In situ chemistry and microbial community compositions in five deep-sea hydrothermal fluid samples from Irina II in the Logatchev field

Mirjam Perner,1* Giorgio Gonnella,2 Stephane Hourdez,3,4 Stefanie Böhnke,1 Stefan Kurtz2 and Peter Girguis5
1Molecular Biology of Microbial Consortia, University of Hamburg, Biocenter Klein Flottbek, Ohnhorststr. 18, 22609 Hamburg, Germany.
2Center for Bioinformatics, University of Hamburg, Bundesstrasse 43, 20146 Hamburg, Germany.
3Genetics of Adaptation to Extreme Environments Group, UMR7144, CNRS, Station Biologique de Roscoff, 29680 Roscoff, France.
4Genetics of Adaptation to Extreme Environments Group, UMR7144, Université Pierre et Marie Curie, Station Biologique de Roscoff, 29680 Roscoff, France.
5Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA.

Summary

We present data on the co-registered geochemistry (in situ mass spectrometry) and microbiology (pyrosequencing of 16S rRNA genes; V1, V2, V3 regions) in five fluid samples from Irina II in the Logatchev hydrothermal field. Two samples were collected over 24 min from the same spot and further three samples were from spatially distinct locations (20 cm, 3 m and the overlaying plume). Four low-temperature hydrothermal fluids from the Irina II are composed of the same core bacterial community, namely specific Gammaphyloproteobacteria and Epsilonproteobacteria, which, however, differs in the relative abundance. The microbial composition of the fifth sample (plume) is considerably different. Although a significant correlation between sulfide enrichment and proportions of Sulfovoron (Epsilonproteobacteria) was found, no other significant linkages between abiotic factors, i.e. temperature, hydrogen, methane, sulfide and oxygen, and bacterial lineages were evident. Intriguingly, bacterial community compositions of some time series samples from the same spot were significantly more similar to a sample collected 20 cm away than to each other. Although this finding is based on three single samples only, it provides first hints that single hydrothermal fluid samples collected on a small spatial scale may also reflect unrecognized temporal variability. However, further studies are required to support this hypothesis.

Introduction

Hydrothermal vents are hallmarked by steep thermal and chemical gradients, which form as ascending hot, reduced hydrothermal fluids mix with entrained cold, oxygenated ambient seawater. Owing to these mixing processes, inorganic reduced energy-rich electron donors and oxidized electron acceptors are both present. Their availability is expected to largely govern microbial community composition (Perner et al., 2010; 2011) and, since these substrates can fuel chemosynthetic metabolism, affect microbial biomass production as well (McCollom, 2007). The emitted hydrothermal fluids and the entrained microorganisms can provide us with an insight into the physicochemical conditions of the subsurface pathways the fluids flow through on the way to the surface.

Chemical signatures of hydrothermal fluids are known to fluctuate over space and time (e.g. LeBris et al., 2006; Rogers et al., 2007; Zielinski et al., 2011), and appear to have an impact on the local microbial diversity (Perner et al., 2010; 2011). As such, it is not surprising to find small-scale (few centimetres to metres) (Nakagawa et al., 2005) and large-scale (Perner et al., 2007; Huber et al., 2010) dynamics in microbial populations. However, while large-scale intra- and inter-field variability has been studied several times (Perner et al., 2007; Davis and Moyer, 2008; Nanoura and Takai, 2009; Huber et al., 2010; Flores et al., 2012), few studies have examined genuine small-scale (Nakagawa et al., 2005) and short-term temporal (Perner et al., 2009) microbial dynamics in hydrothermally influenced provinces. Understanding the variability in hydrothermal vent systems – and the scale at which these variations occur – remains challenging to date. So far, temporal and/or spatial chemical and microbial changes have been ascribed to the type of host rock, degrees of subsurface dilution, precipitation processes,
microbial activities in the subsurface, tidal and bottom currents, seismic events and altered fluid pathways (Perner et al., 2009; and references therein).

To better understand the relationships between microbial ecology (composition, structure, density), physiology (energy metabolism) and geochemistry, recent studies have used high-throughput sequencing technologies and sometimes geochemical measurements (Rocap et al., 2003; DeLong et al., 2006; Huber et al., 2010; Flores et al., 2012). High-throughput sequencing allows unprecedented sampling depth and hence provides new insights into microbial communities (e.g. Sogin et al., 2006). Sequencing of selected phylogenetic markers like 16S rRNA genes can uncover yet unexplored diversity, revealing biological patterns which can be allocated to specific habitat characteristics (Brazelton et al., 2010; Huber et al., 2010). Many such studies have focused on marine microbial ecology, although predominantly in the water column (e.g. Rocap et al., 2003; DeLong et al., 2006). To date, far fewer studies have employed high-throughput sequencing technologies and simultaneously registered geochemical measurements in marine benthic environments, including vents (Huber et al., 2007; 2010; Brazelton et al., 2010).

Applied at vents, this approach would allow examining the relationship between geochemistry and microbial community composition at temporal and spatial scales appropriate to the environment. As mentioned, previous studies have focused largely on this relationship at the regional scale, which can address questions about biogeography. To better understand how microbial community composition relates to temporal and spatial variations in chemistry at the mesoscale (tens of centimetres, over minutes to hours), we conducted a series of experiments to examine short-term temporal and small-scale spatial dynamics of hydrothermally influenced fluids and bacterial community composition using in situ mass spectrometry (ISMS) and pyrosequencing of the 16S rRNA genes – pyrotag sequencing including regions V1, V2 and V3. We collected four low-temperature hydrothermal fluid samples (samples A–D) and one plume water sample (sample E) from the ultramafic-hosted Logatchev field. Samples A through D were recovered from a connected mussel field bordering the main structure Irina II. Two samples (A and B) were collected over 24 min from the same location to investigate short-term temporal variability. Samples C and D were recovered from 20 cm and roughly 3 m, respectively, from the site where samples A and B were retrieved. Sample E originates from the hydrothermal plume at 175 m above Irina II. With these co-registered geochemical and microbial data, we can, for the first time, interrogate in situ chemistry and microbial dynamics and their interconnections at a deep-sea hydrothermal vent.

Results and discussion

To better constrain the significance of any differences among the samples, we worked to characterize the bias associated with our applied methodology. We performed three 10-cycle PCRs of 16S rRNA genes (e.g. 1A, 2A, 3A) for each of the five samples (samples A–E) to gain sufficient material for sequencing. Each of these PCR amplions was then divided into three subsamples (e.g. 1A1, 1A2, 1A3) and finally pyrosequenced. Three runs were performed on equally treated subsamples (for nomenclature of subsamples, see Table S1). With respect to technical biases we found small variations between the different sequencing runs, but not between the above mentioned PCRs of one sample (Figs S1 and S2). While the qualitative diversity means appeared to be generally preserved in our subsamples, the quantitative measure uncovered minimal population skewing within the same sample with respect to different sequencing runs (Fig. S2). Since our communities are distinctly different, this bias does not affect our interpretation. Such skewing, however, may lead to misinterpretation of data when investigating minor variations between microbial communities sequenced in different runs and is thus important to consider.

Time series of hydrothermal fluid samples

The in situ fluid chemistry and associated bacterial community composition was monitored over a time period of 24 min in hydrothermal fluids emitted from the same point (denoted samples A and B). During this time the sampling device was not moved from the location and thus any detected changes are related to intrinsic effects in the system. Within the 24 min the temperature increased from around 6.4°C to a maximum of 11°C (Fig. 1A). There were also significant increases in hydrogen (from 162 μM to max. 327 μM) and methane (from 47 μM to 102 μM) (Fig. 1B) and a small decrease in oxygen (235 μM to 208 μM) (P-value < 0.001 for all gases) (Fig. 1A) suggesting an elevated hydrothermal endmember contribution and thus more reducing conditions. However, magnesium concentrations remained fairly constant around 51 to 52 mM (Fig. 1A). During sampling, the cell numbers decreased by 60% from 5.1 × 10⁵ cells ml⁻¹ to 2.0 × 10⁵ cells ml⁻¹ (Fig. S3), while diversity increased (Shannon diversity indices: sample A 3.3 ± 0.1 and sample B 5.3 ± 0.2) (Fig. S4). Both cell numbers and diversity indices are in the magnitude of what is commonly found in hydrothermal venting regimes (Nakagawa et al., 2005; Perner et al., 2009). The communities from samples A (first 10 min) and B (subsequent 14 min) were significantly different (P-value < 0.05) and a shift in the bacterial community within the 24 min was clearly evident (Fig. 2). During the first 10 min of
sampling (sample A) the majority of 16S rRNA genes were affiliated with Gammaproteobacteria of the genera Alcanivorax and Marinobacter (65–74%) and only few Epsilonproteobacteria (17–24%) were recognized (Fig. 2A). For the 14 min thereafter (sample B) the inverse was the case: Epsilonproteobacteria 61–71% and Gammaproteobacteria 16–27% (Fig. 2A). Additionally, representatives of the thermophilic Aquificae increased during the sampling. The changes in chemistry, from more oxidizing to more reducing conditions, reflect the potential physiologies of the bacteria found in the samples. Alcanivorax and Marinobacter are associated with moderate temperatures and oxygenated conditions (Gonzalesz and Whitman, 2006). In contrast, the Epsilonproteobacteria enriched in sample B, namely thermophilic Caminibacter, and mesophilic Sulfurovum, Arcobacter and other Campylobacterales, are associated with more reducing conditions, where levels of sulphide and hydrogen (that serve as electron donor) are elevated (Campbell et al., 2006). Particularly, the increased representation of thermophilic, anaerobic or microaerobic, hydrogen-oxidizing Caminibacter and Aquificae species coincides with the elevated hydrogen concentrations.

Temporal dynamics have been attributed to a broad range of various effects including tidal currents and local bottom currents, seismic events, precipitation processes, different extents of subsurface dilution, alterations in fluid flow pathways, progressing of vent maturity, microbial activities and biological interactions (Perner et al., 2009; and references therein). Yet, we suggest that major changes in currents, seismic events or gross changes in vent geochemistry cannot explain our observed differences over the 24 min of sampling because the time scales over which these changes are manifest are too short. Our measured temperature and oxygen concentrations compare to those previously determined at Irina II (Zielinski et al., 2011). These data revealed temporal variations in physicochemical conditions (Fig. 1) that are consistent with previously observed highly dynamic changes over short time frames at Irina II (Zielinski et al., 2011). This phenomenon has been observed at other vent locations as well (LeBris et al., 2006). Nevertheless, besides the data presented here (Fig. 2), to our knowledge, only one other study has investigated and reported changes in vent microbial populations on comparably short time scales (in their case 50 min) (Perner et al.,

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, Environmental Microbiology
In the latter study, thermodynamic models predicted that predominant energy metabolisms likely changed during the 50 min of sampling (Perner et al., 2009), and the data in the current study are consistent with that hypothesis. In a previous study at the Irina II habitat, physicochemical fluid variability has, among other possibilities, been attributed to mussel colonization which was hypothesized to cause diversion of fluid flow and influence the degree of oxygenated, ambient seawater mixed with the reduced fluids (Zielinski et al., 2011). In the current study, we collected our fluid samples down among the mussels, and immediately above the zone of emission. In light of our mode of sampling, as well as the temperature data (which suggest a lower representation

![Fig. 2. A. Proportions of bacteria recognized in the three subsamples of the three different PCRs from the four sites and five samples (samples A–E). B and C. PCoA performed on subsamples from five hydrothermally influenced environments around Irina II using Qiime (Caporaso et al., 2010a). (B) Unweighted and (C) weighted normalized algorithms were used. The dots corresponding to the different bacterial communities are colour-coded according to the different sampling sites: sample A is red, sample B is blue, sample C is orange, sample D is green and sample E is purple. The spheres around the points mark the interquartile ranges (jackknifing support). The first three principal coordinates (PC1, PC2 and PC3) for the subsamples of the five environments are plotted. The axes are labelled by the percentage variation explained by the PCs.](image-url)
of ambient seawater when compared with the fluids above the mussels), we suggest that the impact of the mussels is modest. Rather, we posit that the observed changes between the emanated fluids reflect finer-scale alterations in the subsurface flow regime, which influence the proportion and representation of vent and seawater-derived constituents. The availability of these abiotic properties, in turn, have been shown to govern microbial community compositions (e.g. Flores et al., 2011; Perner et al., 2011). Thus, we propose that the shifts in the microbial population in the emanated fluids can be explained by fluid entrainment of microbes from thermally and chemically heterogenous subsurface habitats along the fluid flow pathways.

Spatially divergent hydrothermal fluid samples

Differences in the community compositions on a small spatial scale (few centimetres to metres) have been found for distinct hydrothermal venting sites (e.g. Nakagawa et al., 2005; Davis and Moyer, 2008; Huber et al., 2010). For some locations the small spatial microbial heterogeneity has been attributed to the chemistry and temperature prevailing in the habitat (Davis and Moyer, 2008). The bacterial communities from samples A, B (short-term time series data collected from the same spot) and C (collected within a 20 cm radius) were more similar than populations of the more distantly collected samples D (roughly 3 m away) and E (plume waters 175 m above ground) (Figs 2B and S1A, unweighted algorithm). Remarkably, when considering abundance information, the bacterial population of sample C, collected 20 cm from samples A and B, resembles sample A more in its community composition than samples A and B resemble each other (Figs 2C and S1B). Thus, it appears that fluids with similar microbial communities can emit at different locations – on a small spatial scale – and different population compositions can emanate with fluids at the same point of venting on the time scale of minutes. Consequently, the variability observed herein argues for highly dynamic conditions along the fluid pathways, supportive of heterogenous communities, and likely entails variation in metabolic activity.

To identify the possible abiotic parameters responsible for the variations between the bacterial communities, we took co-registered measurements of the local in situ chemistry alongside the collection of hydrothermal fluid samples at all four sites. The measured in situ chemistry at site C was significantly different to the chemistry of the other low-temperature Irina II samples A, B and D (P-value < 0.05) (Fig. 1). The microbial communities from sample C were also significantly different from those of sample B (P-value < 0.001) and sample D (P-value < 0.01, normalized weighted UniFrac significance). At site C, maximum in situ hydrogen and methane concentrations reached 1666 μM and 486 μM, respectively, oxygen and magnesium decreased to 171 μM and 49 mM, respectively, and temperatures reached a maximum of 24°C (Fig. 1). The elevated values indicate an increased endmember contribution, which is consistent with a greater contribution of vent-derived fluids in the subsurface through which the fluids represented by sample C pass. However, this hypothesis is not supported by the microbial community representation, since thermophilic representatives like Caminibacter or Aquificae, detected in sample B, were hardly present in sample C (Fig. 2A). Instead, in the latter, the majority of bacteria were affiliated to mesophilic Gammaproteobacteria, i.e. Thiomicrospira, Alcanivorax, Marinobacter and Alteromonas. Mesophilic Epsilon proteobacteria, namely Sulfurovum, Sulfurimonas and some Campylobacteria, also made up a considerable proportion of the bacterial community (Fig. 2A). Besides the Campylobacteria, which may be involved in hydrogen cycling (Perner et al., 2011), the other recognized Epsilon proteobacteria can be linked with both sulfur and hydrogen metabolisms (Campbell et al., 2006; Nakagawa et al., 2007). Temperatures at the exit point where fluids from sample C emit (24°C) are higher than those at the other investigated Irina II sites. However, based on the physiology of the identified fluid microbes, it appears that the sample C fluids flow through pathways in the subsurface with a lower temperature than pathways through which the samples A and B fluids pass, possibly indicating subsurface fluid dilution with deep-sea water for these two samples shortly before exiting the seafloor.

Although hydrogen and methane concentrations at site D are comparable with those in samples A and B (hydrogen 170 μM to 303 μM and methane 50 μM to 111 μM), measured temperatures are higher (16°C), and oxygen at the site reached a record low of 152 μM but then climbed back up to 221 μM (Fig. 1). In contrast to the other sites, sulfide concentrations considerably increased from 1–6 μM to a maximum of 15 μM in sample D (Fig. 1B), which is comparable with previous measurements of sulfide concentrations at Irina II (Zielinski et al., 2011). Cell numbers and the diversity index for sample D resembled those of samples B and C (Figs S3 and S4). Epsilon proteobacteria were highly enriched at site D and were mostly affiliated with mesophilic Sulfurovum, Sulfurospirillum, Sulfurimonas and Campylobacterales (Fig. 2A). According to the unweighted algorithm, the microbial community from D was distinctly different to those in samples A, B and C from Irina II (Fig. 2B). This may be a consequence of the distance to the other sites (roughly 3 m), the closer vicinity to the actual main chimney structure and/or the elevated sulfide concentrations.
As expected, the chemical signature from the plume sample E was significantly different to that of all other low-temperature Irina II diffuse fluids, most likely explaining the significant changes (P-value = 0.001–0.01) in the microbial community composition, when compared with samples A, B, C and D (Fig. 2). Hydrogen (14 nM) and methane concentrations (10 nM) were extremely low, sulfide was below the detection limit and oxygen reached the values of the ambient seawater (226 ± 40 μM). Cell counts in the plume dropped to 10% of those in sample A or 20–26% of the counts in samples B, C and D (Fig. S3), and were slightly lower than in other plume samples (Nakagawa et al., 2005). The bacterial diversity was even lower than in sample A (Fig. S4). Sulfurimonas relatives clearly prevailed the bacterial domain of the plume waters (Fig. 2A). Besides Sulfurimonas’ ability to grow chemolithoautotrophically, its metabolic versatility (use of various electron donors, electron acceptors and organic carbon sources), oxygen tolerance and environmental sensing systems (Campbell et al., 2006; Grote et al., 2012) may be its keys to success in this plume water sample. Predominance of a few phylotypes, for example Epsilonproteobacteria, has been observed in hydrothermal plumes before (Sunamura et al., 2004).

(A)biotic interconnections

In the four tested samples from low-temperature diffuse fluids the same core bacterial community was present, consisting of Gammaproteobacteria (Marinobacter, Alcanivorax and Thiomicrospira) and Epsilonproteobacteria (Caminibacter, Sulfurimonas, Sulfurovum and Campylobacterales) (Fig. 2A). Differences between the populations of the distinct samples are visible in the relative proportions of the core genera and orders. However, only few significant correlations are evident here. There is a significant correlation (P-value < 0.05) between the presence of Marinobacter and Alcanivorax on one side and the abundance of Sulfurovum and Campylobacterales on the other side. This suggests that these couples may colonize similar habitats or have syntrophic relationships. There are other significant correlations (P-value < 0.01) between increased hydrogen and elevated methane concentrations, which in turn correlate with reduced magnesium, or increased temperature and reduced oxygen concentrations. These abiotic–abiotic linkages can be explained by the degree of admixed oxygenated ambient seawater affecting temperature, magnesium, oxygen, hydrogen and methane concentrations. The only abiotic feature significantly correlating with the presence of a bacterial group was the interrelationship between elevated sulfide concentrations and Sulfurovum enrichment (P-value < 0.01).

Conclusion

When assessing the microbial community on a temporal (two samples collected over 24 min) and a spatial scale (single samples from four locations) a core bacterial community appears to govern the tested Irina II diffuse fluid emissions. Besides the plume sample with distinctly different abiotic conditions to the low-temperature hydrothermal fluids, all other samples have Gammaproteobacteria associated with Marinobacter, Alcanivorax and Thiomicrospira, as well as Epsilonproteobacteria affiliated with Caminibacter, Sulfurimonas, Sulfurovum and Campylobacterales (Fig. 2A). The changes observed between communities collected at the different time points from the same spot and from different locations can mainly be attributed to shifts in the relative abundance of these core bacterial lineages. The differences in the emanated populations may relate to heterogenous conditions in the subsurface along the fluid pathways. However, besides a significant correlation between sulfide enrichment and proportions of Sulfurovum (Epsilonproteobacteria), no other significant linkages between abiotic factors (temperature, hydrogen, methane, sulfide and oxygen) and bacteria were discerned. This is surprising since in the past interconnections between fluid chemical properties and microbial compositions have been established (e.g. Perner et al., 2010; 2011; Flores et al., 2011). The lack of further correlations between the microbial community composition and geochemistry may be related to differences in the temporal scales of geochemical sampling. Indeed, the temporal resolution of the temperature and chemistry via the Kieler Pump system (KIPS) and the mass spectrometer, respectively, is on the scale of seconds to tens of seconds; the resolution of the microbial community is much lower and is integrated over several minutes to the limitations of existing apparatus. Hence, if collecting samples for analyses of microbial community compositions was possible on the scale of seconds, the highly variable chemistry may be better linkable to the biology. Alternatively, the level of the phylogenetic resolution may be too broad for detecting correlations or 16S rRNA gene phylogenetic analysis may not be able to reveal functional responses of the microbial community to changes in fluid chemistry.

Interestingly, one of the bacterial populations from the two time series samples was in fact more similar to the integrated community sampled 20 cm away than to the population in the sample collected from the same location in the following 14 min. Although replicates are missing, we posit from this data set that small-scale spatial diversity observed within a vent location may also arise – to some yet undetermined degree – from unrecognized temporal diversity changes. Further studies will be required to confirm this hypothesis.
Experimental procedures

Site descriptions and sample collection

Five hydrothermally influenced fluid samples (samples A, B, C, D and E) were collected from the Logatchev field at 14°45′N located at a water depth around 3000 m along the Mid-Atlantic Ridge (MAR) (Perner et al., 2007). Hydrothermal fluids were retrieved during dives with the remotely operated vehicle Kiel ROV 6000 (IFM-GEOMAR, Kiel) during the cruise HYDROMAR VI (January/February 2009) with the RV Maria S. Merian. Detailed sampling procedures are described elsewhere (Perner et al., 2009). Irina II is the main chimney structure in the Logatchev field. It is surrounded by a Bathymodiolus mussel patch, which is colonized by crabs, brittle stars, snails and isolated shrimps. Samples A to D were retrieved from locations where low-temperature hydrothermal fluids emitted from one connected mussel patch by positioning the nozzle of the fluid sampling system deep between the mussels, but not in contact with the substrate. While samples A and B were taken exactly at the same location at different time points (sample A on 4 February 2009, 18:20–18:30 UTC and sample B on 4 February 2009, 18:32–18:46 UTC) and thus represent temporal dynamics, sample C was collected (4 February 2009, 19:02–19:14 UTC) roughly 20 cm away from samples A and B and represents short-range spatial variation. Samples A, B and C were taken roughly 3 m away from the main Irina II structure, and sample D (25 January 2009, 12:47–13:07 UTC) was retrieved just at Irina II’s base. Sample E was taken from the hydrothermal plume 175 m above the ground of Irina II (3 February 2009, around 12:00 UTC).

Fluid chemistry

We used the same methods for ISMS as in Wankel and colleagues (2011) to measure hydrogen and methane concentrations in the field. Briefly, the ISMS unit was configured to sample fluids at temperatures up to 350°C by drawing sample through a titanium heat exchanger prior to passing it by the membrane. A Seabird CTD pump is used to move sample at a rate of ~50 ml min⁻¹. During these deployments, Teflon AF™ backed by an integrated polyvinylidene fluoride sheet (Random Technologies, San Francisco, CA, USA) was used in lieu of a polydimethylsiloxane (PDMS) membrane, due to its consistent performance over a wide range of hydrostatic pressures. Power (24VDC) and communications (RS-232) to the instrument were supplied from the submersible via dielectric oil-compensated junction box and underwater connectors (SubConn, Burwell, NE, USA). Serial communications with the turbo pump (which provided feedback on vacuum status and chamber temperature, etc.) and the RGA mass analyser enabled real time monitoring of instrument response directly from within the control room on board. Using water ion intensity as a proxy for temperature and the associated changes in analyte permeability, thermal artefacts – if any – are removed from the true signal. Once normalized, signal intensity is used to determine concentrations by comparison with laboratory-generated data for each analyte (see Wankel et al., 2011). Because the use of Teflon AF™ can prevent robust oxygen and sulfide quantification, oxygen and sulfide were measured directly on board (ex situ). For these latter molecules, routine procedures were applied: Winkler titration for dissolved oxygen (Carpenter, 1965) and photometry of dissolved sulfide following the methylene blue method (Clene, 1969). Measurements of sulfide concentrations are at the courtesy of H. Strauss (Westfälische Wilhelms-Universität Münster, Germany). Temperature was measured in situ with the KIPS as described previously (Perner et al., 2009). Magnesium in hydrothermal fluids was determined in the home laboratory by inductively coupled plasma-optical emission spectrometry using a CIROS SOP instrument (Spectro, Germany). Temperature and magnesium are at the courtesy of D. Garbe-Schönberg (Christian-Albrechts-Universität, Kiel, Germany). Hydrogen and methane in the plume waters were analysed on board by a gas chromatograph-equipped pulsed discharge detector (PDD) and by applying a purge and trap technique respectively (cf. Perner et al., 2007). Ex situ hydrogen and methane concentrations of the plume are at the courtesy of R. Seifert (University Hamburg). The significance of the chemical differences between distinct samples and the correlations between abiotic properties and microbial community compositions were determined by the Student’s t-test and by Pearson’s correlation (SPSS version 16.0; SPSS, Chicago, IL, USA).

Cell counts, DNA extraction and amplification

Procedures for cell counts of each sample were followed as previously described (Perner et al., 2010). For DNA extraction of each of the samples A, B, C and D, 450 ml of hydrothermal fluids and, for sample E, 1 l of plume waters were concentrated on polycarbonate filters (type: GTTP, pore size 0.2 µm; Millipore, Eschborn, Germany) and kept at ~20°C. DNA was extracted from the five filters using the UltraClean Soil DNA Isolation Kit (MoBio, Solana, CA, USA) according to manufacturer’s instructions. Bacterial 16S rRNA genes were PCR-amplified with the primers 27F and 1492R (Lane, 1991) as described before (Perner et al., 2009) but using only 10 cycles. For each sample three separate PCRs were performed, and the PCR amplicons were further divided into three aliquots. The first PCR aliquot was sequenced in the first sequencing run. The second and third PCR aliquots were sequenced in the second sequencing run and subsamples 1C2 and 1D2 were re-sequenced in a third sequencing run and named 1C4 and 1D4 respectively (for nomenclature of samples, see Table S1).

High-throughput sequencing of 16S rRNA genes

Bacterial tag-encoded FLX amplicon pyrosequencing was performed as described previously (Dowd et al., 2008a,b). Initial generation of the sequencing library utilized a PCR amplification with Hot Start Plus DNA polymerase (Qiagen, Valencia, CA, USA) and fusion primers that include both the ‘tag’ sequence and the adapter. For bacterial library construction, the primers were Gray28F 5’TTTAGCTNTGCGTTCAG (modified from Frias-Lopez et al., 2002) and Gray519r 5’GTNTACNGCGGCKGCT (modified from Manefield et al., 2002). All reactions used template with a concentration of 100 ng µl⁻¹ and employed the following thermal profile: 1 cycle of 95°C for 5 min, 20 cycles of 95°C 30 s, 54°C for 40 s, 72°C for 1 min, 1 cycle of 72°C for 10 min. A secondary PCR
was performed for tag-encoded FLX amplicon pyrosequencing at the Research and Testing Laboratory (RTL; Lubbock, TX, USA) on a Roche 454 FLX instrument (454 Life Science, Branford, CT, USA) with Titanium reagents, following manufacture protocols.

Data analyses

The analysis was performed by combining different programs from the Quantitative insights into microbial ecology (Qime) pipeline (Caporaso et al., 2010a), version 1.4.0, which provides methods for quality filtering of reads, efficient operational taxonomic unit (OTU) picking, taxonomy assignment and analysis of α- and β-diversity. First, all samples were denoised using AmpliconNoise 1.24 (Quince et al., 2011). This procedure allows for the reduction of the sequencing artefacts related to the PCR amplification and the pyrosequencing reactions. Additionally, it allows for the identification and removal of chimeric sequences. Pyrosequencing yielded 428,785 raw reads of length in the range between 32 bp and 844 bp (on average 367 bp, with a median length of 409 bp). Sequences were trimmed to at most 400 bp from the 5′ end, according to the quality scores. After denoising there were 302,280 sequence reads left, with a length ranging from 204 bp to 400 bp (average length: 394 bp, median length: 400 bp). Sequences were clustered de novo into OTUs using uclust (Edgar, 2010) (version 1.2.22) with a 97% similarity threshold. A single representative sequence for each OTU was then extracted and used in subsequent steps. The total number of OTUs picked in the raw data set was 13,640, while in the denoised data set the number of OTUs was 4,796. The sharp decrease is expected and consistent with the finding in Wang and colleagues (2007). The accuracy in the estimation of the correct number of OTUs is increased by the denoising procedure (Reeder and Knight, 2009). All further analyses were performed using the denoised reads. Alpha rarefactions were used to investigate intra-sample variability. A phylogenetic tree relating to the OTUs was constructed as follows. A template-based multiple sequence alignment (MSA) of the set of representative sequences was prepared using PyNAST (Caporaso et al., 2010b) (version 1.1). The GreenGenes core set alignment, from the GreenGenes ribosomal database (DeSantis et al., 2006), provided with Qiime 1.4 was used as template. The alignment of the representative sequence set was filtered to remove gaps and hypervariable positions using the GreenGenes lane mask. From the resulting filtered MSA, an approximate maximum-likelihood phylogenetic tree was constructed using FastTree (Price et al., 2010) (version 2.1.3). The resulting tree was used for the calculation of the unweighted and normalized weighted UniFrac distances (Lozupone and Knight, 2005). β-diversity analyses were performed by principal coordinates analysis (PCoA) applied to the UniFrac distance matrices. To analyse the support of the data, jackknifing replicates were generated by random subsampling and included in the beta analysis. The interquartile ranges of the coordinates obtained from the analysis of the jackknife replicates are visualized as ellipsoids in the PCoA plots. To assess if the differences among the samples observed in the PCoA plots are significant, Bonferroni-corrected P-values were calculated using the UniFrac unweighted and normalized weighted signi-


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Jackknife analysis of Irina II environment clusters using 100 permutations. Calculations were performed using unweighted (A) and weighted normalized (B) UniFrac algorithms. The inner nodes are coloured as follows: red for 75–100% support, yellow for 50–75%, green for 25–50%, blue for < 25% support. For subsample nomenclature, see Table S1.

**Fig. S2.** (A) Unweighted and (B) weighted normalized UniFrac algorithms were used for PCoA with subsamples from Irina II in the Logatchev and clustered according to the sequencing runs. Sequencing run 1 is colour-coded in red, sequencing run 2 in blue and sequencing run 3 in orange. The first three principal coordinates are plotted, and the axes are scaled according to the percentage variation explained by the corresponding coordinate.

**Fig. S3.** Cell counts in the five samples.

**Fig. S4.** Shannon diversity indices calculated from a set of rarefactions of the five samples.

**Table S1.** Nomenclature of the subsamples from the different sites segregated according to the PCR and the sequencing run.