Bioenergetic characterization of a shallow-sea hydrothermal vent system: Milos Island, Greece

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Abstract

Shallow-sea hydrothermal systems, like their deep-sea and terrestrial counterparts, can serve as relatively accessible portals into the microbial ecology of subsurface environments. In this study, we determined the chemical composition of 47 sediment porewater samples along a transect from a diffuse shallow-sea hydrothermal vent to a non-thermal background area in Paleochori Bay, Milos Island, Greece. These geochemical data were combined with thermodynamic calculations to quantify potential sources of energy that may support in situ chemolithotrophy. The Gibbs energies (ΔGr) of 730 redox reactions involving 23 inorganic H-, O-, C-, N-, S-, Fe-, Mn-, and As-bearing compounds were calculated. Of these reactions, 379 were exergonic at one or more sampling locations. The greatest energy yields were from anaerobic CO oxidation with NO₂⁻ (-136 to -162 kJ/mol e⁻), followed by reactions in which the electron acceptor/donor pairs were O₂/CO, NO₃⁻/CO, and NO₂⁻/H₂S. When expressed as energy densities (where the concentration of the limiting reactant is taken into account), a different set of redox reactions are the most exergonic: in sediments affected by hydrothermal input, sulfide oxidation with a range of electron acceptors or nitrite reduction with different electron donors provide 85~245 J per kg of sediment, whereas in sediments less affected or unaffected by hydrothermal input, various S⁰ oxidation reactions and aerobic respiration reactions with several different electron donors are most energy-yielding (80~95 J per kg of sediment). A model that considers seawater mixing with hydrothermal fluids revealed that there is up to ~50 times more energy available for microorganisms that can use S⁰ or H₂S as electron donors and NO₂⁻ or O₂ as electron acceptors compared to other reactions. In addition to revealing likely metabolic pathways in the near-surface and subsurface mixing zones, thermodynamic calculations like these can help guide novel microbial cultivation efforts to isolate new species.
Introduction

Hydrothermal systems are prevalent in tectonically active settings, including plate boundaries and hot spots [1–5]. They are commonly categorized by location and water depth into (1) terrestrial, (2) deep-sea (water depth > 200 m), and (3) shallow-sea (water depth < 200 m) [4, 6, 7]. Due to their accessibility, terrestrial hydrothermal systems (often synonymous with geothermal springs) were the first to be explored [1, 3, 7]. Since the discovery in 1977 of the first deep-sea hydrothermal systems near the Galápagos Islands [8], ~700 hydrothermal vents (with 644 confirmed or inferred to be active) have been reported along the ~60,000 km-long ocean ridge system as well as in back-arc basins in every ocean basin (InterRidge Vents Database: https://vents-data.interridge.org/). Approximately 70 active shallow-sea hydrothermal vent systems have also been identified [6, 9, 10]. Compared to their deep-sea counterparts, they occur in more diverse tectonically active settings, including near submarine volcanoes, island and intra-oceanic arcs, ridge environments, intraplate oceanic volcanoes, continental margins, and rift basins. Corresponding to their setting, the source of water for these systems can be a mixture of meteoric, magmatic, groundwater, and/or seawater. Unlike deep-sea systems, their location in the euphotic zone allows for photosynthetic activity as well [6, 9, 10]. Perhaps because they are influenced by and transitional between terrestrial and off-shore geologic environments, shallow-sea hydrothermal vent systems are typically complex and dynamic, establishing unique microbial ecosystems.

The microbial ecology and physiology in and around hydrothermal systems—terrestrial and marine—have been studied for several decades, but the factors that control community composition and metabolic function remain elusive. What is known, however, is that these systems contain the necessary ingredients for life—carbon sources, chemical energy from thermodynamic disequilibrium, mineral surfaces, and compositional gradients. Because all biological processes, including anabolism, catabolism, growth, development, and reproduction, are dependent on energy transformations [11–17], quantifying the amounts of energy associated with biological processes guides our understanding of ecosystem dynamics. The amount of energy that microorganisms can gain by catalyzing catabolic reactions in their environment can be quantified by calculating the Gibbs energy of redox reactions ($\Delta G_r$), which depends on physicochemical variables, including temperature, pressure, pH, concentrations of products and reactants, and ionic strength. These physicochemical variables and consequently the redox reaction energy yields can vary considerably from one hydrothermal system to the next, with the structure and function of the resident microbial community closely tied to the geologic setting. We can build upon earlier studies that have shown that thermophilic archaea and bacteria in these environments can catalyze a tremendous array of redox reactions to gain energy. Many of these thermophiles are chemolithoautotrophic, i.e., they use metabolic strategies that rely only on inorganic compounds as sources of energy and carbon [14, 18–22]. In fact, a number of studies have quantified the energetic potentials in terrestrial geothermal springs [23–29], deep-sea hydrothermal systems [30–35], and shallow-sea hydrothermal systems [22, 36–42].

Several studies have described the geology, geochemistry and microbiology of the shallow-sea hydrothermal system at Milos [43–49], but the bioenergetic potential there has not been quantified. In this study, we quantify the energetics of 730 inorganic redox reactions in a shallow-sea hydrothermal system of Milos Island, Greece. The reactions include electron donors and acceptors of five major elements (H, C, N, O, S) and three trace elements (Fe, Mn, As) that are commonly enriched in hydrothermal fluids. The thermodynamic calculations can be used to link the energetic potential of microbial communities to molecular evidence of their identities and metabolic capacity.
Materials and methods

Field work and chemical analyses

Samples were collected in May 2014 from Paleochori Bay, Milos Island (Greece) under a permit from the Greek Ephorate of Underwater Antiquities. In this study, we investigated samples from the Saganaki diffuse vent (36°40'24N, 24°30'50E), located under ~12 m of water and ~300 m offshore (Fig 1A and 1B). Milos Island is located in the South Aegean Sea and is part of the Hellenic Volcanic Arc [50, 51] (Fig 1A). The volcanic activity that is responsible for active gaseous hydrothermal venting on and around the island has occurred since the Pliocene. The field sampling, sample preservation, and analytical protocols used in this study were based...
A SCUBA diving team measured *in situ* temperatures and collected fluid (via long pipette) and gas samples from the vent system. Large volumes of fluids with coarse resolution were collected under water through long pipette tips inserted directly into the sediments (~5 cm depth interval, from 5 to 20 cm deep) and attached to 60-mL syringes (Fig 1C and 1D). Free gas samples were obtained with a stainless-steel funnel placed on sites with visible gas bubbles. Glass serum bottles with blue rubber stoppers were filled with seawater before the dive and then connected to the top of the funnel. Once the gas completely replaced the seawater and flushed through for approximately 5 minutes, the valves were closed. Sediment cores (in polycarbonate tubes) were collected and sealed underwater with rubber caps. Sites included the center of a white mat (WM), through a transition zone (TZ) and a sea grass-covered region (SG), ending in a background area (BG) (Fig 1C, 1D, 1E, and 1F).

On shore, waters were carefully transferred into pre-cleaned serum bottles and capped without trapping any air (for dissolved gas analyses) or filtered (0.2 \( \mu \text{m} \)) and stored as described below for other analyses. Rhizons (0.2 \( \mu \text{m} \) filter) were inserted into pre-drilled holes of the polycarbonate tubes at 2 cm intervals from 0 to 20 cm to obtain high-resolution samples while avoiding fluid reflux from different depths (Fig 1G). All porefluids were analyzed for pH and then subsampled and treated for later geochemical measurements. The subsamples were stored in acid-washed polypropylene bottles for organic acid and anion analyses; acidified with 2% ultrapure \( \text{HNO}_3 \) in acid-washed plastic bottles for cation analyses; acidified with 2% HCl and kept in opaque acid-washed glass bottles for arsenic speciation; fixed with zinc acetate solution for sulfide analysis; and stored in acid-washed and combusted glass vials for dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) analyses.

Thermodynamic modeling

The maximum amount of available energy from potential chemolithoautotrophic reactions at the temperature, pressure, and chemical composition of interest is given by the Gibbs energy (\( \Delta G_r \)). Values of \( \Delta G_r \) were calculated using the relation

\[
\Delta G_r = \Delta G_r^0 + RT \ln Q_r
\]

(1)

where \( \Delta G_r^0 \) denotes the standard state Gibbs energy of reaction, \( R \) designates the universal gas constant, \( T \) stands for the temperature in Kelvin, and \( Q_r \) represents the reaction quotient. Values \( \Delta G_r^0 \) were calculated at the temperatures and pressures of interest with the revised-
Helgeson-Kirkham-Flowers (HKKF) [55–57] equations of state using OrganoBioGeoTherm (OBIGT)—which is a user-friendly version of the SCUPCRT92 software package [58]—and thermodynamic data from several sources [56, 59–65].

Values of $Q_r$ were calculated with

$$Q_r = \prod_i a_i^{v_{ir}}$$  \hspace{1cm} (2)

where $a_i$ designates the activity of the $i$th species raised to its stoichiometric reaction coefficient $v_{ir}$ in the $r$th reaction, which is positive for products and negative for reactants. The activities of pure minerals (pyrite (FeS$_2$), elemental sulfur (S$_0$), magnetite (Fe$_3$O$_4$), hematite (Fe$_2$O$_3$), goethite (FeOOH), ferrihydrite (FeOOH), and pyrolusite (MnO$_2$)) and water are taken to be unity ($a_i = 1$). Molalities of the $i$th species in solution ($m_i$) were obtained as noted above, and converted into activities using the individual activity coefficient of the $i$th species, $\gamma_i$:

$$a_i = m_i \gamma_i$$  \hspace{1cm} (3)

Values of activity coefficients were calculated using the program SPEC8 (Geochemist’s Workbench Version 11, Aqueous Solutions LLC) employing the extended Debye-Hückel equation [66]. Aqueous activities of dissolved gases (H$_2$, CH$_4$, O$_2$, CO, CO$_2$, CH$_4$) were calculated from free gas composition data assuming equilibrium. The reactions under consideration include numerous potential electron acceptors (O$_2$, CO, CO$_2$, HCO$_3^-$, N$_2$, NO$_2^-$, NO$_3^-$, pyrite, elemental sulfur, magnetite, hematite, goethite, ferrihydrite, pyrolusite, H$_2$AsO$_4^-$) and donors (H$_2$, CH$_4$, CO, NH$_4^+$, N$_2$, NO$_2^-$, H$_2$S, pyrite, elemental sulfur, magnetite, Mn$^{2+}$, H$_2$AsO$_3^-$) (S1 Table). To permit us to evaluate the energetics of both the forward and reverse direction of every reaction, we also include H$_2$O as a potential electron acceptor (where H in H$_2$O can be reduced) and electron donor (where O in H$_2$O can be oxidized). To facilitate comparisons among reactions, values of $\Delta G_r$ were normalized to the number of moles of electrons transferred in the redox process [14]. In order to scale energy availability to the limiting reactant, the Gibbs energies are also presented in terms of energy densities, $E_r$ [67]. To normalize the $E_r$ on a ‘per kg of venting fluid’ and on a ‘per kg of sediment’ basis, values of $\Delta G_r$ were multiplied by the concentration of the limiting reactant in the fluid. The energy densities in fluid ($E_{fluid}$) were calculated by

$$E_{fluid} = \left[ \frac{\Delta G_r}{V_i} \right] \frac{m_{i, fluid}}{m_{i, fluid}}$$  \hspace{1cm} (4)

where $[m_{i, fluid}]$ refers to the molal concentration of the $i$th limiting electron donor or acceptor per kg of fluid, taking the stoichiometry of the reaction into account. The energy densities in sediment ($E_{sediment}$) were calculated with

$$E_{sediment} = \left[ \frac{\Delta G_r}{V_i} \right] \frac{m_{i, sediment}}{m_{i, sediment}}$$  \hspace{1cm} (5)

where $[m_{i, sediment}]$ is the molal concentration of the $i$th limiting electron donor or acceptor per kg of sediment, considering the porosity of sediments and the density of grains in them (Table 1). We did not evaluate the energy densities for reactions in which solid phases serve as both electron donor and acceptor (Reactions K66-68, L38-43, N17-19, O33-38, P33-38, Q33-38, R16-20, R25-27).
Mixing model

Values of $\Delta G_r$ for all of the reactions listed in S1 Table were also calculated for different mixing ratios of end-member hydrothermal fluid (HF, also referred to as vent fluid) with seawater. The composition of the end-member HF was taken to be the average of white mat samples, and that for seawater was taken from Table 2. The activities of species that are very low in the end-member HF (e.g., oxygen) were taken to be $10^{-9}$. Although the compositions of the mixed fluids are simply proportional to the ratio of end-member fluid to seawater, the temperatures of the mixed fluids ($T_{mix}$) are not a linear combination of the source fluids due to their differing heat capacities. These values were calculated using

$$T_{mix} = \frac{\sum m_i C_{pi} T_i}{\sum m_i C_{pi}}$$

(6)

where $m_i$, $C_{pi}$, and $T_i$ refer to the mass, specific heat capacity, and temperature (K) of the $i^{th}$ fluid. Values of $C_{pi}$ were calculated using the equations of state for water in SUPCRT92 [70, 71]. The temperature of the end-member HF was estimated by extrapolation using the $[\text{Mg}] = 0$ method [72, 73].

Results and discussion

Sample location and geochemistry

The 47 fluid samples from the Saganaki vent area (Fig 1B and Table 2) represent different mixing ratios and physicochemical properties of venting fluids in this system. The hottest area (up to 76.2°C) was covered by a ~1 cm thick, fluffy, white mat (WM) (Figs 1C, 1E, 2 and Table 2). A ~1 m wide transition zone (TZ) separates a flourishing seagrass (SG) area from the diffuse venting site (Fig 1C). Fifteen samples were taken from WM, 15 from TZ, 9 from SG, 7 from background sediment (BG) and 1 from surface seawater (SW).

Fluid measurements (Fig 2 and Table 2) reveal a wide range of temperature (19.1–76.2°C) and pH (4.4–7.4), as well as sharp differences in geochemistry for the five sampling regions. For example, concentrations of $\text{SO}_4^{2-}$ (13.4–34.5 mM), $\text{Na}^+$ (401.2–539.2 mM), and $\text{Mg}^{2+}$ (26.6–59.6 mM) increase as a function of distance from the diffuse vent in the WM area. In contrast, $\text{K}^+$ levels were almost twice as high (22.7–25.9 mM) at WM than at other sites (10.3–12.1 mM). $\text{NO}_3^-$ concentrations were below detection (b.d., 8 μM) in all samples, but $\text{NO}_2^-$ levels were relatively high (0.15–0.23 mM) in several samples. We note that very low nitrate levels (< 0.5–3.5 μM) have previously been reported in Mediterranean surface waters, especially around
### Table 2. Temperature, pH and composition of porefluids and seawater sampled at or near the Siganaki diffuse vent, Milos Island, Greece.

| Date/Time | Depth (cm) | T (°C) | pH | Na+ (mM) | K+ (mM) | Mg2+ (mM) | Ca2+ (mM) | Sr2+ (μM) | Cl− (μM) | Br− (μM) | SO4− (μM) | H2S/HS− | DOC (μM) | cO (μM) |
|-----------|------------|--------|----|----------|--------|-----------|-----------|-----------|----------|----------|----------|----------|--------|--------|
| 140522A-S | G5         | 19.1   | 7.12 | 525.7    | 11.6  | 58.9      | 11.5      | 98.6      | 0.13     | 1.16     | 0.001    | 615.9    | 0.01   | b.d.   |
| 140522A-S | G7         | 19.1   | 5.38 | 538.3    | 12.1  | 59.4      | 11.7      | 99.5      | 0.12     | 1.15     | 0.003    | 619.0    | 0.91   | b.d.   |
| 140522A-S | G15        | 19.1   | 7.05 | 535.6    | 11.9  | 59.3      | 11.7      | 99.3      | 0.13     | 1.16     | 0.16     | 615.9    | 0.01   | b.d.   |
| 140523B-TZ | 11         | 28.0   | 5.19 | 516.2    | 10.4  | 57.9      | 11.6      | 97.9      | 0.35     | 0.37     | 3.42     | 0.84     | 0.001  | 729.9  |
| 140523B-TZ | 10         | 27.2   | 4.88 | 528.0    | 10.8  | 58.4      | 12.4      | 99.2      | 0.41     | 0.28     | 9.46     | 0.04     | 0.002  | 1168.38|
| 140524A-TZ | 15         | 25.9   | 4.80 | 511.2    | 10.1  | 56.8      | 11.6      | 97.1      | 0.36     | 2.03     | 2.39     | 0.18    | 0.000  | 1081.85|

(Continued)
Table 2. (Continued)

| Date-Dive-Type-Depth(cm) | T (°C) | pH | Na⁺ (mM) | K⁺ (mM) | Mg²⁺ (mM) | Ca²⁺ (mM) | Sr²⁺ (μM) | Ba²⁺ (μM) | Fe²⁺ (μM) | Mn²⁺ (μM) | As³⁺ (μM) | As⁵⁺ (μM) | Cl⁻ (μM) | Br⁻ (μM) | NO₂⁻ (μM) | NO₃⁻ (μM) | SO₄²⁻ (μM) | SiO₂⁻ (μM) | H₂S/HS | DOC (μM) |
|--------------------------|--------|----|----------|---------|-----------|-----------|----------|----------|----------|----------|----------|----------|---------|---------|----------|----------|----------|----------|---------|--------|
| 140523B-SG10             | 20.3   | 6.54 | 534.9    | 11.8    | 59.3      | 11.6      | 98.5     | 0.15     | 17.02    | 1.17     | 0.41     | 0.001    | 615.1   | 0.98    | 0.23     | b.d.     | 28.04   | 0.04    | 0.69    | 1210.81 |
| 140523B-SG15             | 20.8   | 6.84 | 530.1    | 11.7    | 58.7      | 11.5      | 98.2     | 0.16     | 20.56    | 1.36     | 0.41     | 0.017    | 609.6   | 0.86    | 0.23     | b.d.     | 28.27   | 0.05    | 2.84    | 1160.12 |
| 140523B-SG20             | 21.3   | 6.93 | 536.4    | 11.9    | 59.3      | 11.7      | 98.8     | 0.18     | 21.20    | 1.51     | 0.45     | 0.011    | 616.9   | 0.90    | 0.20     | b.d.     | 25.69   | 0.05    | 1.89    | 1323.48 |
| **Background**           |        |     |          |         |           |           |          |          |          |          |          |          |         |         |          |          |          |         |        |         |
| 140521B-BG3              | 19.1   | 6.34 | 524.8    | 11.4    | 58.7      | 11.4      | 97.1     | 0.08     | b.d.     | b.d.     | 0.10     | b.d.     | 603.5   | 0.90    | b.d.     | b.d.     | 32.31   | b.d.    | 0.21    | 481.33  |
| 140521B-BG9              | 19.1   | 6.75 | 527.4    | 11.5    | 59.1      | 11.5      | 98.2     | 0.13     | 2.28     | 0.07     | 0.01     | b.d.     | 606.5   | 0.84    | b.d.     | b.d.     | 29.81   | 0.02    | b.d.    | 773.76  |
| 140521B-BG15             | 19.2   | 6.64 | 528.4    | 11.5    | 59.1      | 11.5      | 98.6     | 0.15     | 14.74    | 0.39     | 0.17     | b.d.     | 607.7   | 0.90    | b.d.     | b.d.     | 32.05   | 0.04    | 1.41    | 649.64  |
| 140523B-BG5              | 19.2   | 6.88 | 531.4    | 12.0    | 58.6      | 11.6      | 98.6     | 0.12     | 0.83     | 2.07     | 0.21     | 0.002    | 611.1   | 0.91    | 0.21     | b.d.     | 23.39   | 0.05    | 0.19    | 1198.69 |
| 140523B-BG10             | 19.1   | 6.62 | 529.2    | 11.6    | 58.6      | 11.5      | 97.3     | 0.11     | b.d.     | 0.18     | 0.04     | 0.001    | 608.6   | 0.82    | 0.23     | b.d.     | 30.14   | 0.01    | 0.32    | 1134.78 |
| 140523B-BG15             | 19.1   | 7.09 | 527.7    | 11.6    | 58.5      | 11.5      | 97.6     | 0.13     | 0.34     | 0.05     | 0.06     | 0.002    | 606.9   | 0.81    | 0.20     | b.d.     | 21.71   | 0.02    | 0.06    | 1019.07 |
| 140523B-BG20             | 19.0   | 7.14 | 539.2    | 11.9    | 59.6      | 11.7      | 99.2     | 0.15     | 1.33     | 0.03     | 0.09     | 0.003    | 620.1   | 0.92    | 0.21     | b.d.     | 25.75   | 0.03    | 0.26    | 1089.60 |
| **Seawater**             |        |     |          |         |           |           |          |          |          |          |          |          |         |         |          |          |          |         |        |         |
| 140525A-SW               | 19.0   | 7.4  | 528.5    | 11.6    | 59.2      | 11.6      | 98.5     | 0.06     | b.d.     | 0.2      | 0.1      | b.d.     | 607.8   | 1.01    | 0.23     | b.d.     | 28.4    | 0.01    | 0.10   | 528.85  |
| **Calculated Endmember** |        |     |          |         |           |           |          |          |          |          |          |          |         |         |          |          |          |         |        |         |
| Average                  | 199.41 | 4.12 | 119.98   | 29.56   | 0.00      | 11.57     | 61.04    | 3.75     | 121.82   | 204.17   | 0.79     | 0.01     | 18.09   | 0.00    | 0.00     | 0.00     | 14.73   | 5180.57 |

b.d. = below detection.

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Greece [74], and near mM concentrations of nitrite are known to occur in marine sediments [75]. Concentrations of reduced species, such as H\(_2\)S, Fe\(^{2+}\), and Mn\(^{2+}\) range from b.d. (1 \(\mu\)M, 0.1 \(\mu\)M, and 0.04 \(\mu\)M, respectively) to relatively high levels (93 \(\mu\)M, 78 \(\mu\)M, and 1631 \(\mu\)M, respectively). Consistent with the stable and conservative nature of salinity in seawater (i.e., the principle of constant proportions), and to maintain required charge balance, concentrations of Cl\(^-\) in Table 2 were calculated from a well-established Cl:\(\text{Na}^+\) ratio of 1.15 (e.g., [76]).

**Porefluid energetic potential**

Values of \(\Delta G\) for the 730 reactions listed in S1 Table were calculated with Eqs (1–4) using the fluid compositions and environmental conditions given in Tables 2 and 3. Values of \(\Delta G\), in kJ
per mole of electron transferred are depicted in Fig 3A for the 379 reactions that are exergonic ($\Delta G_r < 0$) in at least one sampling location. The reactions are plotted from top (-162.3 kJ/mole e⁻) to bottom (near 0 kJ/mole e⁻) in order of the average energy yield at all 47 sampling sites. It can clearly be seen that for most reactions, the energy yields vary by 20–50 kJ/mol e⁻ across the different locations and sample depths; in a number of examples, especially reactions with iron (e.g. L34: Fe²⁺/S⁰ and N16: H₂S/Fe₃O₄), the range approaches and even exceeds 80 kJ/mol e⁻. Reactions with nitrite (purple bar) and oxygen (light grey bar) as electron acceptors and with CO as electron donor are the most exergonic, with the top 10 reactions (electron donor/acceptor) being CO/NO₂⁻ (reaction H13, -162.3 kJ/mole e⁻), CO/NO₃⁻ (H14, -151.4 kJ/mole e⁻), CO/O₂ (B5, -147.9 kJ/mole e⁻), CO/NO₃⁻ (I18, -139.9 kJ/mole e⁻), CO₂/O₂ (B6, -142.6 kJ/mole e⁻), CO/MnO₂ (R5, -140.9 kJ/mole e⁻), H₂S/NO₂⁻ (H19, -136.0 kJ/mole e⁻), CO/NO₃⁻ (I19, -131.0 kJ/mole e⁻), CO/MnO₂ (R6, -130.7 kJ/mole e⁻), CO/FeOOH₆⁺ Fe (Q13, -124.9 kJ/mole e⁻) (Fig 3A).

In Fig 3B, energy yields are plotted as energy densities ($E_{fluid}$). Again, the range of energy yields across sites and sample depths vary tremendously, often exceeding 6 orders of magnitude in J/kg H₂O. This broad range is due to the rapid dilution of hydrothermal fluid with seawater, whereby the concentrations of some key redox species change by 2–3 orders of magnitude over short distances (Table 2). For example, the concentration of Mn in WM (28.31–78.30 μM) is 150–400 times that in seawater, and therefore $E_{fluid}$ of Mn-redox reactions point to a much larger energy potential than the corresponding value of $\Delta G_r$ would indicate. In addition, one of the consequences of setting the activities of pure minerals to 1.0 means that the energy densities of reactions involving hematite, goethite, ferricydrate, pyrolusite, pyrite, elemental sulfur, and magnetite are high (100–10,000 J/kg H₂O). Finally, we note that the most exergonic reactions in Fig 3B are not the same as those in Fig 3A. In terms of energy density, the oxidation of sulfide, sulfur, and ammonia are thermodynamically most favorable. A phylogenetic analysis (16S rRNA) of hydrothermal sediments at Milos showed that heterotrophs and sulfur oxidizers were among the most abundant [77]. In fact, several aerobic and anaerobic sulfur oxidizers have been isolated from Milos, including Halothiobacillus Kelly [78], Stetteria hydrogenophila [79], Thiomicrospira sp. Milos-T1 [80], and Gamma Proteobacteria Milos strain OD14G, OBI5, OD116, OBI5 [81]. Values of $\Delta G_r$ and $E_{fluid}$ for the exergonic reactions shown in Fig 3 are re-plotted as colored circles in Fig 4, but with average values for each of the five regions and classified by electron donors (Fig 4A and 4C) and electron acceptors (Fig 4B and 4D). In addition, the total average energy yields for all 47 samples are also plotted (black squares). In terms of $\Delta G_r$, reactions with O₂ as the electron acceptor supplied the most energy, followed by reactions with NO₃⁻, NO₂⁻, and MnO₂, and then reactions with iron minerals, S⁰, As⁵⁺, SO₄²⁻, and inorganic carbon. In units of energy density ($E_{fluid}$), reactions with ammonia and sulfide as electron donors are the most exergonic, especially in the WM and TZ regions (Fig 4C). When color-coded by electron acceptor (Fig 4D), we note that values of $E_{fluid}$ are generally highest (with some exceptions) in the WM region, followed by the TZ, SG, BG, and SW.

### Table 3. Dissolved and free gas composition in white mat (WM), transition zone (TZ) and background (BG) areas.

| Sample                  | H₂   | O₂  | N₂  | CO₂ | CH₄ | CO  |
|-------------------------|------|-----|-----|-----|-----|-----|
| WM Free Gas (%)         | b.d. | 2.72| 18.00| 61.36| 0.299| 0.025|
| WM Dissolved Gas at 10cm (μM) | b.d. | 0.496| 38.5| 11.93| 0.038| 0.076|
| TZ Dissolved Gas at 5cm (μM) | b.d. | 1.001| 76.6| 2.725| 0.089| b.d. |
| BG (μM)                 | b.d. | 2.72| 18.00| 61.36| 0.299| 0.025|

https://doi.org/10.1371/journal.pone.0234175.t003
See LaRowe and Amend (2019) for a discussion on reaction energetics in molal versus density units [82]. This difference can best be seen in reactions with CO, where energy values are -132 to -162 kJ/mol e\(^{-}\), but only 1.7 to 3.5 J/kg H\(_2\)O (e.g. reaction H13). Similarly, for the oxidation of methane, carbon monoxide, and ammonium, values of the energy densities are more
exergonic than the per-electron counterparts reveal, particularly in the WM and TZ regions. Because the energy densities of sulfide oxidation are so variable, it is likely that the importance of this process is highly localized, with notable potential in the more hydrothermally influenced areas (WM, TZ). Energy densities for iron, manganese, and arsenite oxidation reactions are also scattered, while those for the oxidation of elemental sulfur, pyrite and magnetite show no clear trends. Energy densities for the WM and TZ regions appearing on the right hand (i.e., more exergonic) side of Fig 4D indicates that chemolithotrophic primary production has a positive correlation with temperature, or hydrothermal source.

Similar studies of redox reaction energetics for putative chemotrophic metabolisms have been carried out for geochemically diverse hot springs in Yellowstone National Park [26], shallow-sea hydrothermal systems in the Aeolian Islands (Italy) [22, 38] and the continental
subsurface at the Sanford Underground Research Facility (SURF) in South Dakota (USA) [83]. Analogous to the present study at Milos, those communications concluded that reactions with energy yields $>100$ kJ/mol e$^{-}$ are rare and involve $\text{O}_2$ or $\text{NO}_3^-/\text{NO}_2^-$ as electron acceptors. It should be noted that the maximum yields in the Milos system are at $\sim160$ kJ/mol e$^{-}$, while those in the Aeolian Islands are $\sim120$ kJ/mol e$^{-}$, in Yellowstone hot springs are $\sim110$ kJ/mol e$^{-}$, and at SURF are $\sim100$ kJ/mol e$^{-}$. Nine of the most exergonic reactions at Milos are aerobic or anaerobic carbon monoxide oxidation. We note two reasons for the higher $\Delta G_r$ yields at Milos than at other sites: First, CO and $\text{NO}_2^-$ have large and opposite redox potentials, which tends to lead to large Gibbs energies of reaction. Second, we measured and considered a larger range of redox sensitive species than most other studies, which rarely include CO and $\text{NO}_2^-$ because they decay quickly, or their concentrations are below detection limits.

This set of investigations also demonstrated that in a number of examples, changes in chemical composition—with pH being a major driver—can ‘flip’ a reaction from exergonic to endergonic. In other words, the forward reaction may serve as a putative metabolism in some environments, while the reverse direction could do so at very different geochemical conditions. Similarities among these different studies are also observed when energy densities are considered. (Note that studies focused on the shallow-sea vents at Vulcano, Aeolian Islands [22] and Yellowstone hot springs [26] did not provide such results. In each case, the most exergonic reactions in terms of energy density are different from those labeled as most exergonic in $\Delta G$ space.

**Bulk sediment energetic potential**

The calculations summarized above only considered porefluids and not the solid phase minerals that can be used as energy sources. Following the same color scheme as in Fig 3, the amount of energy available per kg of sediment ($E_{\text{sediment}}$, J/kg sediment) is shown in Fig 5A (by electron donor) and 4B (by electron acceptor). The bottom panels reveal total $E_{\text{sediment}}$ for all reactions, while the middle and top panels show values of $E_{\text{sediment}} < 1$ J/kg sediment and $<10$ J/kg.

![Fig 5. Energy densities at all sample locations and depths.](https://doi.org/10.1371/journal.pone.0234175.g005)

Colored bars refer to identities of different electron donors (A) and electron acceptors (B). The upper and middle panels zoom in on reactions with low energy yields ($<1$ J and $<10$ J, respectively). The energy densities refer to either those in 1 kg sediment or 1 kg seawater. See S1 Table for details on the reactions.
sediment, respectively. It can be seen in the bottom panel in Fig 5A that reactions with ammonia, sulfide, sulfur, magnetite, and pyrite as electron donors are the most exergonic; the bottom panel in Fig 5B shows that reactions with nitrate, nitrite, sulfur and sulfate as electron acceptors provide the most energy. These panels reveal three energy-based habitats: (1) hydrothermal-influenced sediments in the WM and TZ regions, (2) seawater-influenced sediments in the SG and BG areas and (3) surface seawater. Energy yields are similar in the WM and TZ regions, increasing with depth at both locations. Conversely, depth is not a correlating factor in the SG and BG areas. Reactions that provide relatively small amounts of energy are shown in the middle and top panels in Fig 5, with patterns of energy yields changing with the distance from the diffuse vent. Both the WM and TZ settings show increasing energy yield with sediment depth, and the dominance of aerobic respiration in seawater-influenced sediments and seawater.

From an energetics perspective, oxygen/nitrate/nitrite and sulfur/sulfide/ammonia should be the main electron acceptors and donors, respectively, to be used by chemolithotrophs at Milos (Fig 5). Based on values of Gibbs energy and elevated levels of abundance, sulfide oxidation coupled to nitrite reduction could support much of the chemolithotrophic primary production. Some evidence, though limited, is available to support this claim: three chemolithotrophic nitrate-reducing sulfate-oxidizing bacteria have been isolated from Milos [81] and isotopic evidence of microbial sulfate reduction in the TZ has been reported [84].

Mixed fluid (SW:HF) energetic potential

The estimated, weighted average temperature of the end-member HF, based on Mg content in 13 WM samples, is 199.4°C (Fig 6A). The curve shown in Fig 6B depicts the calculated temperature of a fluid that results from titrating cold seawater (SW) into 1 kg of this end-member HF, and Fig 6C presents how the activities of major redox-active species change with the mass ratios of SW:HF from 10^3:1 to 10^{-2}:1, as well as a temperature range from 19.0 to 199.4°C. The remaining panels in Fig 6 show how much energy is available from the 17 reactions listed in Table 4 normalized to kJ per mole electron transferred (Fig 6D), J per kg mixed fluid (Fig 6E), and J per kg vent fluid (Fig 6F), where solid lines refer to aerobic processes, dashed lines represent anaerobic processes, and the different colors identify different reactions.

Thermodynamic predictions of redox reaction energetics can change substantially when different normalization schemes are used [85, 86] For instance, when normalized to kJ per mole electron transferred (ΔG/e) for the SW:HF mixing calculations, the energy yields of all reactions decreased with increasing proportion of HF (Fig 6D). When normalized per kg of mixed fluid (Efluid), however, a very different picture emerges (Fig 6E); here, the reactions fall into two groups: three reactions (B14-B16) that can provide >60 J/kg mixed fluid at optimal SW:HF ratios, and the other 14 reactions where energy yields maximize at ~11 J/kg mixed fluid for one example (B24) and ~4 J/kg mixed fluid for the rest. Finally, the potential energy per kg of pure vent fluid is given in Fig 6F. In this normalization, the reactions are most exergonic at a SW:HF mixing ratio of ~100:1, corresponding to a temperature of ~20°C. At these conditions, the energy yields from sulfide oxidation with O2 or NO3 (B14, B15, I29, I30) and aerobic ammonia oxidation (B9) exceed by several orders of magnitude those of the other reactions. A similar story emerges when normalizing values of ΔG per kg mixed fluid and per kg vent fluid for aerobic and anaerobic sulfur/sulfide oxidation in the WM area (Fig 6E and 6F). This can be seen most clearly at temperatures <40°C, where these reactions are 20–50 times as exergonic as the other reactions. As noted above, isotopic data also point to a dynamic sulfur cycle at Milos with microbial sulfate reduction, sulfide oxidation, and rapid recycling of sulfur intermediates that vary with location and time [47, 84].

The curves in Fig 6E (J/kg mixed fluid) and 6F (J/kg hydrothermal fluid) show how the energy densities of the reactions in Table 4 change with HF:SW ratio. The multiple y-axes are
used in Fig 6E and 6F because the results span several orders of magnitude. It can clearly be seen that as the proportion of HF decreases, the energy yields for most reactions also decrease.

Table 4. Catabolic reactions used to define metabolic groups in the analysis shown in Fig 5.

| Metabolic Group          | #  | Reaction                                      |
|--------------------------|----|-----------------------------------------------|
| Hydrogen Oxidizers       | B1 | $2H_2 + O_2 \rightarrow 2H_2O$               |
|                          | I5 | $5H_2 + 2NO_3^{-} + 2H^+ \rightarrow N_2 + 6H_2O$ |
| Methane Oxidizers        | B3 | $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$       |
|                          | I14| $CH_4 + 4NO_3^{-} \rightarrow 4NO_2^{-} + CO_2 + 2H_2O$ |
|                          | M6 | $CH_4 + SO_4^{2-} + 2H^+ \rightarrow H_2S + CO_2 + 2H_2O$ |
| Ammonium Oxidizers       | B9 | $NH_4^+ + 2 O_2 \rightarrow NO_3^{-} + 2 H^+ + H_2O$ |
| Sulfur Oxidizers         | B14| $2H_2S + O_2 \rightarrow 2 S + 2 H_2O$        |
|                          | B15| $H_2S + 2 O_2 \rightarrow SO_2^{2-} + 2 H^+$   |
|                          | B16| $2 S + 3 O_2 + 2 H_2O \rightarrow SO_4^{2-} + 4 H^+$ |
|                          | I29| $5 H_2S + 2 NO_3^{-} + 2 H^+ \rightarrow 5 S + N_2 + 6 H_2O$ |
|                          | I30| $5 H_2S + 8 NO_3^{-} \rightarrow 5 SO_4^{2-} + 4 N_2 + 2 H^+ + 4 H_2O$ |
| Iron Oxidizers           | B24| $4 Fe^{3+} + O_2 + 6 H_2O \rightarrow 4 FeOOH_{Ferricyanide} + 8 H^+$ |
| Manganese Oxidizer       | B28| $2 Mn^{2+} + O_2 + 2 H_2O \rightarrow 2 MnO_2_{Pyrochlocite} + 4 H^+$ |
| Arsenite Oxidizers       | B29| $2 H_3AsO_4 + O_2 \rightarrow 2 H_2AsO_4^- + 2 H^+$ |
| Methanogens              | D3 | $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$         |
| Sulfate Reducers         | M2 | $4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O$ |
| Arsenate Reducers        | S1 | $H_2 + H_3AsO_4^- + H^+ \rightarrow H_2AsO_3^- + H_2O$ |

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especially when these are plotted per kg of vent fluid (Fig 6F). The kinks and inflection points in some of the curves in Fig 6E and 6F are a result of calculating the reaction energies using the concentration of the limiting electron donor or acceptor. These abrupt changes in slope correspond to points where the concentrations of the electron donors and acceptors are equal. The inflection points in Fig 6E also illustrate the temperatures at which the reactions are most exergonic. For example, the sulfur and sulfide oxidation reactions B14 and B15 have inflection points at 27˚C, above which their potential energy yields decrease. Similarly, the limiting reactant for reactions B24 and B28 changes from iron and manganese, respectively, to oxygen as temperature increases (Fig 6E). Our mixing model suggests that microbial metabolic strategies often shift with mixing ratio, and therefore temperature, a notion that is supported by other lines of evidence. Note that cell abundances of sulfur oxidizing bacteria, sulfate reducing bacteria and dissimilatory iron-reducing bacteria at Milos decrease with increasing depth and temperature [87]. Also at Milos, microbial cell numbers are highest in the shallowest sediment [77].

Conclusions

Seawater and porewater chemistries, a fluid mixing model, and thermodynamic calculations were combined to determine the energy yields of more than 700 redox reactions in fluids and sediments of a Milos Island shallow-sea hydrothermal environment. These yields were reported in several normalization schemes—in kJ per mole electrons transferred and in J per kg water or sediment—revealing potential chemolithotrophic microbial metabolisms as a function of depth and distance from a diffuse vent area. We demonstrated that in this system at Milos, analogous to other shallow-sea hydrothermal systems, hot spring environments, and the deep continental subsurface, a large number of inorganic redox reactions can be exergonic, suggesting that diverse chemolithotrophic metabolisms may be occurring simultaneously. Based on modeling of SW:HF mixed solutions in shallow sediments, we also showed that energy yields can change dramatically along the posited steep gradients in temperature, pH, and composition of redox-sensitive aqueous solutes, together with consideration of redox-sensitive minerals. In the Milos hydrothermal system, this applies to a transect from the White Mat area across the Transition Zone to the Seagrass and Background zones and into seawater. An environmental energy framework of the type provided here can help interpret biodiversity data and ecosystem function, and also guide efforts to cultivate dominant as well as important minor members of a chemolithotrophic microbial community.

Supporting information

S1 Table. Redox reactions considered in this study.

S2 Table. Order of reactions shown in Fig 3 from top (1) to bottom (379).

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