

Title: Detection of anaerobic carbon monoxide-oxidizing thermophiles in hydrothermal environments

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Running title: Anaerobic CO-oxidizing thermophiles in hydrothermal environments

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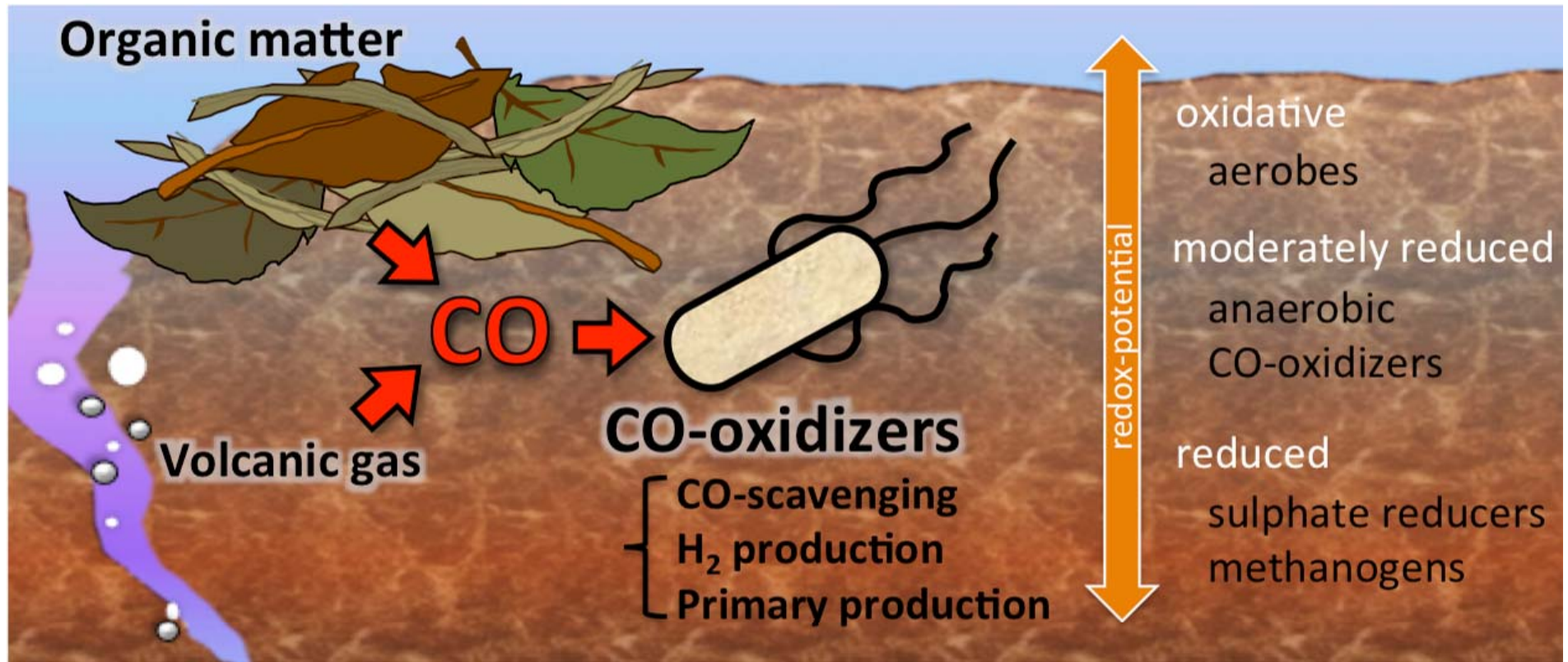
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26 **Abstract**

27 Carboxydophilic anaerobic thermophiles have been isolated from various
28 hydrothermal environments and are considered to be important carbon monoxide (CO)
29 scavengers or primary producers. However, the ecological factors that influence the
30 distribution, abundance and CO-oxidizing activities of these bacteria are poorly
31 understood. A previous study detected the carboxydophilic bacteria *Carboxydotherrmus*
32 spp. in a hot spring sample and found that they constituted up to 10% of the total
33 bacterial cells. In this study, we investigated environmental features, potential microbial
34 CO-oxidation activities and the abundance of *Carboxydotherrmus* spp. in various hot
35 springs to determine environmental factors that affect CO-oxidizers and to see whether
36 *Carboxydotherrmus* spp. are common in those environments. We detected potential
37 microbial CO-oxidation activities in samples that showed relatively high values of total
38 organic carbon (TOC), total nitrogen (TN), oxidation–reduction potential (ORP) and
39 soil-water content. The abundance of *Carboxydotherrmus* spp. did not correlate with the
40 presence of potential microbial CO-oxidation activities; however, *Carboxydotherrmus*
41 spp. were detected in a wide range of environments, suggesting that these bacteria are
42 widely distributed in spite of the relatively low population size. This study implies
43 thermophilic CO-oxidizers occur in a wide range of environments and oxidize CO in
44 somewhat oxidative environments rich in organic matter.



Graphical Abstract Figure

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47 **One-sentence summary**

48 Carbon-monoxide-oxidizing thermophiles are widely distributed in a range of
49 environments, and high potential microbial CO-oxidation activity is associated with the
50 levels of organic matter, oxidation–reduction potential and soil-water content of
51 environments.

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54 **Keywords**

55 carboxydotroph, carbon monoxide, carbon monoxide dehydrogenase, thermophile, hot
56 spring

57

58 **Introduction**

59 It is believed that approximately 2,500–2,600 Tg of carbon monoxide (CO) is produced
60 annually and emitted to environments via the combustion of fossil fuels, oxidation or
61 photochemical degradation of organic compounds, volcanic gas, and metabolism in
62 animals, plants and microbes (King & Weber, 2007). CO concentrations in
63 environments usually occur in trace amounts; e.g., 2–15 nM in seawater (Moran &
64 Miller, 2007), 20–33 nM in hot springs (Kochetkova *et al.*, 2011) and approximately 92
65 ppb global mean mole in the atmosphere (WMO WDCGG, 2014). There is generally
66 equilibrium between CO production and consumption; approximately 90% of
67 atmospheric CO is consumed by reaction with hydroxyl radicals, and CO-oxidizing
68 microbes in soil are considered to be a CO sink accounting for 10% of the total CO
69 consumption (King, 2007).

70 Studies on microbiological CO consumption have been conducted under
71 aerobic conditions using soil, freshwater and seawater (Conrad & Seiler, 1980; Conrad
72 & Seiler, 1982). Aerobic CO-oxidizers utilize molybdenum-containing carbon
73 monoxide dehydrogenases (Mo-CODHs), which can be detected widely in
74 environments such as soils (plant root), sediments, volcanic deposits and the ocean floor
75 (King, 2003; King, 2007; Dunfield & King, 2004; Martin-Cuadrado *et al.*, 2009). In
76 anaerobes, CO oxidation is catalysed by nickel-containing CO dehydrogenases
77 (Ni-CODHs) (Ragsdale, 2004). Ni-CODHs can be divided into the following two major
78 groups: the monofunctional CODH group and the bifunctional CODH/acetyl CoA
79 synthase (ACS) group. Bifunctional CODH/ACSs comprise CODH subdomains and

ACS subdomains and are known to be key enzymes in the Wood–Ljungdahl pathway (reductive acetyl CoA pathway) (Ragsdale, 2004). The CODH subdomains reduce CO₂ to CO, after which the ACS subdomains incorporate CO as a carboxyl group of acetyl-CoA. Monofunctional CODHs catalyse oxidation of CO to CO₂, thereby deriving low potential electrons whose energy can be converted to the transmembrane potential and ATP when they are transferred to some oxidants (Techtmann *et al.*, 2009). In a previous study, monofunctional and bifunctional CODH genes were predicted from their genomic context. Monofunctional Ni-CODH genes are widely found in the prokaryotic genomes of hydrogenogens, methanogens, sulphate reducers, acetogens and gut microbes in animals (Techtmann *et al.*, 2012). CODH genes are found in diverse microbes in various environments; however, only a few studies have reported anaerobic microbial CO oxidation in environmental samples from soil, wetland and hot springs (Conrad & Seiler, 1980; Rich & King, 1999; King, 2007; Kochetkova *et al.*, 2011), and the environmental and physiological significance of anaerobic CO-oxidizers is less understood.

The thermophilic bacterial genus *Carboxydotherrmus* is one of the most studied CO-oxidizing anaerobes. Five species of the genus *Carboxydotherrmus* have been isolated from hot springs and described (Yoneda *et al.*, 2012; Sokolova *et al.*, 2013). Four of the species produce hydrogen via CO-oxidization under 100% CO atmospheric conditions. Because CO is a toxic gas and hydrogen is an important energy source for many microbes in an anoxic environment, CO-oxidizers in the environment are assumed to be important ‘CO scavengers’ or primary producers (Sokolova *et al.*,

2009; Techtman *et al.*, 2009).

The genome of *C. hydrogenoformans* has four genes encoding monofunctional CODH (*cooS-I*, *cooS-II*, *cooS-IV* and *cooS-V*), which have different predicted functions and sequences (Wu *et al.*, 2005). Previously, the quantitative detection of *Carboxydothemus* spp. by targeting the CODH-II gene (*cooS-II*) using real-time PCR showed that these microorganisms can constitute up to approximately 10% of the total bacterial cells in acidic hot spring sediment where *Carboxydothemus pertinax* was previously isolated (Yoneda *et al.*, 2013a). Considering that the optimal growth pH of *Carboxydothemus* spp. is moderately acidic or neutral, the abundance of these bacteria is intriguingly high. This led us to further question whether *Carboxydothemus* spp. are common in other hot springs, where the environment is more suitable for the growth of this genus, and are responsible for CO-oxidation *in situ*. The physiological group of carboxydophilic hydrogenogens has been isolated from various environments, such as hot springs, deep-sea hydrothermal vents, lake sediments and bioreactors (Sokolova & Lebedinsky, 2013). Unfortunately, the range of environments that offer a niche for these microorganisms is still unknown. The aim of the current study was to determine the answer to the above question. In the current study, we measured potential microbial CO-oxidation activities under anoxic conditions and the abundance of *Carboxydothemus* spp. in various hot spring samples.

Materials and methods

Sample collection

Sample sediments were collected from hot springs located in the Kagoshima Pref. (southern Kyushu Island) and Shizuoka Pref., Japan (Fig. 1). The hot springs were Unagi-onsen in the Kagoshima Pref. and Atagawa-onsen (Benzainoyu and Izu Atagawaso), Shimogamo-onsen (Jiunji temple), Mine-onsen (Mine Onsen Fountain Park), Yatsu-onsen (Yakushinoyu) and Yugawara-onsen (Daikan-Soh) in Shizuoka Pref. As references, samples were collected from the Unagi Lake near Unagi-onsen, a water drain in Shimogamo-onsen and the Yamagawa coastal hydrothermal field that had high salinity (Table 1). The Unagi Lake and a water drain near Shimogamo-onsen were non-hydrothermal environments. The surface layer (above a 3.0 cm depth from the water-sediment interface) of the sediments was sampled using a plastic core tube (7.0 cm diameter) or using a metal dipper. Samples were thoroughly mixed using metal spoons and then subsampled into plastic tubes. For measurement of potential microbial CO oxidation activities, samples were placed in a plastic bag with Aneropouch–Anaero (Mitsubishi Gas Chemical, Tokyo, Japan) and sealed immediately. All sample tubes were packed with ice and transported to the laboratory. Potential microbial CO oxidation was measured as soon as possible after return to the laboratory. Samples for DNA extraction were stored at -80°C until use. The other samples were refrigerated at 4°C until use.

Physiochemical analyses of the environment

The temperature, pH and oxidation–reduction potential (ORP) of the samples were measured before sample collection using a portable meter HM-31P (DKK-TOA, Tokyo,

Japan). Soil-water content was calculated on the basis of the weight of the sediments before and after overnight oven-drying at 105 °C. Pore water was extracted by filtration using filter paper (Advantec quantitative ashless filter paper grade no. 5C, Toyo Roshi Kaisha, Tokyo). The total organic carbon (TOC) and total nitrogen (TN) contents of pore water were analysed using TOC-L (Shimadzu, Kyoto, Japan) equipped with a TN meter unit, TNM-L (Shimadzu). The salinity of pore water was measured using a digital salinity probe SS-31A (Sekisui Chemical, Osaka, Japan) in the laboratory at room temperature.

A principal component analysis (PCA) was performed using R 3.0.2 (R Development Core Team, 2008) to compare the environmental features of the samples.

Measurement of potential microbial CO oxidation

Samples were manipulated in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI, USA) under a 5% H₂/ 95% N₂ atmosphere. Approximately 5.0 mL of sediment was placed in each glass vial (63.8 mL), which was then sealed with a rubber stopper. Glutaraldehyde solution (25% v/v), which is reported to effectively inhibit thermophilic microbial CO-oxidation (Slepova *et al.*, 2007), was added to the control samples (5% v/v at final concentration). All of the following manipulations were conducted outside of the anaerobic glove box. Glass vials were vigorously vortexed and pre-incubated at experimental temperatures for 1 h. The experimental temperature for each sample was set as close to the temperature of its origin as possible. Five mL of pure CO gas (CO PURE, Sumitomo Seika Chemicals, Osaka, Japan) was introduced in

each glass vial using a syringe (under atmospheric pressure at room temperature). From the start time at CO injection, 1.0 mL of gas phase was subsampled from each vial using a gas-tight syringe at approximately 24 h intervals over 5 days. CO concentrations in subsamples were measured by gas chromatography as previously described (Yoneda *et al.*, 2012). Potential microbial CO-oxidation in each sample was calculated based on the difference in the average CO concentrations between the samples and controls. The potential microbial CO-oxidation rate was evaluated by regression analysis and regarded as positive when the slope of microbial CO-oxidation increased over the time course ($p < 0.05$). Measurements of CO concentration in samples and controls were performed in triplicate and duplicate, respectively.

DNA extraction and real-time PCR

DNA extraction was conducted using the commercial kit Extrap Soil DNA Kit Plus ver. 2 (NIPPON STEEL and SUMIKIN eco-tech, Tokyo, Japan) following the manufacturer's protocols. Bead-beating steps were carried out using a Beads Crusher μ T-12 (Taitec, Koshigawa, Japan) at a speed of 3,200 r/min for 60 s. Quantitative PCR (qPCR) was performed for the *Carboxydotherrmus* CODH-II gene (*cooS-II*), the bacterial 16S rRNA gene and the archaeal 16S rRNA gene as previously described (Yoneda *et al.*, 2013a). Real-time PCR primers should be designed against conserved regions of closely related strain sequences to cover the target organisms. However, the primer set for *Carboxydotherrmus cooS-II* cooS2_1442F (5'-TGATGCGTCACGGCTTTATGG-3') and cooS2-1606R

(5'-CTAAAGCTACTGCCCCGGGAGT-3') were designed based on the *cooS-II* genes of *C. hydrogenoformans* and *C. pertinax* (Yoneda *et al.*, 2013a) because no other sequences closely related to *Carboxydothemus* species were available. No sequence matches to the primers were found in NCBI nr database using BLASTn search. This primer set can also amplify *cooS-II* from the genomic DNA of *Carboxydothemus siderophilus*, but not *Carboxydothemus ferrireducens*. Therefore, the abundance of CODH copy numbers observed in this study might be underestimated. Bacterial and archaeal 16S rRNA gene primer sets were described previously (Einen *et al.*, 2008). For *cooS-II*, qPCR was conducted in 4 to 7 replicates. The abundance of bacterial and archaeal 16S rRNA genes was detected in triplicate. Some representative samples of *cooS-II* qPCR products were purified, cloned and sequenced as previously described (Yoneda *et al.*, 2013b). The obtained sequences were analysed using the MEGA 6 program (Tamura, 2013).

Results

Sample description

At Unagi-onsen, samples designated UG-20, UG-55 and UG-90 were collected from three points with different temperatures in a hot spring pool. These samples were acidic in the range of pH 2.8-5.3 (Table 1). As a reference, sediments from the Unagi Lake, a freshwater maar lake located approximately 300 m downhill away from the sampling site at Unagi-onsen, were also collected and designated by the label U-Lake. Sample YG-65 was taken from beach sands under hot water flow at Yamagawa coastal

hydrothermal field, where general physicochemical and microbial characteristics have been previously studied (Kawaichi *et al.*, 2013). The hot water of Yamagawa had a high salinity of up to 2.2% (w/v). Hot springs in the Shizuoka Pref. were neutral to slightly alkaline (pH 7.1–8.4) (Table 1). At Atagawa-onsen, deposits from open-air pools of the hot spring well at Benzainoyu and Izu Atagawaso were designated by the labels BZ-65 and AT-90, respectively. At Yakushinoyu in Yatsu-onsen, two samples (samples KS-90 and KS-65) of hot spring deposits and mud were collected from open-air hot pools created by continuous hot water inflow. A sample from Mine-onsen designated by the label ME-90 was composed of white sinters scratched out from a pipe that was used to upwell underground hot water. Sediment samples from Shimogamo-onsen were collected from a trench drain of hot spring water in the grounds of the Jiunji Temple (Samples JI-70 and JI-65). Salinity was as high as 0.7% (w/v) in samples JI-70 and JI-65. Sediment samples designated by the label J-Drain were also collected from a water drain that was next to, but not connected to, the hot water drain in the grounds of the Jiunji Temple. Sample DA-80 was collected from a small hot water container owned by Daikan-soh inn in Yugawara-onsen. White and orange-coloured hot spring deposits were collected from the container, which is usually closed with a cover.

Potential microbial CO-oxidation and environmental factors

Incubation temperature settings for each sample are shown in Table 2. Potential microbial CO-oxidation was observed in UN-55, UN-90, JI-70, JI-65 and J-Drain (Fig. 2). In samples that were positive for potential microbial CO-oxidation activities, ORP,

TOC and TN ranged from -49 mV to 487 mV, 14.7 mg/L to 147.1 mg/L and 3.7 mg/L to 10.8 mg/L, respectively (Table 1). The highest amount of potential microbial CO consumption was observed in JI-65, where as much as 63.6% of initial CO was consumed during the experimental period of 5 days (Table 2). Relatively high CO consumptions were also observed in J-Drain (51.2%), UN-55 (46.1%) and JI-70 (30.4%). Among the hot spring samples from Unagi-onsen (UN-20, UN-55 and UN-90) and Shimogamo-onsen (JI-65 and JI-70), potential microbial CO-oxidation activities were the highest at temperatures of 55 °C and 65 °C, respectively. These results were expected as most of CO-oxidizing thermophilic isolates from terrestrial hot springs show optimal growth at temperatures of approximately 55–65 °C (Sokolova *et al.*, 2009; Sokolova & Lebedinsky, 2013 and references therein). So far, only a few hyperthermophilic carboxydotrophic isolates that can grow at temperature >80 °C have been reported, and they are archaeal species of the genera *Thermococcus* and *Archeoglobus* from marine hydrothermal environments (Sokolova *et al.*, 2004; Henstra *et al.*, 2007; Lee *et al.*, 2008). We observed potential microbial CO-oxidation in sample UN-90 incubated at 90 °C. Previously, microbial CO oxidation has been reported in hot springs as high as 80 °C and 90 °C (Kochetkova *et al.*, 2011). These results suggest the existence of unknown hyperthermophilic CO-oxidizers in terrestrial hot springs.

Significant potential microbial CO-oxidation activities were not observed in the other hot spring samples, such as KS-65 and BZ-65, in which temperature and pH conditions seemed suitable for CO-oxidizers. To investigate the differences between positive and negative samples, principal component analysis (PCA) was carried out

using environmental parameters as shown in Fig. 3. Sample YG-65 was excluded from this analysis because an ORP value was not available. There were no apparent similarities among samples where potential microbial CO-oxidation activities were observed, though they were quite distinct from negative samples (Fig. 3). There was a rough tendency of positive samples to be relatively rich in nutrients (TOC and TN) and have higher ORP values and soil-water contents than negative samples. Among samples associated with the environmental temperature of approximately 65–71 °C and circum-neutral pH, the TOC and TN values of JI-65 and JI-70 (positive samples) were more than double those of KS-65 and BZ-65 (negative samples). ORP values of positive samples ranged from –49 mV to 487 mV and were higher than those of negative samples (Table 1). The OPR values were in a range of ‘reduced’ to ‘moderately reduced’ redox condition that appears too oxidative for sulphate reduction and methanogenesis (DeLaune & Reddy, 2005; Klüpfel *et al.*, 2014). One of the presumed sources of CO in environments is methanogens and sulphate reducers because some of them produce CO under laboratory conditions (Conrad & Thauer, 1983; Lupton *et al.*, 1984; Techtmann *et al.*, 2009). However, in our case, it appeared that the metabolic activity of those microorganisms did not necessarily correlate with the distribution of CO-oxidizing anaerobes. Alternatively, CO is produced from volcanic gas and organic substances (e.g., plant matter and humus). CO production from organic matter is enhanced in the presence of oxygen, higher temperatures and higher water contents (Conrad & Seller 1985; Tarr *et al.*, 1995; Shade *et al.*, 1999; Hellebrand & Shade, 2008). Our result implies that the CO source is likely from organic substances and suggests that

CO-oxidizers prefer environments where high CO productivity can be expected (high ORP values, TOC, TN and soil-water contents).

Abundance of *Carboxydotherrmus* CODH-II genes and 16S rRNA genes

Results of qPCR are shown in Fig. 4. The technical lower detection limit of qPCR for the standard bacterial 16S rRNA gene, archaeal 16S rRNA gene and *cooS-II* were 1.0×10^3 , 1.0×10^3 , and 5.0×10^1 copies/ μ L, respectively. In environmental samples, *Carboxydotherrmus cooS-II* genes were detected in more than two replicates of samples of UN-55, UN-90 and YG-65. *Carboxydotherrmus cooS-II* was also detected in one or two replicates from UN-20, U-Lake, BZ-65, ME-90, JI-70 and JI-65. The highest copy number was 6.05×10^4 copies/g sediment in UN-55 corresponding to $7.95 \times 10^{-4}\%$ of bacterial 16S rRNA. *Carboxydotherrmus cooS-II* copies were detected from samples UN-90 and ME-90, where the environmental temperatures were above growth temperature ($\geq 78^\circ\text{C}$) of *Carboxydotherrmus* spp. (Yoneda *et al.*, 2012). On the other hand, *Carboxydotherrmus cooS-II* was below detection level in sample KS-65 despite its environmental temperature being optimal (65°C) to most *Carboxydotherrmus* spp.

The qPCR series was conducted on CO-incubated samples used for measurement of potential microbial CO-oxidation activities after incubation for a week from the start time of the experiment. *Carboxydotherrmus cooS-II* was detected in more than two replicates in CO-incubated samples UN-20, UN-55, UN-90, KS-90 and KS-65. Interestingly, *Carboxydotherrmus cooS-II* was detected in CO-incubated KS-90 and KS-65 samples where its corresponding environmental samples produced negative

results. In contrast, *Carboxydotherrnus cooS-II* was not detected in CO-incubated JI-70 and JI-65 samples. Abundances of *cooS-II* in environments and in CO incubated samples from UN-55 and UN-90 were compared by t-test. There were no significant differences in *cooS-II* copy numbers in either sample (Fig. 4).

Some of the representatives of qPCR products from environmental samples and CO incubated sediments were sequenced and the inner region of the *cooS-II* primers (123 bp) were compared to *cooS-II* of *C. hydrogenoformans* (CP000141), *C. ferrireducens* (IMG accession no. 2510417609) and *C. pertinax* (AB723512) obtained from DDBJ/EMBL/GenBank or Integrated Microbial Genome (IMG) databases (data not shown). A total of 58 sequences were obtained from the environmental samples. Of these, 31 sequences showed 97–100% similarity to *C. pertinax cooS-II*: UN-20 (4), UN-55 (8), UN-90 (6), U-Lake (3), YG-65 (3), BZ-65 (3), MI-90 (1) and JI-65 (3). A total of 23 sequences showed 98–100% similarity to *C. hydrogenoformans cooS-II* from samples UN-90 (7), U-Lake (1), YG-65 (2), BZ-65 (1), MI-90 (7), JI-70 (4). The remaining four sequences, all from UN-90, showed 94% similarity to both *Carboxydotherrnus ferrireducens* and *C. pertinax cooS-II*. A total of 66 sequences were obtained from CO incubated sediment samples. Of these, 39 sequences were most related to *C. pertinax cooS-II* with 97–100% sequence similarity: U-Lake (5), UN-20 (6), UN-55 (10), UN-90 (5), YG-65 (1), BZ-65 (2), KS-65 (5) and KS-90 (5). The other 27 sequences were most related to *C. hydrogenoformans* with 97–99% similarity: UN-20 (2), UN-55 (2), UN-90 (3), YG-65 (3), BZ-65 (5), KS-65 (8) and KS-90(4). The divergent sequences found in our study sites showed there are variations in

Carboxydotherrmus spp. and suggested that species-level diverse CO-oxidizers co-exist in certain environments.

Discussion

To the best of our knowledge, our work is the first attempt to discover the distribution and abundance of carboxydutrophic anaerobic thermophiles in various hydrothermal environments in relation to environmental factors. Most carboxydutrophic anaerobic thermophiles require strictly anaerobic, reduced conditions for carboxydutrophic growth. However, most of them are not obligate carboxydutrophs and are able to utilize organic substrate and electron acceptors such as ferric iron, AQDS (anthraquinone-2,6-disulfonate) and sulphur compounds (Sokolova *et al.*, 2009; Sokolova & Lebedinsky, 2013). Those alternative metabolic pathways may enable carboxydutrophic bacteria to survive in moderately reduced conditions. We observed potential microbial CO-oxidation in samples where ORP ranged from -49 mV to 487 mV. Within this ORP range, respiration of iron, fumarate and humic substances can take place (Klöpffel *et al.*, 2014), and CO-oxidation may take place as a subsidiary reaction. Our results suggested that anaerobic CO-oxidizing activity is associated with redox conditions, soil-water content, TOC and TN. TOC can be presumed as a CO source where a positive association is conceivable between CO-oxidizing activity and TOC value. Fig. 3 shows that TN is also a putative factor that influences potential microbial CO-oxidation activity. Some CO-oxidizing thermophiles can reduce nitrates as electron acceptors when grown on organic compounds such as yeast extract (e.g.,

Moorella thermoacetica) and lactate (e.g., *C. hydrogenoformans*) (Fröstl *et al.*, 1996; Henstra *et al.*, 2004). Further studies are needed to determine which nitrogen compounds are associated with the distribution or activity of CO-oxidizers. In environments rich in organic matter, the ability to withstand and utilize CO may be an advantage, allowing microbes to compete with fast-growing aerobes, as CO generally inhibits cytochrome oxidase within aerobic respiration (Cooper & Brown, 2008). Potential microbial CO-oxidation activities were detected in relatively high ORP sites, suggesting that CO-oxidizers may have a niche in an intermediate zone of aerobic and anaerobic environments thanks to their utilization of electron acceptors such as nitrate, Fe(III) and fumarate.

The abundance of *Carboxydotherrmus* spp. did not correlate with potential CO-oxidation activities and there were no apparent similarities in the environmental features among samples in which *Carboxydotherrmus* spp. were detected (Fig. 2, Fig. 3 and Fig. 4a). Moreover, no significant changes in the *Carboxdotherrmus* spp. population were observed between environmental samples and CO-incubated samples from UN-55 and UN-90 (Fig. 4a and 4b). In samples JI-65 and JI-70, abundance of *Carboxydotherrmus* spp. dropped below the threshold for detection despite the presence of potential microbial CO oxidation activity. These results indicate that there were other unknown CO-oxidizers responsible for CO-oxidation in some of our samples. Interestingly, the abundance of *Carboxydotherrmus* spp. was maintained or even increased to a detectable level when incubated at a high temperature of 90 °C (in sample UN-90 and KS-90), although sharp decreases in bacterial population were observed. *C.*

hydrogenoformans can produce spores (Wu *et al.*, 2005) that may allow this species to maintain its population in inhospitable environments. *Carboxydotherrmus* spp. can be regarded as rare and robust bacteria that are distributed in a wide range of environments including extremely thermophilic hot springs, non-hydrothermal environments, and coastal environments.

Anaerobic CODH genes are widely distributed in physiologically diverse bacteria and archaea. Their phylogenetic relationships are often incongruent with taxonomy based on 16S rRNA genes, especially clade F including *cooS-I*, *cooS-II* and *cooS-IV* of *C. hydrogenoformans* (Techtman *et al.* 2012). For example, a gene cluster which include *cooS-I* in *C. hydrogenoformans* is closely related with that of *Thermosinus carboxydivorans* indicating horizontal gene transfer of the cluster (Techtman *et al.* 2012). Thus, we could not exclude the possibility of unintended detection of the recipient microorganisms using our *cooS-II* primer set.

Because CO is a common trace chemical in environments, further study on diversity, competition or habitat segregation would be of interest. In addition, further research should include exploration of new habitats (e.g., extremely hot or acidic terrestrial hot springs) for CO-oxidizing anaerobes.

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394 **References**

- 395 **Conrad R & Seiler W (1980)** Role of microorganisms in the consumption and
396 production of atmospheric carbon monoxide by soil. *Appl Environ Microbiol* **40**:
397 437-445.
- 398 **Conrad R & Seiler W (1982)** Utilization of traces of carbon monoxide by aerobic
399 oligotrophic microorganisms in ocean, lake and soil. *Arch Microbiol* **132**: 41-46.
- 400 **Conrad R & Thauer RK (1983)** Carbon monoxide production by *Methanobacterium*
401 *thermoautotrophicum*. *FEMS Microbiol Lett* **20**: 229-232.
- 402 **Conrad R & Seiler W (1985)** Characteristics of abiological carbon monoxide
403 formation from soil organic matter, humic acids, and phenolic compounds. *Environ Sci*
404 *Technol* **19**: 1165-1169.
- 405 **Cooper CE & Brown GC (2008)** The inhibition of mitochondrial cytochrome oxidase
406 by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide:
407 chemical mechanism and physiological significance. *J Bioenerget Biomem* **40**: 533-539.
- 408 **DeLaune RD & Reddy KR (2005)** Redox potential. In *Encyclopedia of Soils in the*
409 *Environment* (pp 366-371) Hillel D (Ed.) Elsevier, Amsterdam, Netherlands.
- 410 **Dunfield KE & King GM (2004)** Molecular analysis of carbon monoxide-oxidizing
411 bacteria associated with recent Hawaiian volcanic deposits. *Appl Environ Microbiol* **70**:
412 4242-4248.

413 **Einen J, Thorseth IH & Øvreås L (2008)** Enumeration of *Archaea* and *Bacteria* in
414 seafloor basalt using real-time PCR quantitative PCR and fluorescence microscopy.
415 *FEMS Microbiol Lett* **282**: 182-187.

416 **Fröstl, JM, Seifritz C & Drake HL (1996)** Effect of nitrate on the autotrophic
417 metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium*
418 *thermoaceticum*. *J Bacteriol* **178**: 4597-4603.

419 **Hellebrand HJ & Schade GW (2008)** Carbon monoxide from composting due to
420 thermal oxidation of biomass. *J Environ Qual* **37**: 592-598.

421 **Henstra AM & Stams AJM (2004)** Novel Physiological Features of
422 *Carboxydotherrmus hydrogenoformans* and *Thermoterrabacterium ferrireducens*. *Appl*
423 *Environ Microbiol* **70**: 7236-7240.

424 **Henstra AM, Dijkema C & Stams AJ (2007)** *Archaeoglobus fulgidus* couples CO
425 oxidation to sulfate reduction and acetogenesis with transient formate accumulation.
426 *Environ Microbiol* **9**: 1836-1841.

427 **Kawaichi S, Ito N, Yoshida T & Sako Y. (2013)** Bacterial and Archaeal Diversity in
428 an Iron-Rich Coastal Hydrothermal Field in Yamagawa, Kagoshima, Japan. *Microbes*
429 *Environ* **28**: 405-413.

430 **King GM (2003)** Molecular and culture-based analyses of aerobic carbon monoxide
431 oxidizer diversity. *Appl Environ Microbiol* **69**: 7257-7265.

432 **King GM (2007)** Microbial carbon monoxide consumption in salt marsh sediments.
433 *FEMS Microbiol Ecol* **59**: 2-9.

434 **King GM & Weber CF (2007)** Distribution, diversity and ecology of aerobic
435 CO-oxidizing bacteria. *Nature Rev Microbiol* **5**: 107-118.

436 **Klöpffel L, Piepenbrock A, Kappler A & Sander M (2014)** Humic substances as fully
437 regenerable electron acceptors in recurrently anoxic environments. *Nature Geosci* **7**:
438 195-200.

439 **Kochetkova TV, Rusanov II, Pimenov NV, Kolganova TV, Lebedinsky AV,**
440 **Bonch-Osmolovskaya EA & Sokolova TG (2011)** Anaerobic transformation of carbon
441 monoxide by microbial communities of Kamchatka hot springs. *Extremophiles* **15**:
442 319-325.

443 **Lee HS, Kang SG, Bae SS, Lim JK, Cho Y, Kim YJ, Jeong JH, Cha S-S, Kwon KK,**
444 **Kim H-T, Park C-J, Lee H-W, Kim SI, Chun J, Colwell RR, Kim S-J & Lee J-H**
445 **(2008)** The complete genome sequence of *Thermococcus onnurineus* NA1 reveals a
446 mixed heterotrophic and carboxydophilic metabolism. *J Bacteriol* **190**: 7491-7499.

447 **Lupton FS, Conrad R & Zeikus JG (1984)** CO metabolism of *Desulfovibrio vulgaris*
448 strain Madison: physiological function in the absence or presence of exogenous
449 substrates. *FEMS Microbiol Lett* **23**: 263-268.

450 **Martin-Cuadrado A.-B, Ghai R, Gonzaga A & Rodriguez-Valera F (2009)** CO
451 dehydrogenase genes found in metagenomic fosmid clones from the deep
452 Mediterranean Sea. *Appl Environ Microbiol* **75**: 7436-7444.

453 **Moran MA & Miller WL (2007)** Resourceful heterotrophs make the most of light in
454 the coastal ocean. *Nature Rev Microbiol* **5**: 792-800.

455 **R Development Core Team (2008)** R: a language and environment for statistical

456 computing. ISBN 3-900051-07-0. URL <http://www.R-project.org>.

457 **Ragsdale SW (2004)** Life with carbon monoxide. *Crit Rev Biochem Mol Biol* **39**:

458 165-195.

459 **Rich JJ & King GM (1999)** Carbon monoxide consumption and production by wetland

460 peats. *FEMS microbiol Ecol* **28**: 215-224.

461 **Schade GW, Hofmann RM & Crutzen PJ (1999)** CO emissions from degrading plant

462 matter. *Tellus B* **51**: 889-908.

463 **Slepova, TV, Rusanov II, Sokolova TG, Bonch-Osmolovskaya EA & Pimenov NV**

464 **(2007)** Radioisotopic tracing of carbon monoxide conversion by anaerobic thermophilic

465 prokaryotes. *Microbiology* **76**: 523-529.

466 **Sokolova TG, Jeanthon C, Kostrikina NA, Chernyh NA, Lebedinsky AV,**

467 **Stackebrandt E & Bonch-Osmolovskaya EA (2004)** The first evidence of anaerobic

468 CO oxidation coupled with H₂ production by a hyperthermophilic archaeon isolated

469 from a deep-sea hydrothermal vent. *Extremophiles* **8**: 317-323.

470 **Sokolova TG, Henstra AM, Sipma J, Parshina SN, Stams AJ & Lebedinsky AV**

471 **(2009)** Diversity and ecophysiological features of thermophilic carboxydotrophic

472 anaerobes. *FEMS Microbial Ecol* **68**: 131-141.

473 **Sokolova T & Lebedinsky A (2013)** CO-Oxidizing Anaerobic Thermophilic

474 Prokaryotes. In *Thermophilic Microbes in Environ Indust Biotechnol* 2nd ed. (pp.

475 203-231). Satyanarayana T, Littlechild J & Kawarabayashi Y (Eds.) Springer,

476 Amsterdam, Netherlands.

477 **Tamura K, Stecher G, Peterson D, Filipski A & Kumar S (2013)** MEGA6: Molecular
478 Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol* **30**: 2725-2729.

479 **Tarr MA, Miller WL & Zepp RG (1995)** Direct carbon monoxide photoproduction
480 from plant matter. *J Geophys Res* **100**: 11403-11413.

481 **Techtmann SM, Colman AS & Robb FT (2009)** ‘That which does not kill us only
482 makes us stronger’: the role of carbon monoxide in thermophilic microbial consortia.
483 *Environ Microbiol* **11**: 1027-1037.

484 **Techtmann SM, Lebedinsky AV, Colman AS, Sokolova TG, Woyke T, Goodwin L &**
485 **Robb FT (2012)** Evidence for horizontal gene transfer of anaerobic carbon monoxide
486 dehydrogenases. *Front Microbiol* **3**: 132.

487 **WMO WDCGG (2014)** Data Summary No. 38. Japan Meteorological Agency. World
488 Meteorological Organization, Tokyo, Japan, 2014.

489 **Wu M, Ren Q, Durkin AS, Daugherty SC, Brinkac LM, Dodson RJ, Madupu R,**
490 **Sullivan SA, Kolonay JF, Nelson WC, Tallon LJ, Jones KM, Ulrich LE, Gonzalez**
491 **JM, Zhulin IB, Robb FT & Eisen JA (2005)** Life in hot carbon monoxide: the
492 complete genome sequence of *Carboxydotherrnus hydrogenofomans* Z-2901. *PLoS*
493 *Genet* **1**: e65.

494 **Yoneda Y, Yoshida T, Kawaichi S, Daifuku T, Takabe K & Sako Y. (2012)**
495 *Carboxydotherrnus pertinax* sp. nov., a thermophilic, hydrogenogenic, Fe (III)-reducing,
496 sulfur-reducing carboxydrotrophic bacterium from an acidic hot spring. *Int J Syst Evol*
497 *Microbiol* **62**: 1692-1697.

498 **Yoneda Y, Yoshida T, Daifuku T, Kitamura T, Inoue T, Kano S & Sako Y. (2013a)**

499 Quantitative detection of carboxydotrophic bacteria *Carboxydotherrnus* in a hot aquatic
500 environment. *Fundam Appl Limnol* **182**: 161-170.

501 **Yoneda Y, Yoshida T, Yasuda H, Imada C & Sako Y. (2013b)** A novel thermophilic,
502 hydrogenogenic, and carboxydotrophic bacterium *Calderihabitans maritimus* gen. nov.,
503 sp. nov. from a marine sediment core of an undersea caldera. *Int J Syst Evol Microbiol*
504 **63**: 3602-3608.

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Figure legends

Fig. 1. Sampling sites in (a) the Kagoshima Pref. in southern Kyushu Island and (b) Shizuoka Pref.

Original maps were obtained from a digital map provided by The Geospatial Information Authority of Japan (GSI) (<http://www.gsi.go.jp/>) and have been edited by the authors.

Fig. 2. Potential microbial CO-oxidation in environmental samples

Each result of regression analysis is shown outside each graph. Lines showing potential microbial CO-oxidation are dotted when negative in the regression analysis.

Fig. 3. Principal component analysis of environmental features

Closed symbols show positive samples for potential microbial CO-oxidation.

Fig. 4. Abundance of the *Carboxydotherrmus cooS-II* and 16S rRNA genes in (a) environmental samples and (b) in CO-incubated samples

CooS-II bars are average values of positive replicates (negative replicates are not included in the analysis). Error bars are shown when the genes were detected in more than two replicates (i.e., detected at least in triplicate).

Fig. 1

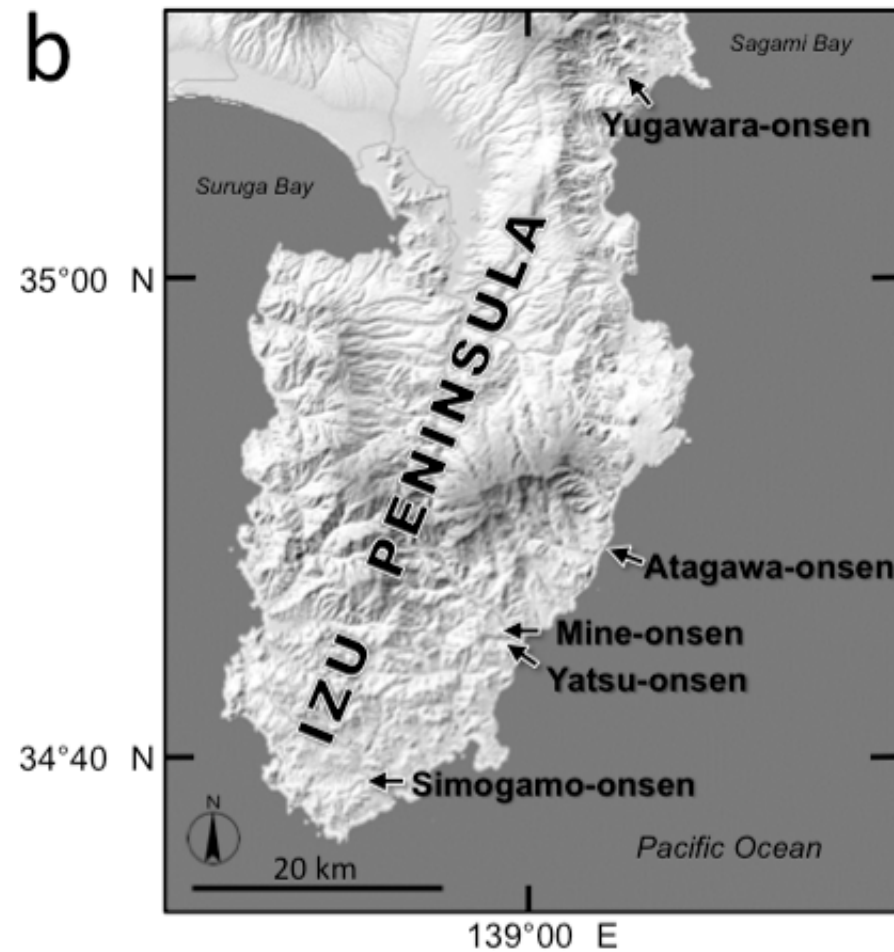
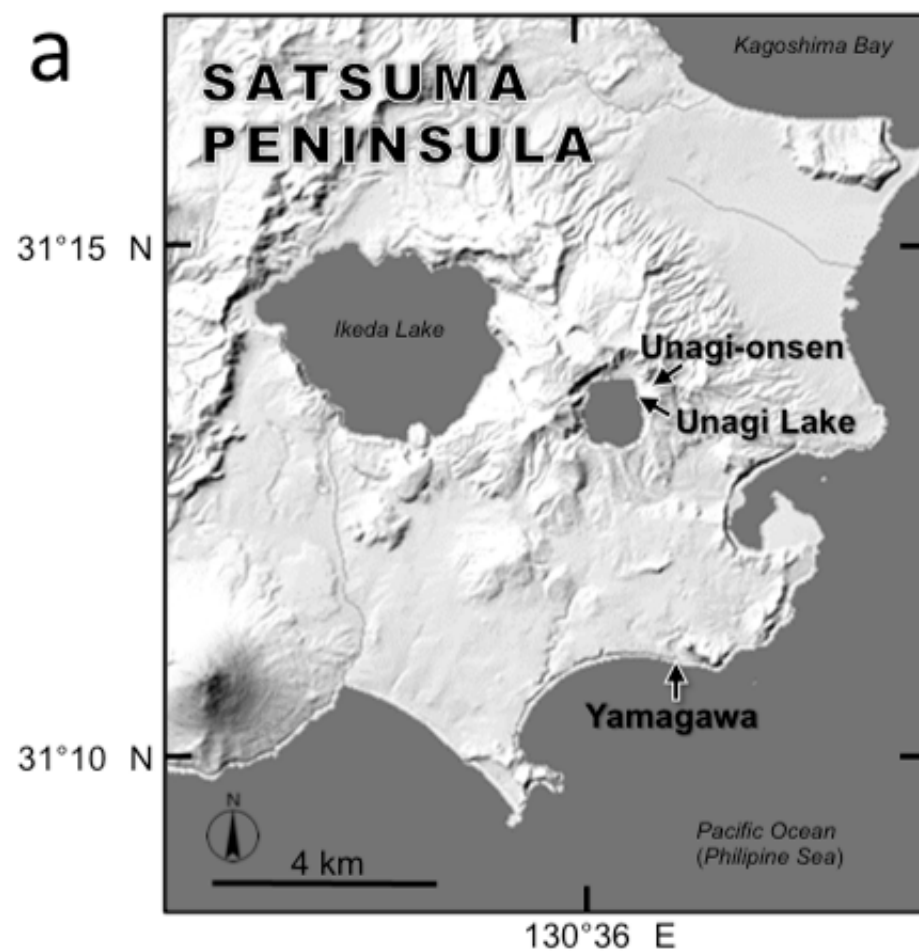


Fig. 2.

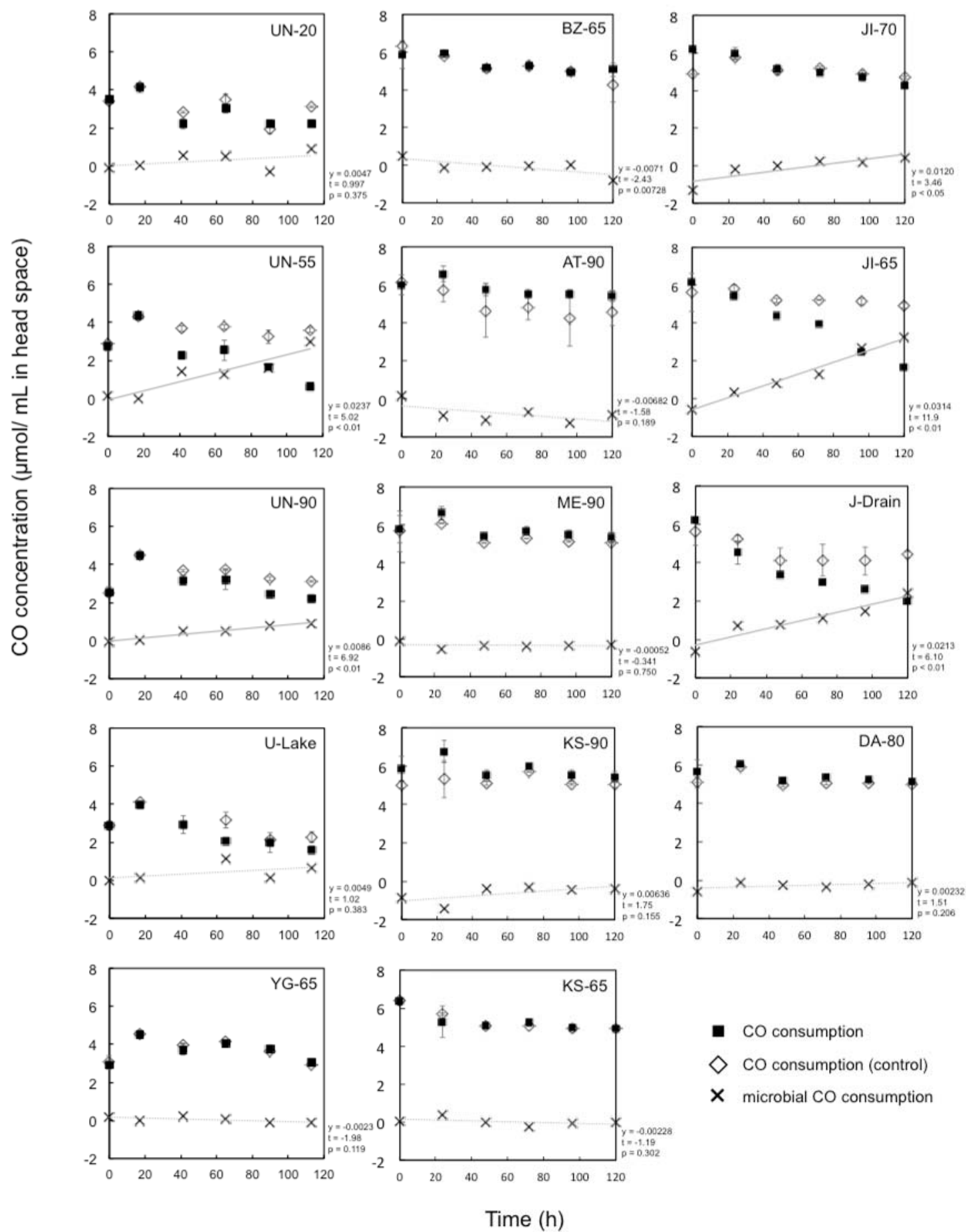


Fig. 3

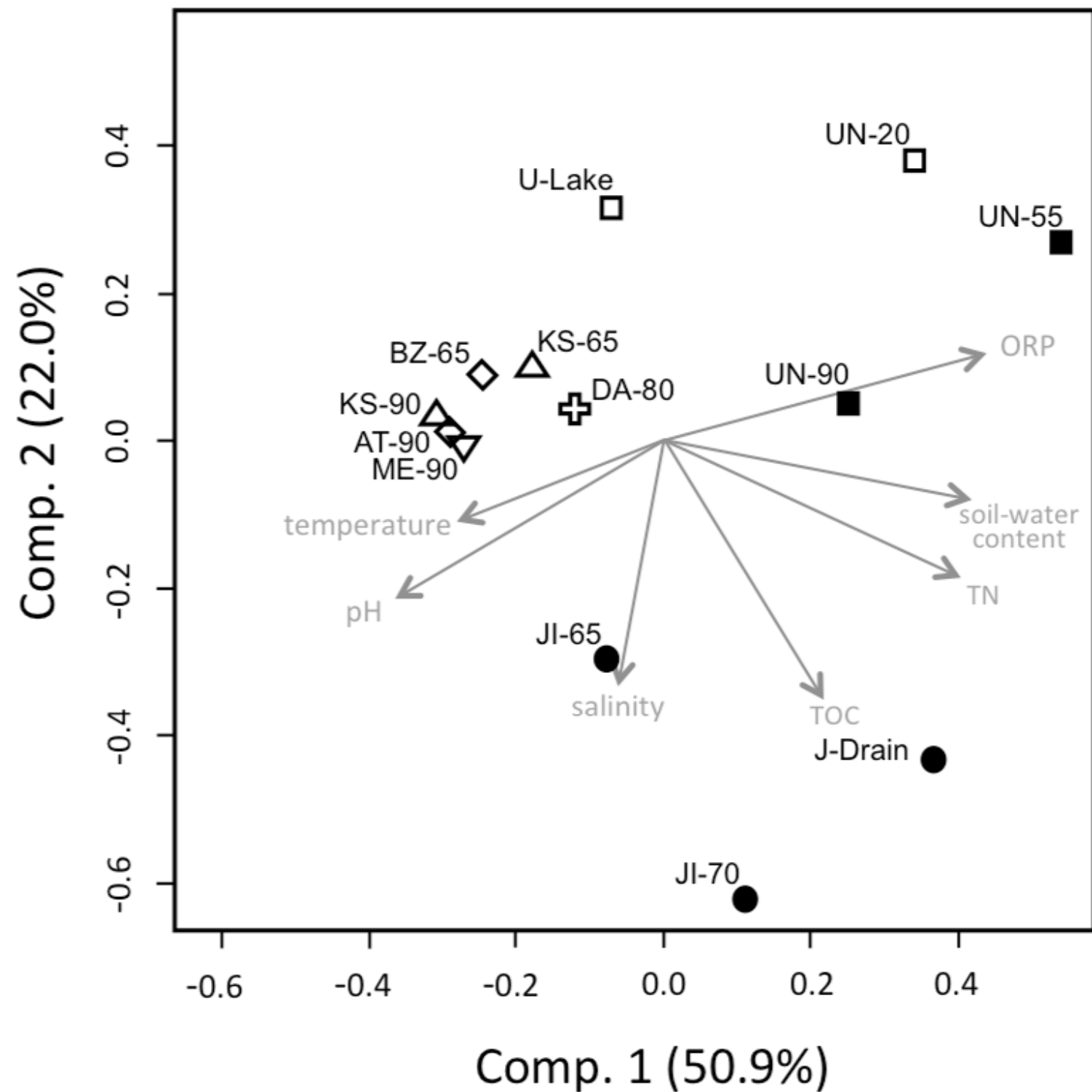
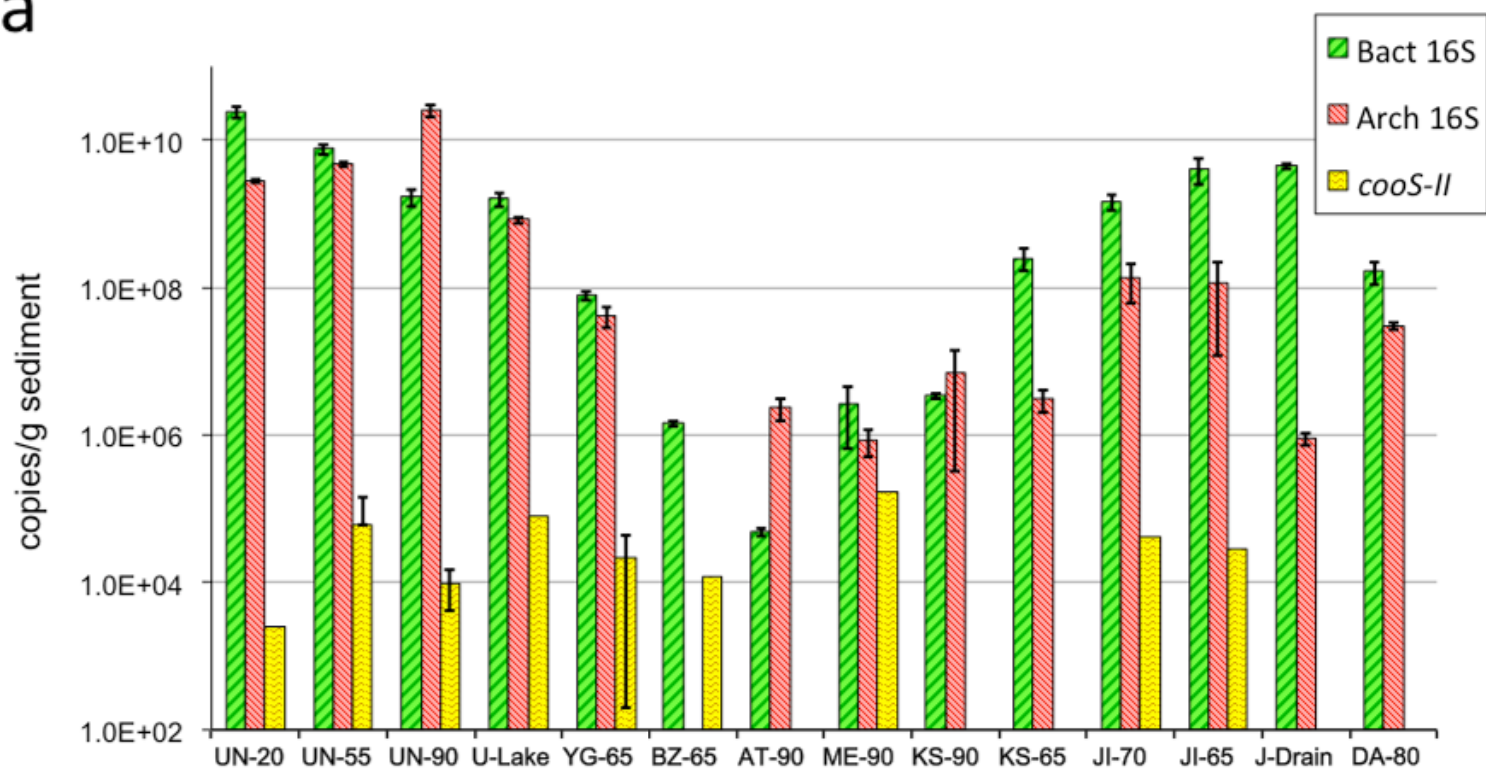


Fig. 4

a



b

