1-V3 region of the bacterial SSU rRNA gene. Singletons (OTUs represented by a single sequence) were removed from subsequent analyses to eliminate potential artefacts, as recommended (Tedersoo et al., 2010; Zhou et al., 2011). The number of reads across libraries was standardized by subsampling based on the lowest number of sequences found in any of the 15 libraries. A share file, describing the number of times each OTU was observed in every library, was used for statistical and phylogenetic analysis. OTUs present in only one library were eliminated as suggested to improve the reliability of our data set (He et al., 2010).

Statistical and phylogenetic analysis

The number of observed OTUs, coverage, Chao1 richness index, and Shannon-Wiener diversity index were calculated for each library. Adult and juvenile trophosome assemblages were compared in terms of membership (*i.e.* absence/presence of OTUs) and structure (*i.e.* relative abundance of each OTUs). Distance matrices calculated from the share file with the Jaccard and Yue and Clayton indices (Yue and Clayton, 2005), describing the dissimilarity between libraries based on membership and structure, respectively, were used for analyses of molecular variance (AMOVAs) and analyses of homogeneity of molecular variance (HOMOVAs). These tests assessed whether the variation in membership and structure of the trophosome assemblages between juveniles and adults was significantly different from that of all libraries pooled together and whether this variation was homogeneous between juveniles and adults, respectively. Nonmetric multidimensional scaling (NMDS) ordination plots were used to visualize the dissimilarity between samples based on the distance matrices. The command "metastats" (White et al., 2009) indicated which OTUs were differentially represented between juvenile and adult tubeworm trophosome assemblages. The same analyses were performed to compare the membership and structure of trophosome assemblages between sites and between High Flow and Low Flow habitats.

Nucleotide sequence accession numbers

Pyrosequence reads were deposited to NCBI's Sequence Read Archive (SRA) under accession number SRA058565.

Results

General characteristics of pyrosequencing data set

We constructed a total of seven pyrosequence libraries, each obtained from the trophosome of five juveniles pooled together, for a total of 169 912 raw bacterial SSU rRNA gene pyrosequence reads. Quality filtering and elimination of singletons left 128 604 reads with an average length of 280 bp representing 649 OTUs. After adding the eight adult libraries and randomly subsampling 10 839 reads from each of the 15 libraries, which corresponded to the lowest number of sequences found in any of the libraries, the number of juvenile OTUs dropped to 598 OTUs. Adult libraries contained a total of 452 OTUs. The total number of OTUs across libraries was 800, of which 250 were shared between juvenile and adult libraries. Eliminating OTUs present in only one library removed a total of 450 OTUs, which accounted for 0.89% of the pyrosequence reads. The final juvenile libraries contained 330 OTUs and adult libraries contained 270 OTUs. Shared OTUs accounted for 99.3% of the pyrosequence reads. The number of OTUs per library varied between 6 and 164, and the coverage between 99.51 and 99.98% (Table 5.2). The Chao1 and Ace richness indices and the Shannon-Wiener diversity index were calculated along with their 95% confidence intervals (Table 5.2). Most juvenile libraries had a higher diversity index than adult libraries from the same site (Table 5.2). Rank abundance curves indicated that a small number of OTUs accounted for the

Table 5.2 Number of observed OTUs, coverage, and richness and diversity estimates at 4% sequence distance for juvenile and adult libraries.

Library[a]	Number of OTUs	Coverage	Chao1 (95% CI)	Ace (95% CI)	Shannon-Wiener (95% CI)
HF10AV2bA	118	99.61%	157 (136-202)	162 (141-202)	1.72 (1.69-1.76)
HF10AV2bNR	107	99.57%	181 (141-267)	160 (135-207)	0.73 (0.70-0.76)
HF10HUbA	100	99.70%	125 (110-159)	130 (114-162)	1.74 (1.71-1.77)
HF10HUbNR	131	99.68%	169 (147-221)	168 (150-205)	3.01 (2.98-3.04)
HF11GRbA	26	99.87%	41 (30-80)	73 (49-122)	0.12 (0.11-0.13)
HF11LBbA	15	99.95%	18 (16-37)	24 (18-42)	0.11 (0.09-0.12)
HF11LBbNR	6	99.98%	7 (6-14)	8 (6-26)	0.04 (0.03-0.05)
LF10AV1bA	82	99.75%	105 (91-142)	107 (93-136)	1.50 (1.47-1.53)
LF10AV1bNR	163	99.74%	181 (170-208)	180 (171-199)	2.97 (2.94-3.00)
LF10AV2bA	75	99.69%	163 (108-306)	197 (155-260)	1.44 (1.41-1.47)
LF10AV2bNR	135	99.83%	143 (137-159)	145 (139-160)	2.41 (2.38-2.45)
LF10HUbA	47	99.83%	69 (54-115)	63 (53-91)	0.32 (0.30-0.34)
LF10HUbNR	164	99.66%	197 (179-239)	196 (181-226)	3.19 (3.16-3.21)
LF10TPbA	131	99.51%	186 (159-241)	255 (216-310)	1.20 (1.16-1.23)
LF10TPbNR	133	99.69%	160 (144-195)	167 (151-199)	2.56 (2.53-2.58)

[a] The name of the library refers to the sampling site ID. "NR" at the end of the name indicates a juvenile library, and “A”, and adult library.

majority of the sequences while most OTUs detected were represented by only a few sequences, but differences in the slope suggested that juvenile trophosome assemblages had a higher evenness (Fig. 5.1). The most abundant OTU in juvenile libraries represented 37.8% of the reads, the 10 most abundant, 78.1%, and the 25 most abundant, 89.5%.

Proteobacteria clearly dominated juvenile libraries, representing 91.0% of the pyrosequence reads, followed by *Bacteroidetes*, accounting for 8.0% (Fig. 5.2). Within

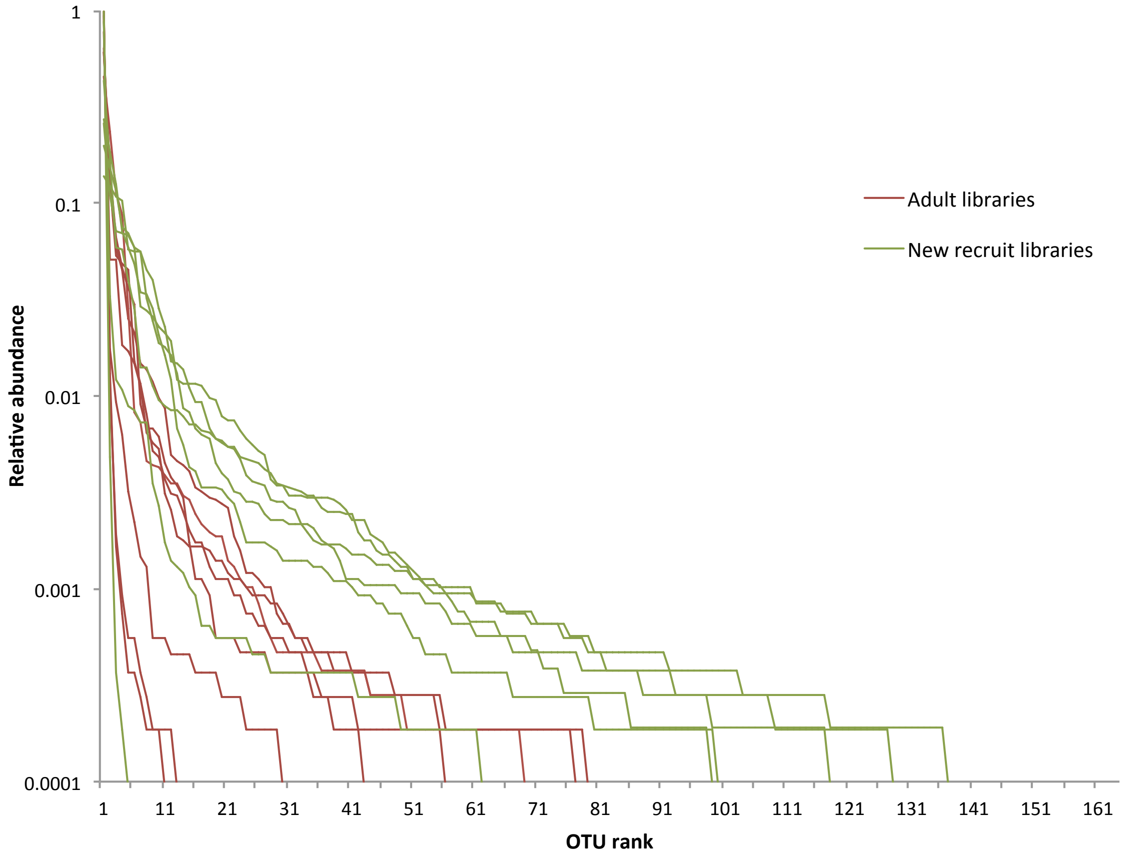

Figure 5.1

Rank abundance curves calculated from 454 pyrosequencing reads for adult and juveniles libraries.

the last 1%, ten other phyla were detected, which were grouped under the category "Others" in Figure 5.2. These phyla included *Chloroflexi*, *Spirochaetes*, *Actinobacteria*, *Chlorobi*, *Deinococcus-Thermus*, *Firmicutes*, *Acidobacteria*, *Cyanobacteria*, and candidate divisions BD1-5 and TM7. Within the *Proteobacteria* phylum, the class *Gammaproteobacteria* was the most abundant (51.4% of the juvenile libraries), followed by *Epsilonproteobacteria* (27.5%), and *Deltaproteobacteria* (9.7%). *Alpha*- and *Betaproteobacteria* accounted for 1.9% and 0.43% of the juvenile libraries, respectively.

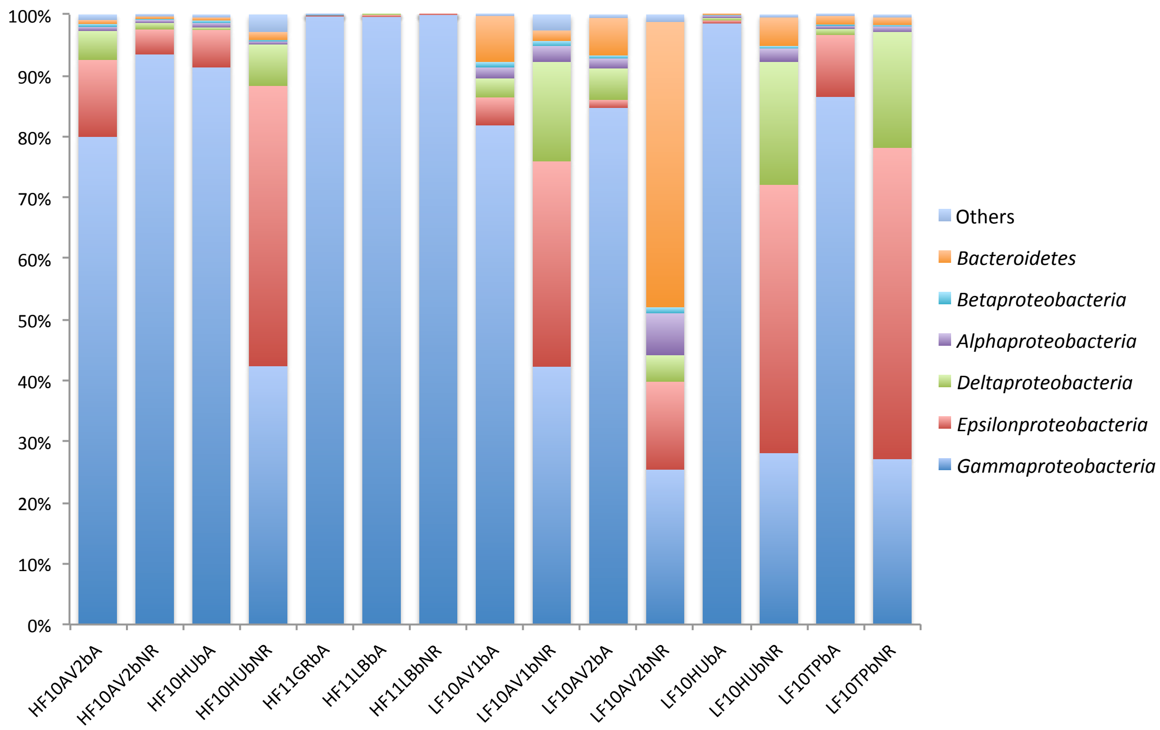

Figure 5.2

Relative abundance of the phyla detected by 454 pyrosequencing for each juvenile and adult libraries. The phylum *Proteobacteria*, dominating all libraries, was divided in the five classes detected. The category “Others” includes phyla representing < 1% of pyrosequence reads. The libraries are grouped by sampling site. The name of the library refers to the sampling site ID. “NR” at the end of the name indicates a juvenile library.

The phylogenetic affiliation of the 25 most abundant OTUs detected within the juvenile pyrosequence libraries is described in Table 5.2. The two most abundant OTUs belonged to the *Gammaproteobacteria* and were classified as members of the genera *Methylomicrobium* and *Halomonas* (Table 5.3). Three other OTUs, members of the genera *Shewanella*, *Nitrosococcus*, and *Methylomicrobium*, were also members of the *Gammaproteobacteria.* The third most abundant OTU belonged to the genus *Sulfurovum* from the *Epsilonproteobacteria*. This genus was assigned to seven of the 25 most abundant OTUs. The epsilonproteobacterial genera *Arcobacter* and *Nitratifractor* were

Table 5.3 Affiliation and occurrence of the 25 most abundant OTUs detected in juvenile pyrosequence libraries. Differentially represented OTUs between juvenile (NR) and adult (A) libraries as well as between sampling sites and High Flow (HF) and Low Flow (LF) habitats were identified ($p < 0.5$; ns = not significant).

OTU rank	OTU ID in Chapter 4[a]	Affiliation[b]	Average in NR	Average in A	NR-A comparison	Sampling site comparison	HF-LF comparison
1	PYRO OTU 1	*Gammaproteobacteria - Methylomicrobium*	37.6 ± 14.8%	75.0 ± 7.1%	x	x	x
2	PYRO OTU 2	*Gammaproteobacteria - Halomonas*	8.2 ± 2.3%	8.8 ± 3.0%	ns	x	ns
3	PYRO OTU 12	*Epsilonproteobacteria - Sulfurovum*	7.1 ± 3.6%	0.4 ± 0.3%	x	ns	ns
4	PYRO OTU 4	*Bacteroidetes - Gaetbulibacter*	6.2 ± 6.2%	1.5 ± 1.0%	ns	x	ns
5	PYRO OTU 18	*Epsilonproteobacteria - Sulfurovum*	4.4 ± 1.5%	0.4 ± 0.3%	x	ns	ns
6	PYRO OTU 11	*Epsilonproteobacteria - Arcobacter*	3.6 ± 1.8%	0.7 ± 0.4%	ns	x	ns
7	PYRO OTU 14	*Epsilonproteobacteria - Sulfurovum*	3.1 ± 1.0%	0.4 ± 0.2%	x	x	x
8	PYRO OTU 3	*Gammaproteobacteria - Shewanella*	2.9 ± 0.8%	3.1 ± 1.1%	ns	x	ns
9	PYRO OTU 20	*Deltaproteobacteria - Desulfuromonadales*	2.5 ± 1.7%	0.4 ± 0.3%	ns	ns	ns
10	na	*Deltaproteobacteria - Desulfocapsa*	2.2 ± 1.8%	> 0.1 ± > 0.1	ns	x	ns
11	na	*Deltaproteobacteria - Desulfobulbus*	1.8 ± 0.8%	> 0.1 ± > 0.1	x	ns	ns
12	na	*Epsilonproteobacteria - Sulfurovum*	1.8 ± 1.6%	0.1 ± > 0.1%	ns	ns	ns
13	PYRO OTU 10	*Epsilonproteobacteria - Sulfurovum*	1.3 ± 0.8%	0.2 ± 0.1%	ns	x	x
14	na	*Epsilonproteobacteria - Nitratifractor*	1.0 ± 1.0%	> 0.1 ± > 0.1	ns	x	ns
15	PYRO OTU 6	*Epsilonproteobacteria - Sulfurovum*	1.0 ± 0.6%	0.7 ± 0.6%	ns	x	x
16	PYRO OTU 8	*Gammaproteobacteria - Nitrosococcus*	1.0 ± 1.0%	1.4 ± 1.4%	ns	x	ns

17	na	*Alphaproteobacteria - Roseobacter*	0.7 ± 0.5%	> 0.1 ± > 0.1	ns	x	ns
18	na	*Deltaproteobacteria - Desulfobulbus*	0.4 ± 0.4%	> 0.1 ± > 0.1	ns	ns	ns
19	na	*Deltaproteobacteria - Desulfobacteraceae*	0.4 ± 0.3%	> 0.1 ± > 0.1	ns	ns	ns
20	na	*Deltaproteobacteria - Desulfarculus*	0.4 ± 0.2%	> 0.1 ± > 0.1	ns	ns	ns
21	PYRO OTU 21	*Epsilonproteobacteria - Sulfurovum*	0.4 ± 0.2%	0.1 ± 0.1%	ns	x	x
22	PYRO OTU 17	*Bacteroidetes - Flavobacterium*	0.4 ± 0.1%	0.2 ± 0.1%	ns	x	x
23	na	*Chloroflexi - Caldilineaceae*	0.4 ± 0.3%	> 0.1 ± > 0.1	ns	ns	ns
24	PYRO OTU 9	*Gammaproteobacteria - Methylomicrobium*	0.3 ± 0.2 %	0.9 ± 0.1%	x	x	x
25	na	*Epsilonproteobacteria - Nitratifractor*	0.3 ± 0.3%	> 0.1 ± > 0.1	ns	ns	ns

[a] na = rare OTU that was not assigned an ID.

[b] Phylum (or proteobacterial class) and lowest confidently assigned level.

also detected. A member of the genus *Gaetbulibacter* from the phylum *Bacteroidetes* was the fourth most abundant OTU detected within juvenile libraries. Another OTU, affiliated to the genus *Flavobacterium*, belonged to the same phylum. Six OTUs were classified as *Deltaproteobacteria.* Deltaproteobacterial genera detected included *Desulfocapsa*, *Desulfobulbus*, and *Desulfarculus*. One OTU belonging to the phylum *Chloroflexi* was also detected within the 25 most abundant juvenile OTUs. For an exhaustive list of the genera detected within the juvenile libraries, see Appendix E, Table E.1.

Comparison of trophosome assemblages between libraries

Pyrosequencing libraries were analyzed based on the membership and structure of trophosome assemblages. For comparison between sampling sites, the adult library HF11GRb was removed from the analyses since no corresponding juvenile library was available. The AMOVA performed on the distance matrix based on the structure of trophosome assemblages suggested that juvenile and adult libraries were significantly different ($p = 0.009$), which can be visualized in the NMDS ordination plot (Fig. 5.3A). No difference was detected between sampling sites or habitats. However, the same test based on the membership showed a significant difference between sites ($p = 0.002$) and between High Flow and Low Flow habitats ($p = 0.014$) (Fig. 5.3B), but no trend between juvenile and adult libraries. The HOMOVA indicated that the variation in the structure was not homogeneous between juvenile and adult libraries ($p = 0.008$; Fig. 5.3), but no difference was detected between sites or habitats. None of the HOMOVA tests were significant when assessing the membership of the libraries. A total of 34 OTUs were differentially represented between juvenile and adult libraries, of which 6 were found

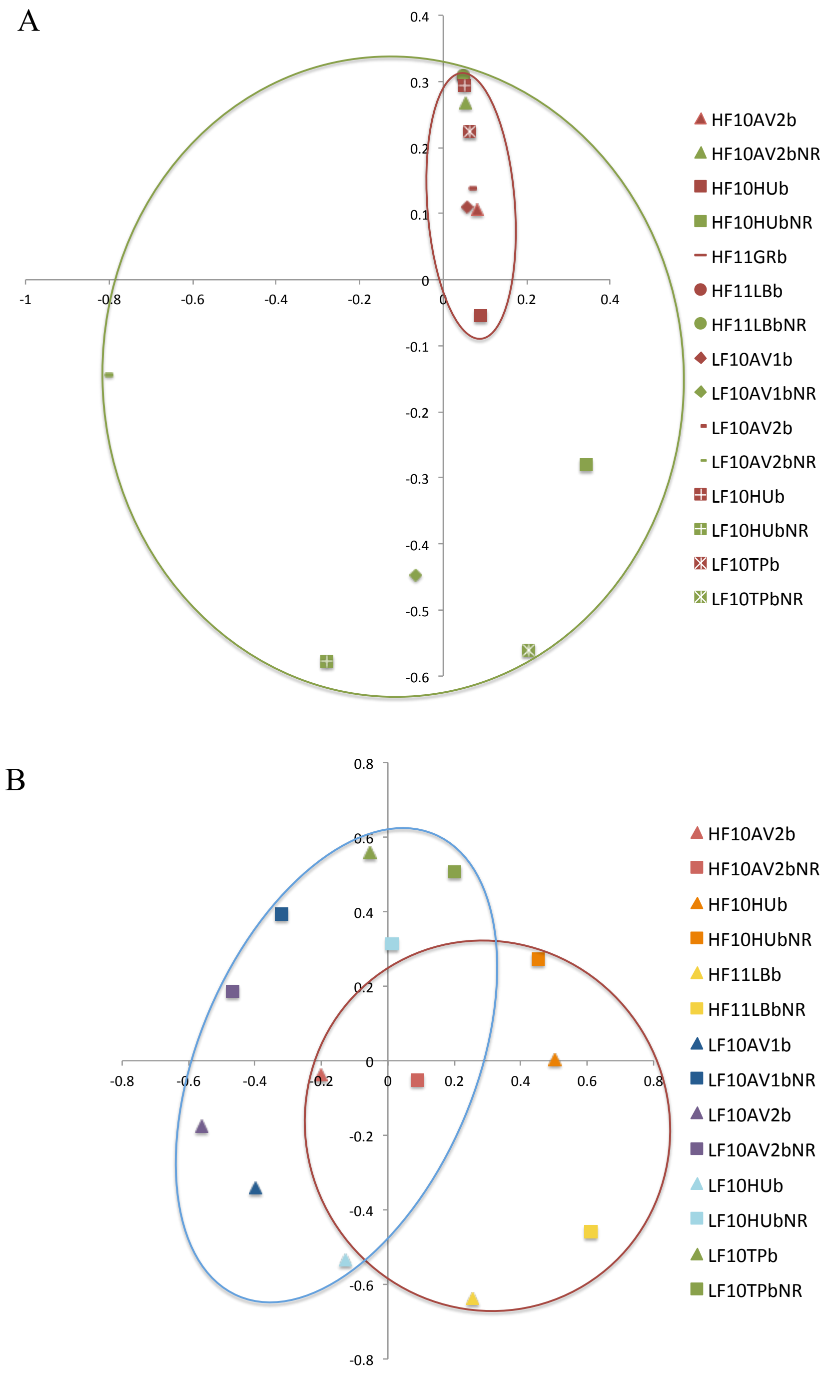

Figure 5.3

NMDS ordination plot constructed from 454 pyrosequencing data describing the dissimilarity of the libraries based on (A) the structure of their trophosome assemblages using the Yue and Clayton index (stress value = 0.11; R^2 = 0.97; samples in red represent adults, and samples in green, juveniles) and (B) the membership of their trophosome assemblages based on the Jaccard index (stress value = 0.32; R^2 = 0.49; samples of the same colour were collected from the same vent site, the blue circle includes Low Flow libraries and the red circle, High Flow libraries).

within the 25 most abundant OTUs detected in juvenile libraries. The other OTUs accounted for ≤ 0.2% of the pyrosequence reads. The most abundant OTU, corresponding to PYRO OTU 1 in Chapter 4, was significantly more abundant in adult libraries (Table 5.3). The third, fifth, seventh and twenty-fourth most abundant OTU, which matched PYRO OTU 12, 18, 14, and 9 in Chapter 4, respectively, as well as the eleventh more abundant OTU, which corresponded to an OTU accounting for < 0.1%, were significantly more abundant in juvenile libraries (Table 5.3). Ten of the other 25 most abundant juvenile OTUs were also found in the 25 most abundant adult OTUs, while nine of them corresponded to minor adult OTUs (Table 5.3). A total of 15 OTUs detected within the 25 most abundant juvenile OTUs were significantly more abundant in one sampling site than another, and seven OTUs were differentially represented between habitats (Table 5.3).

Discussion

Analysis of symbiotic assemblages associated with younger life stages of hosts allows the investigation of the symbiont acquisition and selection processes and provides a better understanding of the basic mechanisms underlying these associations. Hosts harbouring multiple symbiont phylotypes constitute interesting models to explore the variation in the composition of symbiotic assemblages throughout life history. The results of diversity analyses and composition of trophosome assemblages associated with adult individuals slightly differed from those in Chapter 4. This can be explained by the approach used for standardization of the number of pyrosequences across samples; in the first study, subsampling was performed on each sample after removing the three samples with the lowest number of reads. In this study, since we had one juvenile library per site corresponding to five tubeworms pooled together, we pooled the pyrosequences from the five adult samples collected from each sampling site and subsampled a higher number of sequences on these libraries. Furthermore, we excluded any OTU that was only represented in one library in order to improve the quality of our data set.

The most abundant groups detected in juvenile libraries were the same as in adult libraries and were found in similar order of relative abundance. In addition, 250 OTUs out of 350 were shared between the libraries representing the two life stages. The relative abundances of these groups as well as the most abundant OTUs were quite different between adult and juvenile libraries. *Gammaproteobacteria* were considerably less abundant, while *Epsilon*- and *Deltaproteobacteria* were found in higher relative abundance in juvenile libraries. The most abundant OTU detected in adult libraries represented 75.0% of the pyrosequences and was significantly more abundant than in juvenile libraries, in which it accounted for 37.6%. A total of 10 out of the 25 most abundant juvenile OTUs corresponded to minor adult OTUs, implying that 10 relatively abundant adult OTUs were minor members of juvenile trophosome assemblages. Furthermore, the abundance curves suggested a more even distribution of OTUs in juvenile libraries, which was also reflected by higher Shannon-Wiener diversity indices for five out of seven juvenile libraries compared to adult libraries from the same sampling site. Statistical analyses comparing juvenile and adult libraries are consistent with these observations and suggested different structure of trophosome assemblages, but similar membership. The same pattern was observed in the reef-building coral *A. tenuis* hosting two strains of *Symbiodinium* in inverse proportions between juveniles and adults (Little et al., 2004), as well as with the hydrothermal vent shrimp *R. exoculata*, which gamma- and epsilonproteobacterial symbionts remained the same along life cycle, but were differentially represented between first and latter life stages (Guri et al., 2012). A possible explanation might be different physiological requirements of the host along life history, as suggested by Little et al. (2004). However, after investigating the uptake and persistence of symbiont types in *A. tenuis* and *A. millepora*, Abrego et al. (2009) concluded that in early life stages, these corals possess few barriers to the uptake of *Symbiodinium* strains, which leads to the infection and dominance of potentially unsuitable partners. Exploring trophosome bacterial diversity in even earlier life stages of *R. piscesae* might help resolving this question: if trophosome assemblages were more even in younger tubeworms, one could presume that host selection mechanisms are less effective. Examination of the relative abundance of symbiont phylotypes in the free-living communities might constitute another approach: similar structure between very young *R. piscesae* symbionts and free-living communities would reinforce the hypothesis of fewer selection barriers in juveniles.

Our results also indicated that the variation in the trophosome assemblage structure within juveniles was greater than within adult libraries. This result can be explained by a larger gap observed in the representation of the most abundant OTUs within juvenile libraries, suggesting that the occurrence of these bacterial phylotypes varied between sampling sites. Analysis of the membership of trophosome assemblages indicated that libraries from the same sampling sites shared similar OTUs and that the majority of the most abundant OTUs were differentially represented between sampling sites, which corroborates this hypothesis. Our results also showed distinct membership of trophosome assemblages between High Flow and Low Flow habitats. Similar results were obtained when analyzing adult samples between sites and habitats in Chapter 4. A study of other vestimentiferans suggested geographic location as a factor potentially explaining the genetic variation among endosymbionts from different individuals (Di Meo et al., 2000). A correlation between epibiont diversity and geographic distance was also observed in *R. exoculata* (Petersen et al., 2010). Distinctive trophosome assemblage membership of juvenile and adult libraries between sampling sites is coherent with horizontal transmission of symbionts. Many studies have provided evidence for the use of this strategy in vestimentiferans (Jones and Gardiner, 1988; Southward, 1988; Jones and Gardiner, 1989; Nussbaumer et al., 2006). As indicated by Huber et al. (2010) and Forget and Juniper (2013), free-living bacterial communities in hydrothermal vent environments may also be site-specific, most likely depending on physical and chemical conditions. Symbionts acquired *de novo* at each generation from the surrounding free-living communities would be therefore more adapted to the prevailing conditions, and hence, locally more efficient.

In contrast with the variation observed in relative symbiont abundances in *Bathymodiolus* spp. from different vent site (Duperron et al., 2006), we did not detect distinct structure of the trophosome assemblages related to sampling sites or habitats. However, previous results have shown the structure of adult libraries to be site-specific (Chapter 4). In this study, the sampling strategy did not allow comparison of juvenile libraries between sampling sites, but the extent of the variation observed in the structure of trophosome assemblages between juvenile and adult libraries could be masking larger scale spatial patterns.

As mentioned above, a great number of OTUs were differentially represented between sampling sites. Furthermore, ten out of the 25 most abundant juvenile OTUs were minor members of adult libraries, reciprocally implying that 10 of the most abundant adult OTUs were minor members of juvenile libraries. It would be interesting to investigate the role of these symbionts inside *R. piscesae*. For example, in *Bathymodiolus* mussels, the relative abundances of chemoautotrophic and methanotrophic symbionts seem to depend on environmental conditions (Salerno et al., 2005; Duperron et al., 2011). However, in Chapter 4, we noted that the phylogenetic affiliation of the V1-V3 region of the SSU rRNA gene sequences could differ from the classification of near-complete sequences. For example, while the most abundant pyrosequence OTU was assigned to the gammaproteobacterial genus *Methylomicrobium*, the near-complete sequence was classified as *Sedimenticola*. Therefore, the affiliation of our OTUs, and hence, the potential role of detected phylotypes, must be interpreted carefully. Metagenomic analysis will be needed to further understand the complex relationship between *R. piscesae* and its bacterial partners, and the variation in the composition of trophosome assemblages throughout life history.

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Chapter 6 Conclusion

Introduction

Hydrothermal vents are highly variable environments characterized by sharp chemical and physical gradients, providing a variety of habitats for microorganisms (Jannasch *et al.*, 1985; Karl, 1995). Following a general trend in marine microbial ecology, investigators are currently examining how environmental conditions shape the composition of hydrothermal vent microbial communities (Huber *et al.*, 2003; Schrenk *et al.*, 2003; Kormas *et al.*, 2006; Perner *et al.*, 2007; Hodges *et al.*, 2009; Kato *et al.*, 2009; Opatkiewicz *et al.*, 2009; Huber *et al.*, 2010; Perner *et al.*, 2011; Jaeschke *et al.*, 2012; Jorgensen *et al.*, 2012; Sylvan *et al.*, 2012b; Sylvan *et al.*, 2012a; Anderson *et al.*, 2013). My thesis contributed to this area of research by exploring the composition of bacterial communities in two contrasting, hydrothermally active environments differentiated by the main source of energy available for microbial growth (ferric iron and sulphide), employing several molecular tools and using an increasingly sophisticated approach to compare sequence data. The hydrothermal system whose main source of metabolic energy was reducing iron was located on a volcanic arc in the Southwest Pacific. I compared bacterial populations colonizing iron oxide deposits collected from two sites contrasted by depth, iron and photosynthetic pigment concentrations. The workflow developed in this study was subsequently used in the study of the second hydrothermal system, located on a mid-ocean ridge in the Northeast Pacific, in which reducing sulphur species were the main metabolic energy source. There I compared free-living and tubeworm-hosted bacterial assemblages in two contrasting habitats of the vestimentiferan tubeworm *Ridgeia piscesae*. These habitats, referred to as “High Flow” and “Low Flow” habitats, are colonized by faunal assemblages dominated by the most extreme morphotypes of this polychaete, known as the "short-fat" and the "long-skinny" morphotypes (Sarrazin *et al.*, 2002; Urcuyo *et al.*, 2003). My work on samples from these two habitats, which constitutes one of the first systematic studies of free-living bacterial communities associated with vent fauna, makes a major addition to the body of evidence for the co-occurrence of multiple bacterial phylotypes in *R. piscesae* trophosome (de Burgh *et al.*, 1989; Chao *et al.*, 2007), and provides first evidence of the influence of environmental conditions on the composition of trophosome assemblages. This exploration of compositional shifts in bacterial assemblages between habitat types and sites, as well as between two development stages, is without precedent.

In this final chapter, I review the major findings of each study and integrate the results to propose a hypothesis explaining the variation of the bacterial populations in contrasting hydrothermal vent habitats. I then discuss the limitations of my interpretation and provide suggestions for future research. Finally, I provide a summary of the major conclusions of my PhD research.

Major findings

The first study, presented in Chapter 2, compared the composition of microbial mats colonizing iron oxide deposits from hydrothermal vent sites contrasted by depth, the characteristics of iron deposition as well as iron and photosynthetic pigment concentrations. In previous studies of the microbiology of various marine habitats, Sanger sequencing of the SSU rRNA gene has proved to be an effective method for addressing basic questions about the taxonomic diversity of the bacterial communities present and potential sources of energy available for microbial growth. This technique is also useful for comparing microbial communities from different environments in order to identify potential factors influencing the taxonomic structure and diversity and to detect compositional patterns. My results showed that these iron oxide deposits, despite their superficial appearance of being mono-metabolic microbial mats, were colonized by a surprisingly high diversity of bacteria, comparable to other marine environments. Iron-oxidizing *Zetaproteobacteria* were abundant in both clone libraries, but dominated at Volcano 1, where sediments were richer in iron and contained more crystalline forms of iron oxide, suggesting a greater intensity of iron recycling. Internal sulphur cycling was also hypothesized at both sites based on the co-occurrence of sulphur oxidizers and reducers where rare to no reducing sulphur species is thought to be available through hydrothermal venting. Heterotrophic bacteria were more abundant at Volcano 1, the shallower site, where higher concentrations of photosynthetic pigments and organic matter were found. Other sources of energy available, inferred by the phylogenetic affiliations of the operational taxonomic units (OTUs) identified, included methane and nitrogen. While most taxonomic groups revealed in iron oxide deposit samples had also been detected in other hydrothermal vent habitats, their relative abundances as well as the low similarity between our clone libraries and previously published sequences suggested that iron oxide deposits host a unique microflora.

The workflow developed in Chapter 2 was subsequently used to study free-living bacterial communities associated with *R. piscesae* in contrasting hydrothermal vent habitats. These habitats, referred to as “High Flow” and “Low Flow”, differed mainly in the intensity of hydrothermal discharge, which influences local temperature and the availability of reducing sulphur species. In order to account for the variation between vent sites and allow more thorough analysis of the results, I collected four replicate samples of particular detritus from each habitat. In addition to Sanger sequencing, I used 454 pyrosequencing to improve diversity coverage and the detection of rare OTUs. The concurrent use of these two sequencing approaches allowed the comparison and identification of their respective advantages and limitations. The results revealed highly diverse bacterial communities dominated by sulphur-oxidizing *Epsilonproteobacteria*, which was consistent with previous investigations from active hydrothermal vent biotopes and confirmed the importance of reducing sulphur compounds as the main source of energy for bacterial metabolism at vents that support animal life. Statistical analyses indicated a significant relationship between the composition of bacterial communities and habitat type. OTUs differentially represented between habitats included, among others, sulphur- and hydrogen-oxidizing members of the *Epsilonproteobacteria,* which were significantly more abundant in High Flow samples, while diverse heterophophic groups were significantly more abundant in Low Flow samples. These findings suggested that similar to previously identified patterns of habitat specificity in vent faunal assemblages and tubeworm morphotypes, bacterial community composition also follows predictable patterns in High Flow and Low Flow habitats. Differences in temperature, available energy for metabolism, and stability between habitat types were hypothesized to explain these patterns. One sample collected from a Low Flow habitat at Clam Bed vent field was distinct from the other Low Flow samples. The specificity of this sample was attributed to the age of the sampling site, evaluated at ≥ 20 years, compared to the other sites located on hydrothermal edifices, which are very unstable environments. While pyrosequencing eliminated the bias introduced by the cloning step and allowed a deeper screening of the diversity, Sanger sequencing provided longer sequences and therefore higher resolution information about the phylogenetic affiliations and potential ecological roles of the detected OTUs.

A similar approach was then applied to explore the diversity of bacterial assemblages found within trophosome tissue of *R. piscesae* in High Flow and Low Flow habitats. In addition, the experimental design of this study permitted the investigation of variation between sampling sites, with five replicate samples per site collected from four High Flow and four Low Flow vent sites. Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) was used to confirm the presence of *Gamma*- and *Epsilonproteobacteria*, the two most abundant taxonomic groups detected by both Sanger sequencing and pyrosequencing. Other groups detected included *Delta*-, *Alpha*-, and *Betaproteobacteria*, which were found in the same order of relative abundances by both sequencing methods. Pyrosequence libraries also contained members of the *Bacteroidetes* and many other, rarer phyla. Statistical analyses revealed distinct trophosome assemblage membership between High Flow and Low Flow habitats, but no significant difference in terms of assemblage structure. However, both membership and structural properties of the trophosome assemblages were found to be site-specific. All assemblages showed very uneven distributions, with a few OTUs representing most of the sequences and remaining OTUs accounting for a small proportion of the sequence library. These assemblage characteristics led to the conclusion that site-specific environmental conditions shaped the composition of *R. piscesae* trophosome assemblages, resulting in a unique assemblage structure at each vent site, possibly masking the influences of High Flow and Low Flow habitat conditions.

To control for potential pyrosequencing artefacts and environmental contamination in the trophosome bacterial sequences, I applied very strict quality filters and compared the resulting sequence library to pyrosequencing data obtained from the free-living bacterial communities collected from the same habitats. The five most abundant OTUs detected in trophosome tissue were absent from the free-living data set and the four most abundant members of the free-living communities represented minor OTUs in the trophosome library, providing further evidence of the authenticity of the results.

Building on the results obtained from adult *R. piscesae*, I used pyrosequencing to explore the composition of juvenile trophosome assemblages. Juvenile samples were collected together with the previously studied adults, allowing the comparison of trophosome assemblages between two stages of tubeworm development and between sampling sites. Potential patterns influences of High Flow and Low Flow habitat conditions were also investigated. The major taxonomic groups detected in juvenile trophosome assemblages were the same as in adults, in similar order of relative abundance, coherent with a horizontal mode of transmission of bacterial partners and further validating our evidence of multiple bacterial partners in the *R. piscesae* trophosome tissue. Statistical analyses indicated that adults and juveniles from a same vent site shared similar members but in different proportions, suggesting the existence of a selection process during the development of *R. piscesae.* Distinct membership of the trophosome assemblages was observed between sampling sites and habitat types, but no differences in the structure were detected. Variability in the proportions of the most abundant OTUs between adult and juveniles could be masking patterns at the site and habitat levels.

Major conclusions

These studies suggested that local conditions at hydrothermal vents, such as sources of energy available, depth, intensity of hydrothermal discharges, and stability, have detectable and sometimes strong influences on the composition of resident bacterial populations. While general patterns emerged from the comparison of the composition of free-living bacterial communities between contrasting habitats, deeper investigations revealed site-specific characteristics in bacterial populations within the same general habitat type. Similar conclusions were drawn from the exploration of *R. piscesae* trophosome assemblages in both juvenile and adult individuals. Hydrothermal vent ecosystems are characterized by a wide range of physical and chemical conditions and these results suggest that conditions at each vent site are unique, providing a distinctive biotope for bacterial communities. These results will be used by the Canadian Healthy Oceans Network (CHONe) to develop guidelines for the establishment of conservation strategies for the Endeavour Hydrothermal Vents Marine Protected Area.

The comparison of free-living and trophosome bacterial assemblages associated with *R. piscesae* provided insights into the establishment of the relationship between the tubeworm and bacterial partners. Assuming a horizontal mode of symbiont transmission in siboglinids, an active selection of site-specific phylotypes is thought to occur during the process of the ‘colonization’ of *R. piscesae* trophosome tissue by bacteria, as evidenced by our observation that the most abundant members of their trophosome assemblages were rare or undetectable in the free-living bacterial communities. This is followed by a shift in the relative abundance of the bacterial partners, which could be related to shifting physiological requirements during the development of *R. piscesae* or to weaker selection barriers in young tubeworm. Hosting multiple bacterial phylotypes could be advantageous in unstable environments with variable conditions. However, the presence of heterotrophic phylotypes could also indicate an infection of *R. piscesae* trophosome tissue.

Limitations and direction for future research

The main goal of this work was to compare the membership and structure of bacterial communities in contrasting hydrothermal vent environments in order to better understand the influence of habitat on community composition. The sampling strategy employed was adequate for this purpose, but our results also suggested that while some compositional patterns can emerge from the comparison of habitat types, bacterial communities are also site-specific. Experimental designs for future investigations of site-level specificity should include paired sampling of free-living and symbiotic bacterial communities at each vent sites that include the entire range of conditions between the High Flow and Low Flow extremes. Since High Flow vents have been proposed to eventually evolve into Low Flow vents as hydrothermal discharge weakens, such an approach would permit the exploration of bacterial community succession, rather than the simple contrasting of extremes undertaken in this study.

Another avenue for future study that has emerged from this work is the site-specific nature of *R. piscesae* juvenile trophosome assemblages. Such a comparison was not possible with our sampling strategy of harvesting juveniles from adult tubeworms and pooling juvenile tissue to obtain enough material for DNA extraction, since it resulted in only one true replicate per sampling site. Juveniles should be sampled in sufficient number to allow replication at each vent site, so that analytical error can be quantified. As well, the collection of even younger specimens should be investigated since this would permit further exploration of the selection process.

Both sequencing methods used in this project have their advantages and drawbacks. Sanger sequencing offers sequences of sufficient length for confident classification to the species level as opposed to pyrosequencing, which yields shorter reads, which can lead to erroneous phylogenetic affiliation even at the genus level. However, even with species-level taxonomic identification, the low degree of similarity with previously published sequences and the difficulty of successfully cultivating hydrothermal vent bacteria continue to prevent the inference of metabolism and the ecological role of bacteria based on SSU rRNA gene sequence information. Metagenomics or direct RNA sequencing could be used for exploring the presence of key genes and their expression in order to better understand the dynamics of the bacterial populations.

The limited amount of sequences provided by Sanger sequencing was offset by the large data set obtained through pyrosequencing, allowing a better coverage of the diversity and powerful statistical analyses. However, pyrosequencing data should only be considered semi-quantitative. The results obtained for the composition of *R. piscesae* trophosome assemblages could be used to design specific probes targeting the most abundant members to obtain conclusive proof of their presence and abundance in tubeworm tissue using CARD-FISH. Furthermore, these probes could be tested against free-living communities in order to better understand the processes underlying the relationship between *R. piscesae* and its multiple bacterial partners.

Summary

This research project contributed to answering important questions about the microbial ecology of hydrothermal vent ecosystems. The importance of sulphur oxidizing bacteria to vent ecosystems has resulted in many other types of bacterial metabolism being under-appreciated, and little investigated. The surprisingly high taxonomic diversity found in all diffuse flow vent habitats studied here provides a basis for further exploration of the role of other autotrophic, and heterotrophic bacteria in these unique ecosystems.. In addition, my results have expanded our understanding of the role of local habitat conditions in the determination of microbial community composition and diversity. Iron oxide deposits were mainly colonized by iron-oxidizing *Zetaproteobacteria*. Iron concentration and depth were suggested to explain differences observed between two sites. Sulphur-oxidizing *Epsilonproteobacteria* dominated free-living bacterial communities associated with *R. piscesae* in both High Flow and Low Flow habitats. Compositional difference between habitat types and between sampling sites were hypothesized to be the result of specific local conditions. Sulphide was the main source of energy used by bacteria in both High Flow and Low Flow habitats, but the availability of other sources of energy was inferred by the presence of methanotrophs, hydrogen and ammonia oxidizers. Environmental conditions were found to influence the composition of trophosome assemblages in *R. piscesae*, which is thought to actively select bacterial partners from free-living communities during its juvenile phase. Shifting physiological requirements or selection barriers were suggested to explain observed differences in the relative abundance of the major members of the trophosome assemblage between juveniles and adult tubeworms.

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APPENDIX A
Supplementary table for Chapter 2

Table A.1 Sediment characteristics for two iron oxide samples collected on Volcanoes 19 and 1.

Sampling site	Depth (m)	Chl *a* (μg/l)	Phaeo (μg/l)	% total N	% total C	% carb C	% org C
Volcano 19	992	39.0	251.3	0.037	0.874	0.385	0.489
Volcano 1	197	46.4	1687.0	0.064	0.996	0.144	0.853

APPENDIX B
Composition and diversity of microbial mats at shallow hydrothermal vents on Volcano 1, South Tonga Arc

Abstract

Hydrothermal vent-associated microbial mats play an important role in food webs and mineralization processes but their composition and diversity are poorly understood, especially in shallow hydrothermal systems where both light and chemical energy are available. This study compared the bacterial communities of three microbial mat samples collected from Volcano 1 on the Tonga-Kermadec Arc using a PCR-based approach. Small subunit ribosomal RNA gene clone libraries were created from two samples from photic and aphotic depths and found to be highly similar in composition and highly diverse relative to other deep and shallow vent microbial mats. The role of fluctuating hydrothermal discharge in maintaining this high level of diversity is discussed. Both samples were dominated by *Gammaproteobacteria* with *Epsilonproteobacteria* as the second most abundant group. A third sample from a previously characterized Volcano 1 Fe-oxide deposit was used for comparison. The photic and aphotic samples were phylogenetically similar while there was limited similarity between the aphotic and Fe-oxide samples which are both from aphotic depths. This suggests a lesser (if any) role for the presence or absence of light in determining community composition in comparison with the role of hydrothermal conditions.

Introduction

Microbial mats are frequently observed as filamentous biofilms and discontinuous tufts on mineral and animal surfaces at hydrothermal vents and are considered to be important food sources for the vent endemic fauna. Numerous stable isotope and lipid biomarker studies have evaluated the contribution of this microbial material to the diets of vent invertebrates and have shown indications of habitat-specific differences in microbial carbon fixation pathways that feed invertebrate food chains (e.g., Comeault et al., 2010). Organisms present in microbial mats have also been implicated in increased crystallization of iron oxides at hydrothermal vents through biological iron cycling (Langley et al., 2008). However, despite their potential importance to food webs and mineralization processes, our understanding of the composition and metabolism of microbial mats at hydrothermal vents remains rather limited, with few attempts made to explore the biodiversity of these communities using molecular tools. Recent exceptions include studies of filamentous microbial mats that accumulate iron oxide at low temperature vents, which have a surprisingly high diversity of bacteria (Davis and Moyer, 2008; Hodges and Olson, 2009; Forget et al., 2010).

The microflora of microbial mats and sediments has been examined in many other marine environments (reviewed in Cohen and Rosenberg, 1989) and in shallow, hypersaline habitats. In the latter, cm-thick mats host complex communities of microbes that vary across gradients of light, redox conditions and hydrogen sulphide (Guerrero and Mas, 1989; Ley et al., 2006). Water temperature, flow rate, and pH are also known to affect the composition of microbial mats. Diversity within the micro- habitat formed by filamentous mats can be augmented by feedback loops wherein the metabolic bi-products of one species may create favourable metabolites for other species (Guerrero and Mas, 1989). Additional species create a wider variety of metabolites that may support rarer and more specialized microbes.

There are few published studies of microbial communities from shallow hydrothermal systems where both light and chemical energy sources are available. Hirayama et al. (2007) used molecular tools to characterize a very diverse microbial mat collected from a shallow sub- marine vent system near Taketomi Island, Japan, and concluded that the dominant pathway for carbon fixation in these photic zone microbial mats was chemosynthesis rather than photosynthesis. Here we examine the composition and phylogenetic diversity of two filamentous microbial mats growing at active hydrothermal sites on Volcano 1 on the South Tonga Arc. One of the mats was sampled at vents within the photic zone, at the summit of a volcanic cone (100-115 m depth), while the other was collected in areas of diffuse venting below the photic zone (189-197 m depth). Both mats had a typical white filamentous appearance as has been described from sulphidic vent sites elsewhere. Bacterial mat composition at a non-sulphidic, Fe-oxide deposit on Volcano 1 was previously described by Forget et al. (2010). Data from this third hydrothermal site are compared with results from the present study to provide further perspective on the influence of habitat on the composition of bacterial mats.

Materials and Methods

In the Southern Pacific Ocean between New Zealand and Fiji, a 425 km long segment of the Tonga-Kermadec Arc known as the South Tonga Arc spans from 21°-25°S (Massoth et al., 2007) and is a focus of submarine hydrothermal and volcanic activity (Fig. A.1). Volcano 1 is the northernmost volcano in this segment. The most intense hydrothermal venting on the South Tonga Arc has been recorded at Volcano 1 where there are several sites of widespread, diffuse venting on the Southern rim of the caldera (Massoth et al., 2007).

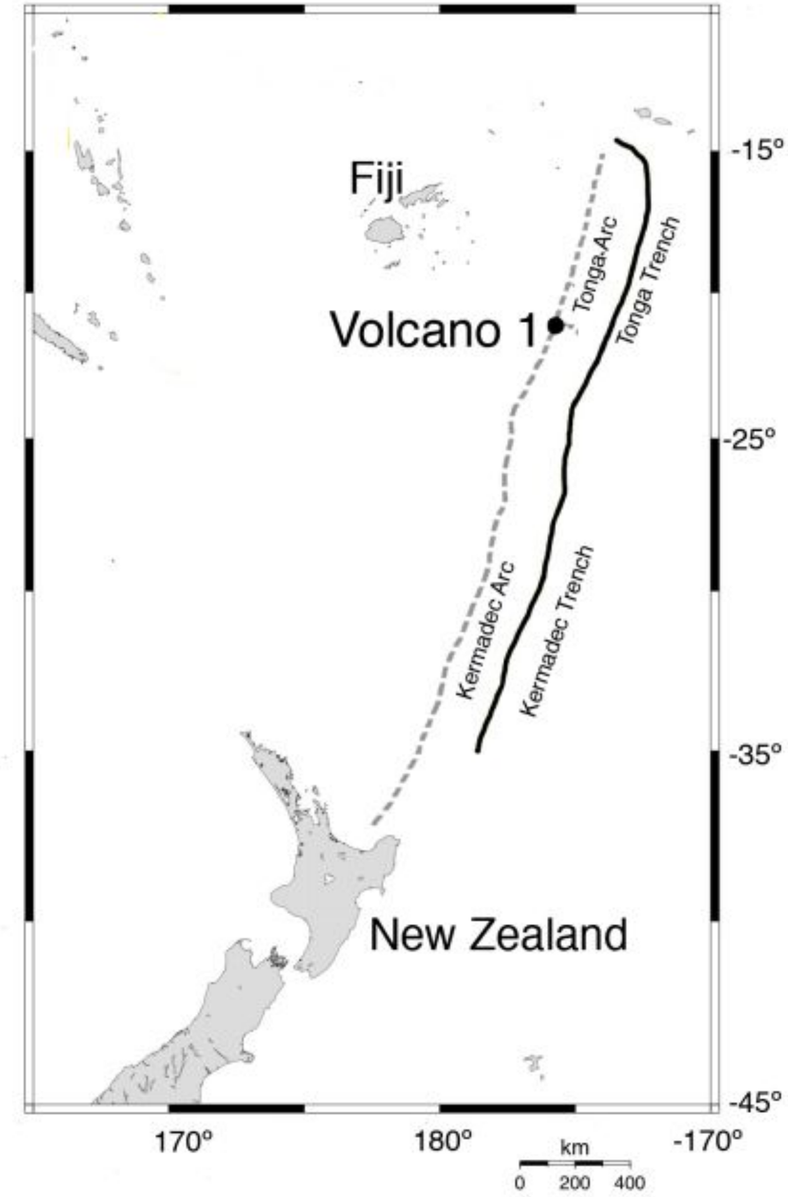

Figure B.1

Map showing the location of Volcano 1 on the Tonga section of the Tonga-Kermadec Arc, which runs between New Zealand and Fiji. The dashed line indicates the arc and the solid line indicates the trench.

Our photic zone sample was collected from the summit of a post caldera scoria cone (175°45.529'W-21°8.592'S) at a depth of 115.3 m where penetration of light was visually apparent and red and green algae shared the substrata with the microbial mat.

Ambient seawater temperature was 19.2°C and sediment temperature was 20°C. Bacterial mat occurred as macroscopic white tufts that formed a continuous coating on large boulders. The aphotic sample was taken from the floor of an explosion crater on the caldera rim (175°44.6392'W-21°09.3468'S) at a depth of 189.1 m, where ambient temperature was 16.7°C and sediment temperature was 16.9°C. Here, the bacterial mat was also white but was less extensive and grew as a thin coating on individual particles of volcaniclastic gravel. The iron-rich microbial mat described in Forget et al. (2010) was collected approximately 500 m away from the aphotic sample at a depth of 197 m. All three samples were collected during the SONNE 192/2-MANGO cruise (2007) by the remotely operated vehicle ROPOS using a suction sampler. After collection, samples were stored at -80°C.

DNA was extracted from the photic and aphotic samples using the UltraClean™ Microbial DNA Isolation Kit by MO BIO. Bacterial 16S ribosomal RNA (rRNA) libraries were constructed and analysed from each sample as described by Forget et al. (2010). Chao-1 species richness and abundance-based coverage estimators (ACE) were measured using DOTUR (Schloss and Handelsman, 2005) based on the distance matrices obtained with the PHYLIP software package, v3.68 (Felsenstein, 2004). Fast UniFrac (Hamady et al., 2010), an online tool for comparing gene sequences, was used to determine phylogenetic distances and to perform a phylogenetic (P) test, a Jackknife sample cluster analysis, and a principal coordinates analysis (PCoA) for all three samples (this study plus the Volcano 1 sample from Forget et al. (2010)).

The 16S gene sequences representative of each phylotype in the photic and aphotic samples were deposited in the GenBank database under the accession numbers GU369872-GU369937 and HQ153833-HQ154017.

Results

After the removal of poor quality sequence data and chimeras, 268 clones from the photic mat and 262 clones from the aphotic mat remained for a total of 530 clones. The clones from the photic and aphotic mats were divided among 127 and 125 phylotypes, respectively, using a 97% similarity cutoff for the phylotype definition. Neither rarefaction curve reached an asymptote (data not shown) and diversity estimators (Chao-1 and ACE) revealed a high level of diversity when compared to other vent-associated mat and sediment samples (Table A.1).

Proteobacteria in the photic and aphotic mats comprised 66% and 72% of the total number of clones, respectively. In both samples, the *Gammaproteobacteria* were the most abundant group (33% of photic, 35% of aphotic) followed by the *Epsilonproteobacteria* (17% of photic, 19% of aphotic). Clones belonging to the *Alphaproteobacteria* were slightly more abundant than those belonging to the *Deltaproteobacteria* (a- 13%, d- 5%) in the aphotic mat, whereas in the photic mat these two groups were found in similar proportions (8% each). Bacteroidetes made up 10% and 11% of the aphotic and photic clones, respectively, and ten other phyla were detected at much smaller proportions (Fig. A.2). The majority of clones (311 of 530) were detected in both mats and distributed among only 34 phylotypes (Fig. A.2). Phylotypes containing the largest numbers of clones were those shared between sites and

Table B.1 Comparison of bacterial diversity estimates from similar microbial mat and sediment samples. SMBSC = Southern Mariana Back-Arc Spreading Centre.

Environment	Diversity estimators		Dominant 16S group	Reference
	ACE	Chao-1		
Iceberg Vent, NW Rota-1	2	2	*Epsilonproteobacteria*	Davis and Moyer, pers. comm.
Champagne Vent, Eifuku	20	19	*Epsilonproteobacteria*	Davis and Moyer, pers. comm.
Tangaroa Floc	54	49	*Epsilonproteobacteria*	Hodges and Olson, 2009
Clark Floc	59	61	*Epsilonproteobacteria*	Hodges and Olson, 2009
Fryer Site 1W, SMBSC	111	97	*Deltaproteobacteria*	Davis and Moyer, pers. comm.
Fryer Site 2W, SMBSC	120	112	*Nitrospira*	Davis and Moyer, pers. comm.
Tangaroa Sediment	151	160	*Epsilonproteobacteria*	Hodges and Olson, 2009
Volcano-1 Iron Oxide Sediment	281	241	*Zetaproteobacteria*	Forget et al, 2010 and pers. comm.
Volcano 1 Photic Mat	**482**	**398**	***Gammaproteobacteria***	**This study**
Volcano 1 Aphotic Mat	**614**	**509**	***Gammaproteobacteria***	**This study**
Taketomi Island fluids	ND	ND	*Gammaproteobacteria*	Hirayama et al, 2007
Taketomi Island mats	ND	ND	*Gammaproteobacteria*	Hirayama et al, 2007

corresponding phylotypes held similar numbers of clones indicating a similar community structure between mats. These phylotypes were generally related clones retrieved from other hydrothermally influenced sources (i.e. sediments, mats or symbionts of vent organisms) or marine and gas hydrate sediments.

Compositional differences between the two mats were the result of a high number of rare or infrequent phylotypes (90 phylotypes containing 109 clones in the photic zone sample and 88 phylotypes containing 110 clones for the aphotic sample, Fig. A.2). The UniFrac distance and the P test value measured for each pair of samples showed that the composition of the microbial communities detected in the Volcano 1 iron oxide sediment sample of Forget et al. (2010) was significantly different from the photic and aphotic

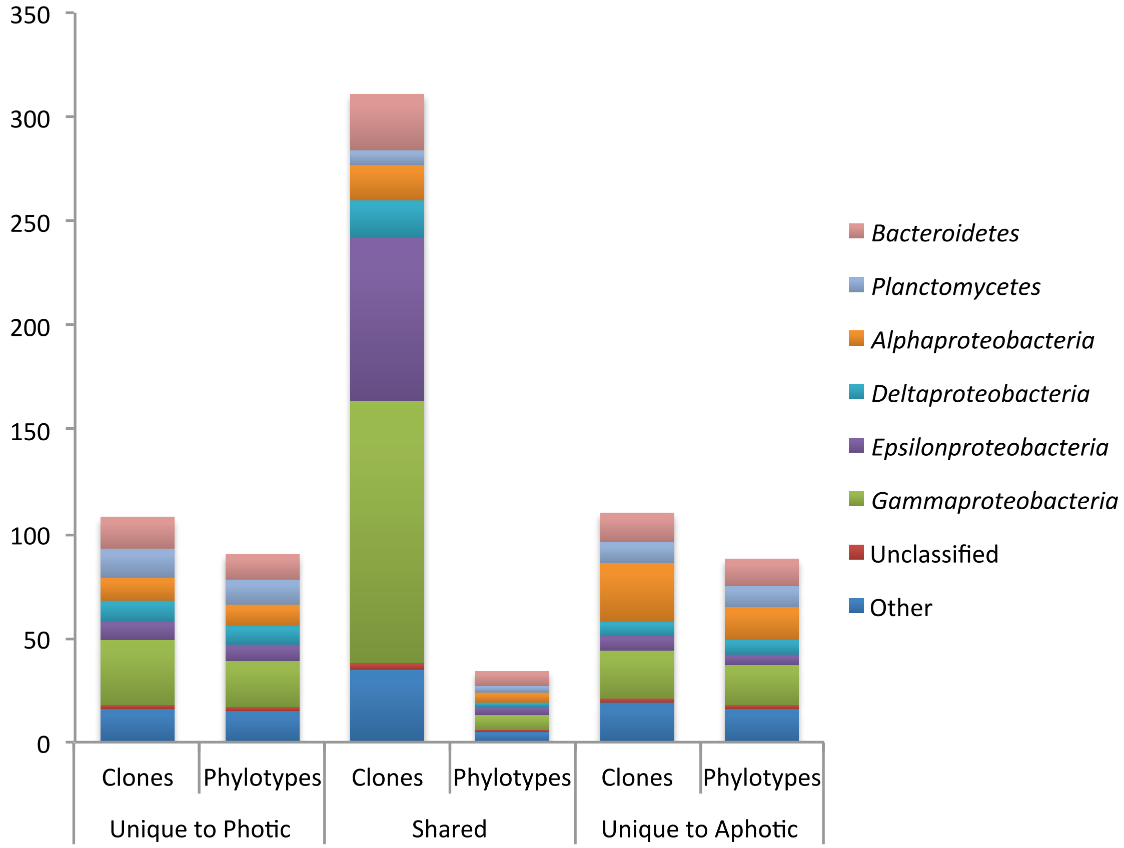

Figure B.2

Taxonomic composition of clones and phylotypes which are shared between samples or unique to each sample. Clones belonging to phyla which contributed less than 5% to the total composition of the samples are contained in the group "Other" and include *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Deferribacteres*, *Spirochaetes* (in the photic sample only) and *Verrucomicrobia* (in the aphotic sample only). The unclassified group refers to clones whose closest GenBank database match was too distant to confidently assign it to a phylum.

zone samples, while these latter two samples were not significantly different from one another (Table A.2). Similarly, a Jackknife sample cluster analysis (data not shown) grouped our samples together with a Jackknife value of 100%, while the iron oxide sediment sample from Volcano 1 was positioned outside of this group.

The phylotype dominating both mats was a *Gammaproteobacterium* whose closest match was retrieved from an iron oxide mat at Clark Seamount (Hodges and Olson, 2009). This phylotype grouped within the order *Thiotrichales*, and shared 90% sequence similarity with the isolate *Leucothrix mucor* which is capable of lithoheterotrophy by means of sulphite and thiosulphate oxidation (Grabovich et al., 1999). The order *Thiotrichales* also contains the thiosulphate-oxidizing *Sulfurivirga caldicuralii*, which was isolated from the shallow hydrothermal system off Taketomi Island, Japan (Takai et al., 2006). Clones belonging to this order represented 17% and 19% of the photic and aphotic samples, respectively and the dominant phylotype accounted for 28% of the 311 clones shared between sites.

The second largest shared group, which contained 4 phylotypes classified under the epsilonproteobacterial genus *Sulfurovum*, accounted for 24% of the shared clones. These phylotypes shared 94-95% sequence similarity with the isolate *Sulfurovum lithotrophicum*. Isolated from hydrothermal sediments, *S. lithotrophicum* is a facultative anaerobic chemolithoautotroph that oxidizes elemental sulphur and thiosulphate using oxygen or nitrate as electron acceptors (Inagaki et al., 2004).

Purple sulphur bacteria of the gammaproteobacterial order *Chromatiales* were found in both the photic and aphotic mats and accounted for 12% of the shared phylotypes. This order contains both photo- and chemosynthetic organisms as well as some phototrophs that can revert to chemotrophy in dark conditions (Imhoff, 2005). Four phylotypes belonging to this order were found in both mats with 92% similarity to *Thioprofundum*

Table B.2 UniFrac distances and P test values obtained with 1000 permutations.

Comparison	UniFrac distance	P-value
Photic zone vs Iron oxide mat	0.62	< 0.001
Aphotic zone vs Iron oxide mat	0.66	< 0.001
Photic zone vs Aphotic zone	0.33	> 0.01

lithotrophica, a sulphur-oxidizing chemolithoautotroph isolated from a hydrothermal vent chimney (Takai et al., 2009).

Other lineages known to contain phototrophic organisms, such as the Chloroflexi, Cyanobacteria, and certain orders belonging to the *Alphaproteobacteria*, were detected in small numbers and in similar abundances from both photic and aphotic libraries.

Discussion

Although the photic and aphotic mats showed a great degree of similarity in their microbial communities, there was a much greater abundance of mat material at the shallower site. The photic zone bacterial mat was thick enough to be visible in video imagery whereas the mat at the deeper site could only be seen microscopically as a filamentous coating on gravel particles. This macroscale difference could be related to hydrothermal fluid chemistry or substratum effects which were not included in this study. However, video observations showed the hydrothermal vent crab *Xenograpsus ngatama* to be considerably more abundant at the deeper site than on the photic zone mats. While it is not possible to quantify grazing pressure at either site, stable isotope and lipid biomarker analysis identified this crab as a consumer of microbial mats at both sites (Comeault et al., 2010).

Comparison of the Volcano 1 mat samples to other hydrothermally influenced mats and sediments revealed a relatively high level of diversity (Table A.1). With few available comprehensive studies of shallow hydrothermal microbial communities it is difficult to say whether this level of diversity is unusual in such environments. An investigation of the shallow hydrothermal system off Taketomi Island by Hirayama et al. (2007) sequenced a small number of clones compared to the present study, and did not provide diversity estimators. However, the large number of phylotypes found in their mat sample (57 in 78 clones) suggests a level of diversity comparable to the communities at Volcano 1.

The microbial communities of Volcano 1 and Taketomi Island are also similarly distinct from other microbial mat samples in their composition. Whereas the *Epsilonproteobacteria* are widely reported to be the dominant group in hydro- thermal vent microbial mats and fluids, both the Volcano 1 and Taketomi mats were dominated by members of the *Deltaproteobacteria* (Table A.1). Some insight into possible habitat influences on the dominance of the *Epsilonproteobacteria* in hydrothermal vent mats can be found in an extensive survey of microbial mats along the Mariana Island arc and back-arc system by Davis and Moyer (2008). This study identified three distinct clusters of T-RFLP bacterial community fingerprints. The so-called Mariana Cluster II was only comprised by samples from shallow and mostly lower temperature mats. It was highly diverse and dominated by phylotypes associated with seawater and sediments (i.e. non-hydrothermal environments). In contrast, Mariana Cluster III contained samples from warmer vents, and clone libraries from Cluster III samples were dominated by *Epsilonproteobacteria*.

Clone libraries from Volcano 1 did contain some clones that were most closely related to database sequences with a non-hydrothermal affiliation (i.e. Mariana Cluster II). In addition, *Epsilonproteobacteria* were the second most abundant group in these samples and a large portion of clones was related to sequences from hydrothermally influenced environments. One explanation for these affinities with both Mariana clusters II and III is that there may have been considerable environmental variability at the Volcano 1 vents, resulting in the accumulation of phylotypes favoured by both weaker and stronger hydrothermal conditions. Fluctuating hydrothermal discharge was proposed by Langley et al. (2008) to explain advanced crystallization of Fe-oxides observed in an iron oxide sample from Volcano 1. They suggested that waxing and waning of vent discharge could create alternating cycles of oxidizing and reducing conditions for biological iron cycling. In another study of these same iron oxide sediments, Forget et al. (2010) also invoke fluctuating hydrothermal conditions to explain the presence of 16S gene sequences related to both iron oxidizing and iron reducing bacteria.

Previous investigations of Volcano 1 observed local high temperature venting up to 152°C (Stoffers et al., 2006). No high temperature venting was observed during the 2007 MANGO cruise. Instead, we found only widespread diffuse venting and gas discharge. At the time of collection, the photic and aphotic mats were not exposed to direct hydrothermal discharge and there were no significant temperature anomalies associated with the samples. If hydrothermal activity on Volcano 1 is indeed quite variable, it is possible that these microbial mats could be periodically exposed to conditions ranging from ambient seawater to anoxic hydrothermal fluid, resulting in varying availability of reducing chemical species. Davis and Moyer (2008) report a shift in diversity of microbial mats at a similarly dynamic setting on N.W. Rota-1 Volcano on the Mariana Arc. The shift corresponded to a drop in temperature and hydro- thermal discharge at the Iceberg site over a two-year sampling period, and they propose the dynamic nature of the N.W. Rota-1 volcano as a potential reason for the change in diversity. Similarly, the high diversity mats observed at Volcano 1 may host groups of organisms that can take advantage of more or less intense hydrothermal conditions, as well as others exploiting bi-products of their metabolisms. In addition, the metabolically diverse *Gammaproteobacteria*, which dominated both photic and aphotic samples, include both photo- and chemotrophic groups as well as groups that can opportunistically switch between utilization of the two energy sources (Imhoff, 2005), providing a unique advantage in ephemeral or marginal hydrothermal vent habitats found in the photic zone. Evidently this latter capability does not explain their presence in our sample from below the photic zone.

Based on culture-dependent and –independent analyses, which showed high abundances of organisms involved in sulphur metabolisms, Hirayama et al. (2007) concluded that the microbial community in the shallow hydrothermal system off Taketomi Island is sustained by chemosynthesis rather than photosynthesis, despite an abundance of sunlight. At Volcano 1, the high degree of phylogenetic similarity between the photic and aphotic samples and low degree of similarity between the aphotic and Fe-oxide samples (Table A.2), which are both from aphotic depths, similarly suggests that the presence or absence of light plays a lesser (if any) role in determining diversity and community composition than do the available chemical species. This is supported by the dominance of clones in both photic and aphotic libraries with a high level of relatedness to organisms involved in sulphur metabolisms, and a paucity of those belonging to phototrophic lineages.

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APPENDIX C
Supplementary tables for Chapter 3

Table C.1 454 pyrosequencing summary.

Sample ID	Total reads	Quality control[a]	Unique[b]	OTUs
HF8SMb	37226	28693	4010	1192
LF8SMb	38546	28301	4224	1235
HF8GH1b	55230	42024	4562	1253
LF8GH1b	24732	17864	3521	1600
HF8GH2b	3795	2130	917	476
LF8GH2b	4953	2923	1056	518
HF8CBb	10364	7883	1105	364
LF8CBb	12549	10201	1091	412

[a] See the Experimental Procedures for the description of the quality filters.

[b] Number of sequences after removing identical sequences.

Table C.2 Occurrence of the gammaproteobacterial genera within the eight sequence libraries. The genera in bold represented more than 1% of the total number of sequences.

Genus	HF8SMb	HF8GH1b	HF8GH2b	HF8CBb	LF8SMb	LF8GH1b	LF8GH2b	LF8CBb
Alkanindiges						x		
Aquimonas		x			x	x		x
Aquisalimonas						x		
Azorhizophilus			x					
Bermanella						x		x
Caedibacter						x		
Carnimonas						x	x	
Chromatium	x	x			x	x		
Cobetia							x	
Cycloclasticus		x	x		x	x		x
Dasania	x				x	x	x	x
Ectothiorhodosinus	x	x	x		x	x	x	x
Endozoicomonas	x	x	x	x	x	x	x	
Enhydrobacter						x		
Escherichia/Shigella	x							
Fangia					x	x	x	
Granulosicoccus		x			x	x	x	x
Haliea								x
Halospina						x		
Halovibrio					x			
Hydrogenovibrio	x							
Idiomarina						x		
Kangiella					x	x	x	
Leucothrix	x	x	x		x	x	x	x
Litoricola						x		x
Marinimicrobium						x		
Methylobacter					x	x		
Methylohalomonas		x				x		x
Methylomonas					x	x	x	

Methylonatrum					x			
Methylosarcina	x	x	x		x	x	x	x
Methylosoma						x		x
Methylosphaera					x		x	
Modicisalibacter		x						
Neptuniibacter	x					x	x	
Neptunomonas						x	x	x
Nitrosococcus		x					x	
Oceanobacter					x	x		
Oleispira								x
Photobacterium						x		
Piscirickettsia						x		
Plesiomonas						x		
Pseudomonas		x						
Psychrobacter	x							
Rhabdochromatium								x
Saccharophagus		x					x	
Saccharospirillum		x			x	x		
Sedimenticola		x				x		
Simiduia								x
Solimonas			x					
Spongiibacter						x	x	x
Spongiispira			x			x		
Stenotrophomonas	x					x		
Thermithiobacillus		x						
Thioalkalibacter	x				x	x		x
Thioalkalispira		x			x	x	x	
Thioflavicoccus						x		
Thiohalocapsa						x		
Thiohalophilus	x	x			x	x		x
Thiohalospira						x		
Thiorhodospira						x	x	
Zooshikella	x	x				x	x	x

Table C.3 Occurrence of the deltaproteobacterial genera within the eight sequence libraries. The genera in bold represented more than 1% of the total number of sequences.

Genus	HF8SMb	HF8GH1b	HF8GH2b	HF8CBb	LF8SMb	LF8GH1b	LF8GH2b	LF8CBb
Anaeromyxobacter		x				x		
Archangium								x
Bacteriovorax	x	x	x	x	x	x		x
Bdellovibrio						x		
Byssovorax		x				x		
Chondromyces		x						
Desulfacinum	x							
Desulfarculus		x				x	x	
Desulfatiferula	x	x	x		x	x		x
Desulfatirhabdium					x	x		
Desulfobacca	x				x	x		
Desulfobacterium					x		x	x
Desulfobacula								x
Desulfobotulus			x			x		x
Desulfobulbus	x	x	x	x	x	x	x	x
Desulfocapsa	x	x	x	x	x	x	x	x
Desulfocella				x				
Desulfococcus						x		
Desulfocurvus	x					x		
Desulfofustis	x	x			x	x		
Desulfoglaeba	x					x		
Desulfoluna	x	x	x	x	x	x	x	
Desulfomonile		x				x		
Desulfonatronovibrio			x					
Desulfonema	x		x	x	x	x	x	
Desulfopila	x		x		x	x		
Desulforegula		x				x		x
Desulforhabdus	x	x		x		x	x	
Desulforhopalus	x			x	x	x		x

Desulfospira	x					x		
Desulfotalea				x	x	x		x
Desulfovibrio		x						
Desulfovirga	x					x	x	
Desulfurivibrio							x	
Desulfuromonas	x	x	x		x			
Desulfuromusa	x	x	x	x	x	x	x	x
Enhygromyxa		x						
Geopsychrobacter	x	x	x	x	x	x	x	x
Hippea	x					x	x	x
Hyalangium	x							
Kofleria	x				x	x		
Lawsonia	x	x				x		
Malonomonas	x		x			x		x
Melittangium	x	x	x	x	x	x	x	
Nannocystis		x						
Peredibacter		x						
Phaselicystis						x		
Plesiocystis			x		x	x	x	x
Pyxidicoccus	x		x				x	
Smithella		x				x		x
Sorangium	x	x			x	x		
Syntrophorhabdus					x	x		

Table C.4 Occurrence of the alphaproteobacterial genera within the eight sequence libraries.

Genus	HF8SMb	HF8GH1b	HF8GH2b	HF8CBb	LF8SMb	LF8GH1b	LF8GH2b	LF8CBb
Afifella		x	x		x	x		
Afipia						x		
Ahrensia					x	x		x
Albimonas	x							
Amaricoccus								x
Amorphus								x
Anderseniella						x		x
Angulomicrobium						x		
Bradyrhizobium	x							
Caenispirillum						x		
Catellibacterium								x
Cohaesibacter							x	
Croceicoccus					x			x
Cucumibacter		x			x	x		
Devosia								x
Filomicrobium	x	x		x		x		x
Hellea		x				x	x	
Henriciella			x					
Hirschia		x			x	x		
Hoeflea						x		
Hyphomicrobium		x			x	x		
Jannaschia		x						
Kordiimonas						x		x
Leisingera						x		x
Loktanella						x		
Lutimaribacter						x		
Maritalea					x	x	x	x
Methylocapsa	x					x		
Methylocystis								x
Methylorhabdus						x		

Neoasaia			x					
Nereida			x			x		x
Oceanibaculum								x
Oceanibulbus						x		
Oceanicola	x							
Octadecabacter		x	x			x		x
Orientia						x		
Pannonibacter						x		
Paracoccus					x			
Parvibaculum		x				x	x	
Pelagibius					x	x		
Pelagicola								x
Ponticoccus						x		
Porphyrobacter					x			
Prosthecomicrobium						x		
Rhodopila	x	x				x		
Rhodoplanes							x	
Rhodospira						x		
Robiginitomaculum			x			x		
Roseomonas				x				
Rubellimicrobium					x			
Sandaracinobacter								x
Sandarakinorhabdus						x		
Shimia		x				x		x
Sinorhizobium						x		x
Sphingopyxis								x
Sphingosinicella								x
Starkeya		x						
Sulfitobacter	x					x		
Terasakiella						x		
Thalassobacter								x
Thalassobius						x		x
Thalassospira						x		

Tistrella							x
Zhangella	x			x	x	x	x

Table C.5 Occurrence of the betaproteobacterial genera within the eight sequence libraries.

Genus	HF8SMb	HF8GH1b	HF8GH2b	HF8CBb	LF8SMb	LF8GH1b	LF8GH2b	LF8CBb
Brachymonas								x
Leptothrix					x			
Methylibium	x							
Methylotenera	x	x			x	x		
Oxalicibacterium					x			
Rubrivivax					x			
Sutterella		x						
Thermothrix	x							
Variovorax	x							

APPENDIX D
List of primers used for pyrosequencing

To achieve the PCR amplifications of the bacterial SSU rRNA gene hypervariable regions V1-V3, a general reverse primer (R519) combined with B primer (Roche) was used in combination with a unique to tagged forward primer (F63-targeted) combined with A primer (Roche):

519R CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGWATTACCGCGGCKGCTG

F63-1 CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTCAGGCCTAACACATGCAAGTC

F63-2 CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACACAGGCCTAACACATGCAAGTC

F63-3 CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACGCACTCCAGGCCTAACACATGCAAGTC

F63-4 CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGCAGGCCTAACACATGCAAGTC

F63-5 CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGCAGGCCTAACACATGCAAGTC

F63-6 CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGCAGGCCTAACACATGCAAGTC

F63-7 CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTACAGGCCTAACACATGCAAGTC

F63-8 CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCCAGGCCTAACACATGCAAGTC

F63-9 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGCAGGCCTAACACATGCAAGTC

F63-10 CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTCAGGCCTAACAC
ATGCAAGTC

F63-11 CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGCAGGCCTAACAC
ATGCAAGTC

F63-12 CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACCAGGCCTAACAC
ATGCAAGTC

F63-13 CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTACAGGCCTAACAC
ATGCAAGTC

F63-14 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTACAGGCCTAACAC
ATGCAAGTC

F63-15 CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACCAGGCCTAACAC
ATGCAAGTC

F63-16 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTACGTAGCCAGGCCTAACAC
ATGCAAGTC

F63-17 CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCCAGGCCTAACAC
ATGCAAGTC

F63-18 CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGCAGGCCTAACAC
ATGCAAGTC

F63-19 CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGCAGGCCTAACAC
ATGCAAGTC

F63-20 CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGAGTATGCAGGCCTAACAC
ATGCAAGTC

F63-21 CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGCAGGCCTAACAC
ATGCAAGTC

F63-22 CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGCAGGCCTAACAC
CATGCAAGTC

F63-23 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTCGCTCGCAGGCCTAACACATGCAAGTC

F63-24 CCATCTCATCCCTGCGTGTCTCCGACTCAGACATACGCGTCAGGCCTAACACATGCAAGTC

F63-25 CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGAGTATCAGGCCTAACACATGCAAGTC

F63-26 CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTCAGGCCTAACACATGCAAGTC

F63-27 CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACAGTCAGGCCTAACACATGCAAGTC

F63-28 CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACTATACTCAGGCCTAACACATGCAAGTC

F63-29 CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCGTCGTCTCAGGCCTAACACATGCAAGTC

F63-30 CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTACGCTATCAGGCCTAACACATGCAAGTC

F63-31 CCATCTCATCCCTGCGTGTCTCCGACTCAGATAGAGTACTCAGGCCTAACACATGCAAGTC

F63-32 CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGCTACGTCAGGCCTAACACATGCAAGTC

F63-33 CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGTAGACGTCAGGCCTAACACATGCAAGTC

F63-34 CCATCTCATCCCTGCGTGTCTCCGACTCAGCGACGTGACTCAGGCCTAACACATGCAAGTC

F63-35 CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACACACTCAGGCCTAACACATGCAAGTC

F63-36 CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACGTGATCAGGCCTAACACATGCAAGTC

F63-37 CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGATCGTCAGGCCTAACACATGCAAGTC

F63-38 CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGCTGTCTCAGGCCTAACACATGCAAGTC

F63-39 CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTGTAGATCAGGCCTAACACATGCAAGTC

F63-40 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATCACGTCAGGCCTAACACATGCAAGTC

F63-41 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCACTAGTCAGGCCTAACACATGCAAGTC

F63-42 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGCGACTCAGGCCTAACACATGCAAGTC

F63-43 CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATACTATCAGGCCTAACACATGCAAGTC

APPENDIX E
Supplementary tables for Chapter 4

Table E.1 List of genera detected within the pyrosequence library.

Phylum	Class	Order	Family	Genus	Rel. abund. in seq. library
Acidobacteria	*Acidobacteria*	*Acidobacteriales*	*Acidobacteriaceae*	Unclassified	0.003%
Acidobacteria	*Holophagae*	*Holophagales*	*Holophagaceae*	*Geothrix*	0.001%
Actinobacteria	*Actinobacteria*	*Acidimicrobiales*	*Iamiaceae*	*Iamia*	0.001%
Actinobacteria	*Actinobacteria*	*Acidimicrobiales*	Microthrix family	*Candidatus Microthrix*	0.001%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*	0.023%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Dermabacteraceae*	*Brachybacterium*	0.002%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Dermacoccaceae*	*Kytococcus*	0.003%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Dietziaceae*	*Dietzia*	0.001%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Fodinicola*	Unclassified	0.006%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Intrasporangiaceae*	*Janibacter*	0.002%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Intrasporangiaceae*	*Ornithinimicrobium*	0.001%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Candidatus Planktoluna*	0.001%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Curtobacterium*	0.005%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Leifsonia*	0.002%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Microbacterium*	0.005%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Arthrobacter*	0.003%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Kocuria*	0.002%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Micrococcus*	0.005%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Nesterenkonia*	0.001%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micromonosporaceae*	*Actinoplanes*	0.007%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micromonosporaceae*	*Luedemannella*	0.001%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Mycobacteriaceae*	*Mycobacterium*	0.003%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	0.006%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardioidaceae*	*Nocardioides*	0.003%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Propionibacteriaceae*	*Propionibacterium*	0.107%
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Streptosporangiaceae*	*Microtetraspora*	0.001%

Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Tsukamurellaceae*	*Tsukamurella*	0.001%
Actinobacteria	*Actinobacteria*	*Rubrobacteridae*	*Conexibacteraceae*	*Conexibacter*	0.001%
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	0.002%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Cryomorphaceae*	*Fluviicola*	0.001%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Aquimarina*	0.001%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	0.280%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Gaetbulibacter*	2.039%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Galbibacter*	0.001%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Lutibacter*	0.061%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Lutimonas*	0.130%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Persicivirga*	0.018%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Pibocella*	0.003%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Polaribacter*	0.090%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Psychroserpens*	0.004%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Sandarakinotalea*	0.002%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	Unclassified	0.010%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Segetibacter*	0.009%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagaceae*	*Cytophaga*	0.002%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Cytophagaceae*	*Spirosoma*	0.001%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Flammeovirgaceae*	*Fulvivirga*	0.009%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Mucilaginibacter*	0.001%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Nubsella*	0.001%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	Unclassified	Unclassified	0.036%
Bacteroidetes	Unclassified	Unclassified	Unclassified	Unclassified	0.002%
BD1-5	Unclassified	Unclassified	Unclassified	Unclassified	0.033%
Chlorobi	*Chlorobia*	*Chlorobiales*	Unclassified	Unclassified	0.005%
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	Unclassified	0.001%
Chloroflexi	*Caldilineae*	*Caldilineales*	*Caldilineaceae*	*Caldilinea*	0.001%
Chloroflexi	*Caldilineae*	*Caldilineales*	*Caldilineaceae*	Unclassified	0.034%
Cyanobacteria	SubsectionIV	SubgroupII	*Rivularia*	Unclassified	0.002%
Cyanobacteria	Unclassified	Unclassified	Unclassified	Unclassified	0.002%
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	0.001%
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Salirhabdus*	0.002%

Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Ureibacillus*	0.001%
Firmicutes	*Bacilli*	*Bacillales*	*Family_XI_Incertae_Sedis*	*Gemella*	0.001%
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Incertae_Sedis*	0.002%
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	0.017%
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Natronincola*	0.001%
Firmicutes	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Anaerococcus*	0.001%
Firmicutes	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Finegoldia*	0.005%
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Veillonella*	0.008%
Lentisphaerae	*Lentisphaeria*	Unclassified	Unclassified	Unclassified	0.004%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*	*Hirschia*	0.001%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*	*Robiginitomaculum*	0.021%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Beijerinckiaceae*	Unclassified	0.001%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Bradyrhizobium*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Rhodopseudomonas*	0.004%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	Unclassified	0.002%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Brucellaceae*	*Pseudochrobactrum*	0.008%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Ancalomicrobium*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	0.014%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Ahrensia*	0.010%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	*Afifella*	0.001%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	*Anderseniella*	0.012%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	*Parvibaculum*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Loktanella*	0.002%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Octadecabacter*	0.001%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*	0.002%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Pseudovibrio*	0.002%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Roseobacter*	0.072%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Sediminimonas*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Sulfitobacter*	0.002%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unclassified	0.013%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Yangia*	0.001%

Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Roseospira*	0.015%
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Thalassospira*	0.009%
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*Rickettsiaceae*	*Rickettsia*	0.011%
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	Unclassified	Unclassified	0.319%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Erythrobacteraceae*	*Erythrobacter*	0.002%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Erythrobacteraceae*	*Porphyrobacter*	0.005%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*	0.272%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	0.025%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	Unclassified	0.005%
Proteobacteria	*Alphaproteobacteria*	Unclassified	Unclassified	Unclassified	0.082%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Achromobacter*	0.005%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*	0.058%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Cupriavidus*	0.007%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*	0.075%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	0.008%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Aquincola*	0.001%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Caenimonas*	0.002%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	0.001%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Delftia*	0.003%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Diaphorobacter*	0.002%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*	0.002%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Ideonella*	0.002%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Leptothrix*	0.002%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Methylibium*	0.002%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Pelomonas*	0.016%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Rhodoferax*	0.003%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Sphaerotilus*	0.001%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Variovorax*	0.042%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Collimonas*	0.001%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herbaspirillum*	0.004%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Janthinobacterium*	0.005%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	Unclassified	0.008%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Undibacterium*	0.055%

Proteobacteria	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*	0.001%
Proteobacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*	*Formivibrio*	0.001%
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Dechloromonas*	0.005%
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Ferribacterium*	0.002%
Proteobacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Bdellovibrio*	0.003%
Proteobacteria	*Deltaproteobacteria*	*Desulfarculales*	*Desulfarculaceae*	*Desulfarculus*	0.014%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfatiferula*	0.001%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfonema*	0.001%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	Unclassified	0.013%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	0.218%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfocapsa*	0.009%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	Unclassified	0.063%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromusa*	0.018%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geopsychrobacter*	0.002%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	Unclassified	Unclassified	1.748%
Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	Unclassified	Unclassified	0.002%
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Desulfomonile*	0.001%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*	1.958%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Campylobacter*	0.003%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Sulfurospirillum*	0.021%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	Unclassified	0.007%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfurimonas*	0.014%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfurovum*	3.502%
Proteobacteria	*Epsilonproteobacteria*	*Nautiliales*	*Nautiliaceae*	*Nitratifractor*	0.116%
Proteobacteria	*Epsilonproteobacteria*	*Nautiliales*	*Nautiliaceae*	*Thioreductor*	0.014%
Proteobacteria	*Epsilonproteobacteria*	Unclassified	Unclassified	Unclassified	0.001%
Proteobacteria	*Gammaproteobacteria*	*Aeromonadales*	*Succinivibrionaceae*	*Succinivibrio*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Alteromonadaceae*	Unclassified	0.001%
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	0.167%
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Psychromonadaceae*	*Psychromonas*	0.048%
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*	3.171%
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	*Chromatiaceae*	*Nitrosococcus*	1.054%
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	*Granulosicoccaceae*	*Granulosicoccus*	0.002%

Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	Sedimenticola family	*Sedimenticola*	0.009%
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	Thiobios family	*Candidatus Thiobios*	0.031%
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	Unclassified	0.001%
Proteobacteria	*Gammaproteobacteria*	*Legionellales*	*Legionellaceae*	*Legionella*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	*Methylococcaceae*	*Methylomicrobium*	73.762%
Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	*Methylococcaceae*	Unclassified	0.004%
Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	Unclassified	Unclassified	0.004%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Halomonas*	8.962%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Modicisalibacter*	0.002%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Oceanospirillaceae*	*Nitrincola*	0.011%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Oleiphilaceae*	*Oleiphilus*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	Ruthia family	*Candidatus Ruthia*	0.026%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Saccharospirillaceae*	*Saccharospirillum*	0.017%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	Unclassified	Unclassified	0.350%
Proteobacteria	*Gammaproteobacteria*	*Pasteurellales*	*Pasteurellaceae*	*Haemophilus*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	0.025%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Enhydrobacter*	0.008%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	0.005%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.240%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Fangia*	Unclassified	0.002%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*	*Thioalkalimicrobium*	0.004%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*	Unclassified	0.022%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	*Leucothrix*	0.016%
Proteobacteria	*Gammaproteobacteria*	Unclassified	Unclassified	Unclassified	0.019%
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Sinobacteraceae*	Unclassified	0.001%
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Arenimonas*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Aspromonas*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Pseudoxanthomonas*	0.001%
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Stenotrophomonas*	0.011%
Spirochaetes	*Spirochaetes*	Unclassified	Unclassified	*Candidatus Cloacamonas*	0.018%
TM6	Unclassified	Unclassified	Unclassified	Unclassified	0.001%
TM7	Unclassified	Unclassified	Unclassified	Unclassified	0.004%

Table E.2 Diversity estimates for the pyrosequencing data for each sampling site and for High Flow (HF) and Low Flow (LF) samples.

	Average in HF10AV2b	Average in HF10HUb	Average in HF11GRb	Average in HF11LBb	Average in LF10AV1b	Average in LF10AV2b	Average in LF10HUb	Average in LF10TPb	Average in HF samples	Average in LF samples
Number of OTUs	55 ± 36	51 ± 3	10 ± 5	9 ± 8	54 ± 17	50 ± 17	28 ± 13	72 ± 35	29 ± 27	51 ± 26
Coverage	99.4 ± 0.4%	99.7 ± 0.1%	99.9 ± 0.1%	99.9 ± 0.1%	99.4 ± 0.3%	99.5 ± 0.1%	99.6 ± 0.2%	99.2 ± 0.2%	99.7 ± 0.3%	99.5 ±0.3 %
Chao1	78 ± 47	58 ± 5	14 ± 9	12 ± 14	81 ± 38	66 ± 14	43 ± 28	114 ± 44	37 ± 36	76 ± 40
ACE	98 ± 65	61 ± 4	24 ± 31	19 ± 30	104 ± 48	94 ± 40	64 ± 52	115 ± 37	47 ± 47	94 ± 45
Shannon-Wiener	1.33 ± 0.77	1.63 ± 0.34	0.11 ± 0.05	0.11 ± 0.10	1.55 ± 0.46	1.37 ± 0.75	0.32 ± 0.08	1.19 ± 0.77	0.72 ± 0.79	1.08 ± 0.73
SHEI	0.33 ± 0.16	0.42 ± 0.09	0.05 ± 0.02	0.06 ± 0.02	0.39 ± 0.10	0.34 ± 0.17	0.10 ± 0.02	0.27 ± 0.14	0.19 ± 0.18	0.27 ± 0.16

Table E.3 P-value for statistical analyses performed on each pair of sampling sites.

Comparison of sampling sites	Parsimony		Amova		Homova
	Membership	Structure	Membership	Structure	Structure
HF10AV2b-HF10HUb	0.018	ns[a]	<0.001	ns	ns
HF10AV2b-HF11GRb	0.009	0.008	0.001	<0.001	<0.001
HF10AV2b-HF11LBb	0.012	0.012	0.01	<0.001	0.005
HF10HUb-HF11GRb	0.012	0.013	<0.001	0.002	<0.001
HF10HUb-HF11LBb	0.007	0.004	0.008	<0.001	0.009
HF11GRb-HF11LBb	ns	ns	ns	ns	ns
LF10AV1b-LF10AV2b	ns	ns	ns	ns	ns
LF10AV1b-LF10HUb	0.012	ns	0.01	0.002	0.006
LF10AV1b-LF10TPb	0.012	ns	<0.001	ns	ns
LF10AV2b-LF10HUb	0.005	ns	<0.001	0.014	ns
LF10AV2b-LF10TPb	0.005	ns	<0.001	ns	ns
LF10HUb-LF10TPb	ns	0.007	<0.001	<0.001	<0.001
HF10AV2b-LF10AV1b	ns	0.013	ns	ns	ns
HF10AV2b-LF10AV2b	ns	ns	0.02	ns	ns
HF10AV2b-LF10HUb	ns	ns	<0.001	<0.001	0.011
HF10AV2b-LF10TPb	ns	ns	<0.001	ns	ns
HF10HUb-LF10AV1b	0.023	ns	0.005	ns	ns
HF10HUb-LF10AV2b	0.011	ns	<0.001	ns	ns
HF10HUb-LF10HUb	0.011	0.008	0.005	0.004	0.004
HF10HUb-LF10TPb	0.012	ns	<0.001	0.031	ns
HF11GRb-LF10AV1b	0.009	ns	0.01	0.001	<0.001
HF11GRb-LF10AV2b	0.004	0.003	0.003	0.004	<0.001
HF11GRb-LF10HUb	ns	0.005	<0.001	<0.001	ns
HF11GRb-LF10TPb	0.006	ns	<0.001	0.002	<0.001
HF11LBb-LF10AV1b	0.009	0.006	0.007	<0.001	0.008
HF11LBb-LF10AV2b	0.007	ns	<0.001	0.004	<0.001
HF11LBb-LF10HUb	ns	ns	0.011	0.009	ns
HF11LBb-LF10TPb	0.004	0.005	<0.001	0.001	<0.001

[a] ns = not significant

Table E.4 Occurrence of the OTUs representing 0.1% of the pyrosequence library for each sampling site. The p-value indicates whether each OTU is differentially represented between each pairwise comparison. (A) Comparison between High Flow sampling sites. (B) Comparison between Low Flow sampling sites.

(A)

OTU ID	Average proportion of OTU per sampling location				Comparison of sampling sites (p-value)					
	HF10AV2b	HF10HUb	HF11GRb	HF11LBb	HF10AV2b-HF10HUb	HF10AV2b-HF11GRb	HF10AV2b-HF11LBb	HF10HUb-HF11GRb	HF10HUb-HF11LBb	HF11GRb-HF11LBb
1	59.1 ± 17.2%	47.7 ± 5.9%	98.2 ± 0.4%	98.1 ± 0.8%	ns[a]	0.015	0.013	<0.001	<0.001	ns
2	14.1 ± 5.5%	21.2 ± 2.5%	0.1 ± 0.1%	0.1 ± 0.1%	ns	0.005	0.005	<0.001	<0.001	ns
3	5.2 ± 2.2%	8.8 ± 0.3%	0.1 ± < 0.1%	0.1 ± < 0.1%	ns	0.015	0.012	<0.001	<0.001	ns
4	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	0.0%	0.0%	ns	ns	ns	ns	ns	ns
5	0.9 ± 0.5%	0.3 ± 0.1%	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	ns	0.037	0.031	<0.001	0.005	ns
6	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	0.0%	0.0%	ns	ns	ns	ns	ns	ns
7	9.2 ± 7.9%	0.8 ± 0.4%	0.1 ± < 0.1%	0.4 ± 0.4%	ns	ns	ns	0.038	ns	ns
8	0.0%	8.9 ± 5.8%	0.1 ± < 0.1%	0.0%	<0.001	<0.001	ns	ns	ns	0.021
9	1.0 ± 0.4%	0.4 ± 0.1%	1.2 ± 0.2%	1.0 ± 0.1%	ns	ns	ns	0.008	0.004	ns
10	0.6 ± 0.4%	0.4 ± 0.3%	0.0%	0.0%	ns	ns	ns	<0.001	ns	ns
11	0.9 ± 0.8%	0.4 ± 0.2%	< 0.1 ± < 0.1%	0.1 ± 0.1%	ns	ns	ns	<0.001	ns	0.007
12	0.2 ± 0.1%	3.9 ± 3.4%	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	ns	ns	ns	<0.001	ns	ns
13	0.2 ± 0.2%	0.0%	0.0%	0.0%	ns	ns	ns	ns	ns	ns

14	0.2 ± 0.2%	< 0.1 ± < 0.1%	0.0%	0.0%	ns	ns	ns	0.031	ns	ns
15	< 0.1 ± < 0.1%	0.0%	0.0%	0.0%	ns	ns	ns	ns	ns	ns
16	0.4 ± 0.1%	0.6 ± 0.2%	0.0%	0.0%	ns	<0.001	<0.001	<0.001	0.014	ns
17	0.5 ± 0.1%	0.4 ± 0.1%	0.0%	< 0.1 ± < 0.1%	ns	<0.001	<0.001	<0.001	<0.001	ns
18	1.1 ± 0.8%	0.9 ± 0.4%	< 0.1 ± < 0.1%	0.0%	ns	ns	ns	<0.001	0.023	ns
19	0.5 ± 0.1%	0.5 ± 0.1%	< 0.1 ± < 0.1%	0.0%	ns	0.001	0.001	<0.001	0.002	ns
20	1.3 ± 1.2%	0.1 ± 0.1%	0.0%	0.0%	ns	ns	ns	0.002	ns	ns
21	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	0.0%	0.0%	ns	ns	ns	ns	ns	ns
22	0.0%	0.0%	0.0%	0.0%	ns	ns	ns	ns	ns	ns
23	< 0.1 ± < 0.1%	0.0%	0.0%	0.0%	ns	ns	ns	ns	ns	ns
24	0.3 ± 0.1%	0.1 ± < 0.1%	0.0%	< 0.1 ± < 0.1%	ns	0.006	0.007	0.004	0.002	ns
25	0.0%	< 0.1 ± < 0.1%	0.0%	0.0%	ns	ns	ns	ns	ns	ns

[a] ns = not significant

(B)

OTU ID	Average proportion of OTU per sampling location				Comparison of sampling sites (p-value)					
	LF10AV1b	LF10AV2b	LF10HUb	LF10TPb	LF10AV1b-LF10AV2b	LF10AV1b -LF10HUb	LF10AV1b-LF10TPb	LF10AV2b-LF10HUb	LF10AV2b-LF10TPb	LF10HUb-LF10TPb
1	48.1 ± 11.2%	57.3 ± 13.3%	94.6 ± 0.8%	67.7 ± 15.7%	ns[a]	< 0.001	ns	0.003	ns	0.044
2	19.5 ± 6.2%	15.8 ± 6.1%	2.1 ± 0.8%	4.4 ± 1.3%	ns	0.003	0.009	0.014	0.032	ns
3	5.5 ± 1.3%	5.6 ± 2.5%	0.6 ± 0.2%	1.6 ± 0.5%	ns	< 0.001	0.003	0.025	ns	0.042

4	11.8 ± 7.1%	4.8 ± 3.0%	< 0.1 ± < 0.1%	0.5 ± 0.4%	ns	ns	ns	ns	ns	ns
5	4.9 ± 3.9%	6.2 ± 3.5%	0.1 ± 0.1%	< 0.1 ± < 0.1%	ns	ns	ns	0.050	0.038	ns
6	0.1 ± < 0.1%	0.0%	< 0.1 ± < 0.1%	10.1 ± 9.2%	ns	ns	ns	0.017	ns	ns
7	0.6 ± 0.3%	0.4 ± 0.3%	< 0.1 ± < 0.1%	0.2 ± 0.1%	ns	0.019	ns	ns	ns	0.009
8	0.0%	0.0%	0.0%	< 0.1 ± < 0.1%	ns	ns	ns	ns	ns	ns
9	0.4 ± 0.1%	0.9 ± 0.2%	0.8 ± 0.1%	0.5 ± 0.1%	0.027	0.010	ns	ns	0.041	ns
10	< 0.1 ± < 0.1%	0.1 ± 0.1%	0.3 ± 0.1%	2.6 ± 1.2%	ns	0.026	0.016	ns	0.018	0.032
11	2.2 ± 1.2%	0.5 ± 0.5%	0.0%	0.1 ± < 0.1%	ns	0.041	0.040	ns	ns	0.000
12	0.0%	0.0%	< 0.1 ± < 0.1%	0.2 ± 0.1%	ns	0.034	0.006	0.033	0.004	0.032
13	0.4 ± 0.2%	1.9 ± 1.6%	0.1 ± 0.1%	0.1 ± < 0.1%	ns	ns	ns	ns	ns	ns
14	0.1 ± 0.1%	< 0.1 ± < 0.1%	0.1 ± 0.1%	2.0 ± 1.5%	ns	ns	ns	ns	ns	ns
15	1.2 ± 0.8%	1.1 ± 0.6%	0.1 ± < 0.1%	0.2 ± 0.1%	ns	ns	ns	ns	ns	0.048
16	1.0 ± 0.5%	0.3 ± 0.1%	0.1 ± < 0.1%	0.1 ± < 0.1%	ns	0.024	0.025	ns	ns	ns
17	0.8 ± 0.3%	0.3 ± 0.2%	< 0.1 ± < 0.1%	0.1 ± < 0.1%	ns	0.001	0.003	ns	ns	0.033
18	0.1 ± 0.1%	0.0%	0.1 ± 0.1%	0.1 ± < 0.1%	ns	ns	ns	< 0.001	0.015	ns
19	0.4 ± 0.2%	0.4 ± 0.2%	0.1 ± < 0.1%	0.1 ± < 0.1%	ns	ns	ns	0.027	ns	ns
20	0.0%	0.3 ± 0.3%	0.1 ± 0.1%	0.0%	< 0.001	< 0.001	< 0.001	ns	ns	ns
21	0.0%	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	1.3 ± 1.0%	ns	0.034	ns	ns	ns	ns
22	0.0%	0.0%	0.0%	1.2 ± 0.6%	ns	ns	0.024	ns	0.025	0.030
23	< 0.1 ± < 0.1%	0.0%	< 0.1 ± < 0.1%	1.0 ± 0.9%	ns	ns	ns	ns	ns	ns
24	0.1 ± < 0.1%	0.3 ± 0.2%	< 0.1 ± < 0.1%	< 0.1 ± < 0.1%	ns	0.001	0.006	0.021	0.028	ns
25	0.0%	0.6 ± 0.5%	0.0%	< 0.1 ± < 0.1%	< 0.001	ns	ns	ns	ns	ns

[a]ns = not significant

APPENDIX F
Supplementary table for Chapter 5

Table F.1 List of genera detected within the pyrosequence library.

Phylum	Class	Order	Family	Genus	Rel. abund in seq. library
Acidobacteria	*Acidobacteria*	*Acidobacteriales*	*Acidobacteriaceae*	Unclassified	0.014%
Acidobacteria	*Holophagae*	Unclassified	Unclassified	Unclassified	0.028%
Actinobacteria	*Actinobacteria*	*Actinobacteridae*	Unclassified	Unclassified	0.007%
Actinobacteria	*Actinobacteridae*	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*	0.021%
Actinobacteria	*Actinobacteridae*	*Actinomycetales*	*Microbacteriaceae*	*Microbacterium*	0.014%
Actinobacteria	*Actinobacteridae*	*Actinomycetales*	*Micrococcaceae*	*Arthrobacter*	0.014%
Actinobacteria	*Actinobacteridae*	*Actinomycetales*	*Nakamurellaceae*	*Nakamurella*	0.104%
Actinobacteria	*Actinobacteridae*	*Actinomycetales*	*Propionibacteriaceae*	*Propionibacterium*	0.062%
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	0.007%
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Marinilabiaceae*	Unclassified	0.083%
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	Unclassified	Unclassified	0.007%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Bergeyella*	0.007%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	0.353%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Gaetbulibacter*	5.359%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Leeuwenhoekiella*	0.014%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Lutibacter*	0.651%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Persicivirga*	0.014%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Polaribacter*	0.145%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Ulvibacter*	0.042%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	Unclassified	0.159%
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	Unclassified	Unclassified	0.021%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	*Saprospiraceae*	*Saprospira*	0.007%
Bacteroidetes	*Sphingobacteria*	*Sphingobacteriales*	Unclassified	Unclassified	0.048%

Bacteroidetes	Unclassified	Unclassified	Unclassified	Unclassified	0.048%
BD1-5	Unclassified	Unclassified	Unclassified	Unclassified	0.021%
Chlorobi	*Chlorobia*	*Chlorobiales*	*Chlorobiaceae*	*Chlorobaculum*	0.028%
Chlorobi	*Chlorobia*	*Chlorobiales*	*Chlorobiaceae*	*Chloroherpeton*	0.028%
Chlorobi	*Chlorobia*	*Chlorobiales*	Unclassified	Unclassified	0.007%
Chloroflexi	*Caldilineae*	*Caldilineales*	*Caldilineaceae*	Unclassified	0.388%
Cyanobacteria	*Chloroplast*	Unclassified	Unclassified	Unclassified	0.014%
Cyanobacteria	*SubsectionI*	*Synechococcus*	Unclassified	Unclassified	0.007%
Deinococcus-Thermus	*Thermales*	*Thermaceae*	*Vulcanithermus*	Unclassified	0.028%
Firmicutes	*Bacilli*	*Bacillales*	*Paenibacillaceae*	Unclassified	0.007%
Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Isobaculum*	0.007%
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Natronincola*	0.014%
Firmicutes	*Clostridia*	*Clostridiales*	Family_XI_Incertae_Sedis	*Tissierella*	0.014%
Firmicutes	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	Unclassified	0.007%
Proteobacteria	*Alphaproteobacteria*	*Adriatic90*	Unclassified	Unclassified	0.042%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	0.021%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	Unclassified	0.014%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*	*Hellea*	0.028%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*	*Hirschia*	0.042%
Proteobacteria	*Alphaproteobacteria*	*Caulobacterales*	*Hyphomonadaceae*	*Woodsholea*	0.055%
Proteobacteria	*Alphaproteobacteria*	*Kordiimonadales*	*Kordiimonadaceae*	Unclassified	0.014%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Beijerinckiaceae*	*Beijerinckia*	0.014%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	Unclassified	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*FukuN57*	Unclassified	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Ancalomicrobium*	0.222%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	0.222%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylocystaceae*	Unclassified	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Ahrensia*	0.083%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Hoeflea*	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	*Afifella*	0.048%

Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	*Parvibaculum*	0.083%
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*	Unclassified	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Loktanella*	0.062%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Octadecabacter*	0.014%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Roseobacter*	2.119%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Roseovarius*	0.014%
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unclassified	0.159%
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*	*Teichococcus*	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Roseospira*	0.048%
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	Captivus family	*Candidatus Captivus*	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	Hepatincola family	*Candidatus Hepatincola*	0.007%
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*Rickettsiaceae*	*Rickettsia*	0.021%
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	Unclassified	Unclassified	0.132%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*	0.159%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	0.014%
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	Unclassified	0.007%
Proteobacteria	*Alphaproteobacteria*	Unclassified	Unclassified	Unclassified	0.215%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Achromobacter*	0.028%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*	0.069%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Cupriavidus*	0.014%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*	0.090%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	0.007%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Pelomonas*	0.014%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Rhodoferax*	0.007%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	Unclassified	0.055%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Variovorax*	0.021%
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Undibacterium*	0.048%
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Zoogloea*	0.014%
Proteobacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Bdellovibrio*	0.007%
Proteobacteria	*Deltaproteobacteria*	*Desulfarculales*	*Desulfarculaceae*	*Desulfarculus*	0.409%

Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfatiferula*	0.035%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfonema*	0.152%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	Unclassified	0.339%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobacterium*	0.035%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	3.427%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfocapsa*	2.403%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfofustis*	0.021%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulforhopalus*	0.042%
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	Unclassified	0.173%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromusa*	0.069%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geopsychrobacter*	0.007%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geothermobacter*	0.014%
Proteobacteria	*Deltaproteobacteria*	*Desulfuromonadales*	Unclassified	Unclassified	2.887%
Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	*Sorangiineae*	*Polyangiaceae*	0.007%
Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	Unclassified	Unclassified	0.007%
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Desulfomonile*	0.007%
Proteobacteria	*Deltaproteobacteria*	Unclassified	Unclassified	Unclassified	0.007%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*	3.074%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Campylobacter*	0.042%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Sulfurospirillum*	0.159%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	Unclassified	0.035%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfurimonas*	0.035%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Sulfurovum*	23.312%
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	Unclassified	0.014%
Proteobacteria	*Epsilonproteobacteria*	*Nautiliales*	*Nautiliaceae*	*Nitratifractor*	1.793%
Proteobacteria	*Epsilonproteobacteria*	*Nautiliales*	*Nautiliaceae*	*Thioreductor*	0.062%
Proteobacteria	*Epsilonproteobacteria*	*Nautiliales*	*Nautiliaceae*	Unclassified	0.104%
Proteobacteria	*Epsilonproteobacteria*	Unclassified	Unclassified	Unclassified	0.014%
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	0.062%
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*	2.389%

Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	*Chromatiaceae*	*Nitrosococcus*	1.094%
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	*Chromatiaceae*	Unclassified	0.028%
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*	Thiobios family	*Candidatus Thiobios*	0.083%
Proteobacteria	*Gammaproteobacteria*	Marine Group E01-9C-26	Unclassified	Unclassified	0.007%
Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	*Methylococcaceae*	*Methylomicrobium*	36.897%
Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	Unclassified	Unclassified	0.007%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	Ruthia family	*Candidatus Ruthia*	0.021%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Halomonas*	7.443%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Saccharospirillaceae*	*Saccharospirillum*	0.298%
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	Unclassified	Unclassified	0.194%
Proteobacteria	*Gammaproteobacteria*	*Pasteurellales*	*Pasteurellaceae*	*Haemophilus*	0.014%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	0.021%
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.201%
Proteobacteria	*Gammaproteobacteria*	*Thiobacillus*	Unclassified	Unclassified	0.014%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*	Unclassified	0.069%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*	*Thioalkalimicrobium*	0.007%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*	Unclassified	0.007%
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	*Leucothrix*	0.007%
Proteobacteria	*Gammaproteobacteria*	Unclassified	Unclassified	Unclassified	0.048%
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Stenotrophomonas*	0.014%
Proteobacteria	Unclassified	Unclassified	Unclassified	Unclassified	0.104%
Spirochaetes	*Spirochaetes*	Unclassified	Unclassified	*Candidatus Cloacamonas*	0.374%
SR-1	Unclassified	Unclassified	Unclassified	Unclassified	0.007%
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Roseibacillus*	0.035%

APPENDIX G
List of abbreviations and acronyms

AMOVA	Analysis of molecular variance
ATP	Adenosine triphosphate
CARD	Catalyzed reporter deposition
CHONE	Canadian Healthy Oceans Network
DNA	Deoxyribonucleic acid
FISH	Fluorescence *in situ* hybridization
HF	High Flow
HOMOVA	Homogeneity of molecular variance
HRP	Horseradish peroxidase
LF	Low Flow
MEF	Main Endeavour vent field
MPA	Marine Protected Area
MPC	Magnetic Particle Concentrator
NCBI	National Centre for Biotechnology Information
NMDS	Nonmetric multidimensional scaling
NPMLE	Non parametric maximum likelihood estimator
NSERC	Natural Sciences and Engineering Research Council
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PYRO	Pyrosequencing
RDP	Ribosomal Database Project
ROV	Remotely-operated vehicle
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
R/V	Research vessel
SHEI	Shannon’s evenness index
S&M	Smoke & Mirrors
SNG	Sanger sequencing

SRA	Sequence Read Archive
SSU	Small subunit
t-RFLP	Terminal restriction fragment length polymorphism
UPGMA	Unweighted Pair Group Method with Arithmetic Mean