Impact of protists on a hydrocarbon-degrading bacterial community from deep-sea Gulf of Mexico sediments: a microcosm study

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Abstract

In spite of significant advancements towards understanding the dynamics of petroleum hydrocarbon degrading microbial consortia, the impacts (direct or indirect via grazing activities) of bacterivorous protists remain largely unknown. Microcosm experiments were used to examine whether protistan grazing affects the petroleum hydrocarbon degradation capacity of a deep-sea sediment microbial community from an active Gulf of Mexico cold seep. Differences in *n*-alkane content between native sediment microcosms and those treated with inhibitors of eukaryotes were assessed by comprehensive two-dimensional gas chromatography following 30-90 day incubations and analysis of shifts in microbial

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community composition using small subunit ribosomal RNA gene clone libraries. More biodegradation was observed in microcosms supplemented with eukaryotic inhibitors. SSU rRNA gene clone libraries from oil-amended treatments revealed an increase in the number of proteobacterial clones (particularly γ-proteobacteria) after spiking sediments with diesel oil. Bacterial community composition shifted, and degradation rates increased, in treatments where protists were inhibited, suggesting protists affect the hydrocarbon degrading capacity of microbial communities in sediments collected at this Gulf of Mexico site.

Keywords:
Gulf of Mexico, deep-sea, sediment, hydrocarbons, grazing, protists

1. Introduction

Marine sediments can be locally impacted by hydrocarbon contaminants introduced into the environment through naturally occurring seeps or via anthropogenic activities (Bauer et al., 1988; Oros et al., 2007). Not only can petroleum products damage surrounding ecosystems, they also can constitute a carbon and energy source for diverse groups of hydrocarbon-degrading microorganisms present in water columns and sedimentary habitats (Slater et al., 2006).

The biodegradation of hydrocarbons is known to occur by cyanobacteria, fungi, and some algae (Dalby et al., 2008), however bacteria are currently considered by most to be the primary consumers of hydrocarbons in the environment (Atlas, 1981; Leahy and Colwell, 1990). Although present in hydrocarbon-containing environments, Archaea have not been

48 observed to degrade hydrocarbons (Röling et al., 2004a). The molecular composition of
49 petroleum varies with each deposit and can contain a complex mixture of different
50 hydrocarbons derived from four classes: aromatics, asphaltenes, saturates, and resins.
51 Although hydrocarbon-degrading bacteria are ubiquitous, with as many as 79 known genera
52 (Dalby et al., 2008), the ability to fully degrade all of the compounds found in oil is thought
53 to be beyond the capability of any single species. Microbial consortia preferentially degrade
54 *n*-alkanes, branched-alkanes, and aromatics (Prince et al., 2007), however, due to their high
55 carbon and low nutrient content, hydrocarbon degradation is typically limited by availability
56 of nitrogen and phosphorus (Swannell et al., 1996). For this reason, fertilization has proven
57 an effective treatment for stimulating hydrocarbon-degrader growth and consequently
58 hydrocarbon degradation (Gertler et al., 2012; Röling et al., 2002).
59 The responses of marine bacterial communities to the presence of hydrocarbons has
60 been well documented in a number of bioremediation studies of seawater and beach sand
61 mesocosms (Gertler et al., 2012) and microcosms (Jung et al., 2010; Röling et al., 2002), as
62 well as field studies of beach sands (Röling et al., 2004; Kostka et al., 2011). Although these
63 studies generally agree that the oil-degrading microbial community size and composition is
64 variable, site-specific members of a group of microbes collectively known as obligate
65 hydrocarbonoclastic bacteria (OHCB) are universally detected. Despite low natural
66 abundances, genera of OHCB such as *Alcanivorax*, *Cycloclasticus*, and *Marinobacter* are
67 known to rapidly proliferate and outcompete other indigenous bacteria upon introduction of
68 petroleum into the environment (reviewed in Yakimov et al., 2007).
69 While significant evidence implicates OHCB as important environmental
70 hydrocarbon degraders, much less is known about responses of the protistan community to

71 petroleum in the environment. Furthermore, relatively little is known about the impact
72 protists have on hydrocarbon degradation either directly or indirectly as a result of their
73 grazing activities on hydrocarbon-degrading bacteria including members of OHCB. For
74 instance, predation can be detrimental to bioremediation efforts if bacteria integral to the
75 degradation process are removed due to predation (e.g., Gurijala and Alexander, 1990). The
76 possibility that protists may make significant direct contributions to hydrocarbon
77 degradation in certain environments has not been adequately tested. Much of what we know
78 about protistan grazing comes from studies of planktonic bacteria (e.g., Sherr and Sherr,
79 2002). Aquatic bacterial communities are subject to population structure regulation by
80 environmental factors that include nutrient availability, viral lysis, and predation by
81 bacterivorous protists. Grazing, in particular, is thought to be one of the most important
82 regulators of bacterial community composition and net production (Sherr and Sherr, 2002).
83 Nutrient substrates released directly from heterotrophic protists or indirectly as a result of
84 their predatory activities (sloppy feeding or digestive wastes) may stimulate accelerated
85 growth of otherwise nutrient-limited bacteria, thus affecting the physiological condition of
86 the community. Additionally, size or morphology-selective grazing can shift the taxonomic
87 composition of bacterial communities to the benefit or detriment of individual species
88 (reviewed in Hahn and Höfle, 2001). Although these processes are much less understood in
89 sediments, recent evidence suggests that soil and sediment communities under grazing
90 pressure may be similarly impacted (Murase et al., 2006), that bacterial communities under
91 protozoan grazing pressure have lower abundances of bacteria, and grazing often stimulates
92 the rate of decomposition of organic matter (Fenchel and Harrison 1976). Studies of the
93 interactions between protists, bacteria and organic contaminants in sedimentary

environments are providing indications that protists can often have significant positive impacts on degradation processes, however the nature of these impacts appears to depend at least in part on the habitat and/or specific microorganisms investigated (e.g., Kinner et al. 2002; Kota et al.1999; Mattison and Harayama 2001; Mattison et al. 2005; Schmidt et al. 1992; Tso and Taghon 2006). Among potential organic contaminants, petroleum hydrocarbons are of particular concern given recent increases in drilling and extraction activities over the past decade.

A previous microcosm study found that common planktonic eukaryotes, including *Paraphysomonas foraminifera,* limited bacterial growth through increased grazing in oil-polluted seawater (Dalby et al, 2008). However, in a hydrocarbon-rich natural environment where there is a higher likelihood of protist communities already adapted to hydrocarbon exposure, protist grazing activities may help maintain higher rates of bacterial hydrocarbon degradation by releasing needed nitrogen and phosphorus. To test this hypothesis specifically for deep marine sediments, we conducted a set of laboratory-based microcosm experiments using surface sediments from a Gulf of Mexico hydrocarbon seep site to determine whether the presence of indigenous protists 1) enhances the biodegradation of hydrocarbons performed by *in situ* microbes in these deep-sea hydrocarbon-seep sediments and 2) if protists alter the *in situ* bacterial community composition over a 12-week period. The degradation of fossil diesel spiked into these microcosms was monitored by gas chromatography. Changes in eukaryotic and bacterial *in situ* communities were analyzed by molecular methods.

2. Materials and methods

2.1. *Sediment collection*

Surface sediment samples were collected with a slurp gun attached to the HOV *Alvin* from the Rudyville site at the Mississippi Canyon Federal Lease Block 118 (MC118) long-term observatory in the Gulf of Mexico. MC118 is an active gas-hydrate and oil seep located on the continental slope of the northern Gulf of Mexico (28-51.114N, 88-29.521W) and is about 15 km from the source of the 2010 *Deepwater Horizon* oil spill. Soft, 5.5°C sediments were retrieved using the HOV *Alvin* (Dive #4658) on December 1, 2010 from 900-m depth. Samples were mixed 50/50 with bottom seawater and the resulting slurry was stored at 4^{o}C during transport to the lab where experiments were initiated ten days later.

2.2. *Microcosm experiments*

Experiments were conducted to examine the effect of protistan grazing on bacterial community composition and hydrocarbon degradation under combinations of conditions including nutrient addition, oxic vs. anoxic incubations, and addition of protist inhibitors (Table 1). Glass vials (60-ml volume) were used for all microcosms. Forty milliliters of the MC118 sediment/seawater slurry were dispensed into each of 42 sterile glass tubes and each tube was spiked with the addition of 25 microliters (~20µg) of 100% fossil diesel fuel oil (WP 681 USEPA Standard Oil). This diesel fuel oil was selected because: (1) microbial biodegradation of compounds present in this mixture is easily detected by gas chromatography and (2) it has a great capacity to mix into the water column (Reddy and

Quinn, 2001) and sorb to sediments (Reddy et al., 2002; Peacock et al., 2007). Oxygen was removed from anaerobic treatments by nitrogen infusion of the slurry prior to capping, while aerobic incubations vials were loosely plugged with sterile cotton. All treatment combinations were conducted in triplicate. Oxic and anoxic control microcosms of sterilized (autoclaved) slurry (with or without nutrients added) were sacrificed on day 0 (6 tubes), 30 (oxic microcosms), and 90 (anoxic microcosms) to monitor for non-biological loss of hydrocarbons. Remaining tubes were divided into 8 experimental treatment groups based on combination of incubation conditions and additions each received (Table 1). At T_0 a sample of the sediment slurry used for live experiments was terminated with MTBE immediately following the addition of oil, and used for a control comparison of microbial community and hydrocarbon composition with samples following the microcosm incubations.

Treatment groups included all possible combinations of: 1) presence or absence of added nutrients (NH_4 Cl and K_3 PO_4 to final concentrations of 5 and 0.9 $mmoll^{-1}$ respectively; DeMello et al., 2007) 2) presence or absence of a mixture of protist inhibitors cyclohexamide and colchicine (added to 0.5 mg/ml; Adamczewski et al., 2010) and 3) presence or absence of oxygen. Vials were placed onto a shaker table, covered with foil, and agitated at 60 revolutions per minute (rpm) at ambient temperature (15 °C) for the duration of the experiment. All incubations were terminated by the addition of 10 ml of methyl *tert*-butyl ether (MTBE) after 30 days for aerobic treatments or 90 days for anaerobic treatments. After termination, 1 ml of each microcosm sample was collected into sterile 1.5 ml tubes and stored at -80°C for molecular analysis.

2.3. *Analysis by gas chromatography*

Control (sterile, autoclaved) samples and replicate treatments were analyzed as previously described (DeMello et al., 2007; Slater et al., 2006). Extracts were analyzed by traditional gas chromatography with a flame ionization detector (GC-FID) on a non-polar column and comprehensive two-dimensional gas chromatography (GC×GC-FID) (Nelson et al., 2006). For more details on the operation on the latter technology, refer to a recent review by Eiserbeck et al., 2012. Each instrument was calibrated with a wide range of saturate and aromatic hydrocarbons purchased from Aldrich Chemical, Chiron, Cambridge Isotopes, and Ultra Scientific.

2.4. *Nucleic acid extraction and PCR*

Total genomic DNA was extracted from 1.0 ml of slurry from all replicates using the PowerSoil DNA Extraction Kit (MoBio) following experiment termination. Small-subunit rRNA (SSU rRNA) gene fragments were PCR amplified from selected DNA extracts using the eukarya-specific primers 360F (Medlin et al., 1988) and U1391R (Lane, 1991) as well as bacteria-specific primers 8F and 1492R (Lane, 1991). Archaeal amplifications were not attempted. PCR conditions were: 95°C for 5 min followed by 35 cycles of 95°C for 60 seconds, 50° C (bacteria) or 55°C (eukarya) for 60 seconds, and 72°C for 90 seconds followed by 72°C for 7 minutes. PCR products were visualized on a 1% agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research). Purified products were ligated and cloned into the pCR4 vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen).

186

187 2.5. *Sequencing and sequence analysis*

188

189 Forty-eight clones from each selected treatment (T_0, Oxic + nut, Oxic +nut +inhib)
190 were Sanger sequenced at the W. M. Keck Ecological and Evolutionary Genetics Facility
191 (MBL) on a 3730XL capillary sequencer (ABI) with the universal M13F primer. Sequences
192 were trimmed of vector data and unreliable base calls using the sequence editing software
193 Sequencher (Gene Codes Corporation, Ann Arbor, MI). Furthermore, due to a combination
194 of the unknown orientation of the vector insert and single primer sequencing strategy it was
195 necessary to partition the sequences based on 5' or 3' sequence data prior to analysis.
196 Sequences were clustered into operational taxonomic units (OTUs) sharing 97% sequence
197 identity using UCLUST. Representative sequences from each OTU were compared against
198 the GenBank nt database using BLASTn searches to obtain taxonomic affiliation using
199 JAguc (Nebel et al., 2010). Taxonomic histogram plots displaying the abundance of
200 sequences affiliating with different taxa across samples were created using the
201 summarize_taxa_through_plots.py command in QIIME (Caporaso et al., 2010).

202

203 **3. Results**

204

205 3.1. *Hydrocarbon consumption*

206

207 Hydrocarbon degradation was assessed by measuring changes in sediment
208 hydrocarbon content using both GC-FID and GC×GC-FID. Straight chain alkanes, such as

209 *n*-C_{17} are known to biodegrade more rapidly than similarly sized branched alkanes, such as
210 pristane. Therefore,*n*-alkane biodegradation has historically been inferred from an increase
211 in the *n*-pristane/ *n*-C_{17} and phytane/ *n*-C_{18} ratios (Blumer et al., 1973; Jones et al., 1983).
212 We similarly focused only on changes in pristane /*n*-C_{17} and phytane/*n*-C_{18} ratios over the
213 experimental time period in both our sterilized controls and our live incubations, all of
214 which received the same diesel oil spike at T_0.
215 Measurements of starting and ending n-alkane ratios for all replicate microcosm
216 experiments are shown in Supplementary Table 1, and averages of triplicate microcosms for
217 each treatment are plotted in Figures 1 and 2. Abiotic (sterile, autoclaved sediments)
218 microcosms showed little loss of *n*-alkanes regardless of oxygen availability. Oxygen was an
219 important factor for biodegradation among our live treatments. All aerobic microcosms
220 showed evidence of early hydrocarbon biodegradation, while all anaerobic treatments did
221 not, in comparison to the control samples. A typical GC×GC-FID chromatogram for the
222 aerobic biodegradation (reduction in peak size) of the *n*-C_{17} and *n*-C_{18} observed in this study
223 is shown in Figure 3. The level of biodegradation varied within aerobic treatments with
224 those receiving the protist inhibitors generally displaying slightly elevated levels of
225 biodegradation compared to those that did not. Surprisingly, nutrient supplementation did
226 not significantly increase hydrocarbon loss in any of the oxic treatments. Changes in
227 pristane /*n*-C_{17} and phytane/*n*-C_{18} ratios over the experimental time period for all treatments
228 and sterilized controls are shown in Figures 1 and 2. It should be noted that there were
229 differences within treatment replicates regarding pristane /*n*-C_{17} and phytane/*n*-C_{18} ratios.
230 We consider incomplete slurry homogenization and minor dispensing variations to be the
231 most likely causes of slight replicate discrepancies.

3.2. *Bacterial community composition*

Bacterial SSU rRNA gene clone libraries were constructed from a single replicate from each of two oxic treatments (OxicN and OxicNI) at the end of 30 days and a day 0 (T_0) sample. While obtaining SSU rRNA data from all three replicates for each treatment group would have been ideal, this was not feasible within the scope of this project. Forty-eight clones representing partial SSU rRNA gene fragments amplified from each of the 3 samples were sequenced and grouped into operational taxonomic units (OTUs) at 97% sequence identity. Taxonomic histogram plots displaying the abundance of OTUs affiliating with different bacterial taxa are shown in Figure 4.

The *Gammaproteobacteria* represented the highest percentage of OTUs present in all 3 of our samples examined (Figure 4). Other less abundant taxonomic groups shared among our libraries include *Epsilonproteobacteria* and *Firmicutes*. The T_0 clone library contained rRNA sequences with broad taxonomic distribution, including sequences affiliating with the *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, and Candidate division OP9 uncultured hydrocarbon seep bacterium (Figure 4). Within the *Proteobacteria*, phylotypes of the *Gammaproteobacteria* were the most numerous, accounting for roughly 33% of the total number of clones recovered on day 0. That number increased to more than half the total number of sequenced OTUs after 30 days in oxic microcosms after receiving the diesel oil spike. Nutrient supplementation without addition of protist inhibitors (OxicN) resulted in a slightly higher total number of gammaproteobacterial clones, but lower overall bacterial diversity relative to the T_0 sample. Members of only seven bacterial classes were

recovered from the OxicN clone library compared to 11 from the T_0 sample. Taxonomic groups such as *Fusobacteria* and *Chloroflexi*, which were present on day 0, were entirely absent from the nutrient amended treatment on day 30. The *Deltaproteobacteria*, which were relatively abundant in the T_0 sample were absent in the nutrient amended treatment without protist inhibitors after 30 days. *Betaproteobacteria* and *Alphaproteobacteria*, which were not detected at T_0 were recovered after 30 days in the treatment receiving nutrients but no inhibitors of protists (Figure 4a).

When both nutrients and inhibitor were included (OxicNI), the percentage of gammaproteobacterial clones was also elevated relative to the T_0 sample after 30 days. Additionally, the overall bacterial diversity (number of different taxonomic groups detected) was more similar to the T_0 sample than in the nutrient amended treatment without inhibitors at day 30, however composition (identity of those taxonomic groups) shifted (Figure 4a). Various OTUs affiliated with *Bacteroidetes* were recovered from the OxicNI treatment after 30 days but were absent from our other libraries. OTUs affiliating with Clostridia were less abundant in the OxicN but not in the OxicNI treatment after 30 days relative to T_0, suggesting that protists may be selectively grazing upon them.

Many important known OHCB belong to the *Proteobacteria*. Since our SSU rRNA gene clone libraries were dominated by proteobacterial sequences, particularly *Gammaproteobacteria*, we constructed a second taxonomic histogram plot at the family level that included only the proteobacterial sequences recovered in this study (Figure 4b). Although *Gammaproteobacteria* are the most abundant, *Epsilonproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, and a single phylotype of the *Betaproteobacteria* are also represented. Sequences derived from the family *Alteromonadaceae* are the only γ-

proteobacterial sequences present in all three of our treatments. Members of the *Alcanivoracaceae*, which includes the genus *Alcanivorax*, were detected in both nutrient amended treatments at the end of the experiment, but not at T_0. At the family level, differences were also observed in taxonomic composition between oxic treatments with and without protist inhibitors, suggesting that addition of inhibitors impacted community composition (Figure 4b).

3.3. *Eukaryotic community composition*

Eukaryotic SSU rRNA genes were PCR amplified and cloned from the same three treatments as the bacterial libraries. Histograms showing OTU distributions (97% OTUs) are shown in Figure 5. The most abundant taxa differed for each library and there were no taxa recovered from all three samples. The T_0 library contained many OTUs belonging to ciliates affiliating with Intramacronucleata as well as OTUs from Rhizaria (Gromiidae) and various stramenopile families. Addition of nutrients alone to the slurry (OxicN) resulted in a shift from ciliates to stramenopiles as the dominant group. Additionally, Gromiidae and Cercozoa that were present at T_0, were not present in clone libraries at the end of the experiment, while a number of Dinophyceae groups appeared in clone libraries only at the end of the experiment. When nutrients and protist inhibitors were added, a significant shift was observed after 30 days, as expected. The only eukaryotic group recovered from the OxicNI treatment clone library was the Cercozoa. Cercozoa were present in the T_0 clone library but they were not the dominant group (7% of OTUs) and appeared to disappear completely from the OxicN treatment.

4. Discussion

4.1. *Biodegradation measurements*

The GCxGC-FID method is extremely sensitive, and enabled us to follow the degradation of trace additions of diesel fuel added to our microcosm experiments. Since Bowles et al. (2011) was not able to detect alkanes in similar oily sediments from the same site, we chose to utilize the more sensitive GCxGC approach, which successfully tracked degradation of diesel compounds over the timeframe of our study (Figure 3). Our study suggests that the presence of protist grazers had a detrimental impact on hydrocarbon degradation in these sediments under this set of conditions. This result contrasts with some other studies of protist impacts on biodegradation, indicating that further investigation of the underpinnings of protist-prokaryote dynamics in hydrocarbon contaminated sediments using an expanded experimental regime is warranted.

The Rudyville site at the Mississippi Canyon Federal Lease Block 118 (MC118) is characterized by 108-576µM NH_4^+, 14-44 µM NO_x^-, and 0.7-21µM PO_4^{3-} (Bowles et al. 2011). The addition of 900 µM of K_3 PO_4 and 5000 µM of NH_4 Cl to each microcosm as a nutrient spike is far in excess of *in situ* concentrations. While we don't believe that microbial communities in our microcosms became nutrient limited during the time of our experiment, we cannot rule out this possibility. Prior to initiating our experiments, small (~5-100µm) protists were observed in our sediment slurries using microscopic examination (data not shown). Living protists were not observed two hours after addition of protist

inhibitors in sediment slurries, and few intact protists, suggesting most protists in those samples were lysed. Lysis of protists would have further increased nutrient concentrations in those microcosms. The amount of diesel oil we added to each microcosm (~20µg) was low relative to *in situ* concentrations of petroleum hydrocarbons. At the MC118 site aliphatic hydrocarbons at 1cm depth were 477 µg/g wet sediment, and at 7 cm depth were 1541 µg/g wet sediment (Bowles et al. 2011). Bowles et al. (2011) noted that these sediments contained an unresolved complex mixture of hydrocarbons with few resolved compounds like normal alkanes. This is supported by our T_0 analysis of hydrocarbon compositions in the sediments, which did not detect a normal alkane concentration above the levels of our added spike. Microorganisms in these sediments may have already degraded most of the more easily accessible hydrocarbons, and entered a state of very low activity. When we added the trace diesel spike, however, GCxGC mass spectrometry allowed us to observe the normal alkanes in this spike were quickly utilized by the sediment community (Figures 1 and 2).

Many previous studies of the composition and activity of petroleum-associated protistan communities have been conducted under mesocosm enrichment conditions (Gertler et al., 2012; Dalby et al., 2008; Jung et al., 2010). Laboratory incubations allow a means of controlling variables in complex experimental designs, however such experiments have been criticized for not adequately reproducing environmental microbial ecosystems (Carpenter, 1996), and this microcosm study shares the same limitations. The biodegradation of hydrocarbons in marine sediments is likely impacted by a number of environmental factors including temperature, hydrocarbon content, nutrient availability, and oxygen levels. In this study, we maintained our microcosms at 15°C, which is higher than original *in situ* temperatures around 5°C. This likely caused alterations in the sediment microbial

community structure, and while all treatments experienced the same temperature regime, we can't rule out the possibility that differences such as this could have altered our community structure and composition. Since all treatments were handled the same, this does not interfere with a comparison of results, however we cannot rule out the possibility that the increased temperature had detrimental effects on some microbiota in our samples. Any detrimental impacts on some hydrocarbon degraders would have been experienced in all our treatments, thus this would not have interfered with our findings for the taxa that remained. However, hydrocarbon degradation may have been higher in all treatments had they been run at 5°C, although, studies have shown hydrocarbon degradation to increase with temperature (e.g., Coulon et al. 2005). Furthermore, in spite of our microscope observation of living protozoa in inhibitor-free treatments at the end of our study, we acknowledge that the higher than *in situ* temperature may have negatively impacted some key protist grazers of hydrocarbon degrading bacteria. In that case, their positive influences on hydrocarbon degradation may have been underestimated.

By testing combinations of +/- nutrient addition, and +/- protist inhibitor addition, under oxic and anoxic conditions, we were able to compare microbial degradation of hydrocarbons under various conditions in the presence and absence of protists. The fossil diesel fuel added to our microcosms exhibited microbial biodegradation only under aerobic conditions. This was not surprising, as it is widely accepted that rapid hydrocarbon degradation proceeds most rapidly under oxic conditions. Although anaerobic biodegradation has been proposed in the deep subsurface (Head et al., 2003) and oil-degrading bacteria have been isolated from beach sands under anaerobic conditions (Kostka et al., 2011), we observed no detectable activity in anaerobic microcosms over the course of

90 days. The biodegradation of *n*-alkanes observed in the microcosms is assumed here to result almost exclusively from the activity of bacteria and not protists, although some eukaryotes have been reported to degrade hydrocarbons (Raikar et al., 2001; Kaska et al., 1991).

The rapid increase in biodegradation activity typically observed from bacterial populations following hydrocarbon exposure probably results from the sudden increased availability of both a labile carbon and energy source. But this activity may not be sustained due to limited availability of nutrients such as nitrogen and phosphorus. Researchers often supplement laboratory incubations to stimulate biodegradation. We similarly included nutrient amended microcosms in our experiment (OxicN), however our results suggest that the addition of nutrients to aerobic incubations had a limited effect on biodegradation. The highest levels of biodegradation in this study as indicated by elevated pristane/*n*-C_{17} and phytane/*n*-C_{18} ratios relative to their ratios at T_0, resulted from the two oxic microcosm groups that received the protist inhibitors cycloheximide and colchicine (OxicI), followed by oxic treatments that received the inhibitors plus added nutrients (OxicNI). This suggests that nutrients had a lesser stimulatory effect on hydrocarbon degradation than the addition of protist inhibitors.

Elevated biodegradation of hydrocarbons in our oxic treatments that received protist inhibitors is likely due to reduced grazing pressure on active hydrocarbon-degrading bacteria. Some evidence was seen suggesting selective grazing of certain bacterial groups known to participate in hydrocarbon degradation. For example, OTUs affiliating with Clostridia were less abundant in the OxicN but not in the OxicNI treatment after 30 days relative to T_0. *Clostridium* sp. has been described to degrade chlorinated hydrocarbons in lindane-amended,

393 flooded soils (e.g., Sethunathan and Yoshida, 1973). Selective grazing of hydrocarbon
394 degrading bacteria may have contributed to lower observed degradation in the presence of
395 protist grazers in our study. Decreased grazing may also allow hydrocarbon degraders to
396 start growing more rapidly that may have otherwise maintained low activity levels as a
397 defense mechanism against predation, as suggested previously (del Giorgio et al., 1996).
398 However, it cannot be ruled out that, increased organic and inorganic nutrient availability
399 resulting from the consumption of inhibitor-killed protists may have also stimulated
400 bacterial activity, although we don't expect this was significant given that our separate
401 nutrient additions did not produce a significant increase in hydrocarbon degradation. Our
402 results are in contrast to other studies that found a positive influence of protists on bacterial
403 metabolic activity (e.g., Biagini et al. 1998; Fenchel 1987). Tso and Taghon (2006) found
404 that protist grazing enhanced bacterial mineralization of naphthalene in field-contaminated
405 estuarine sediment up to four times over treatments that received the grazing inhibitor
406 cytochalasin B. Similarly, during in silico culture bottle experiments, the impact of grazing
407 by the soil flagellate *Heteromita globosa* on the aerobic degradation of benzene (Mattison et
408 al. 2005) and toluene (Mattison and Harayama 2001) by a *Pseudomonas* sp. the soil was
409 investigated. Both studies revealed enhanced consumption of hydrocarbons by
410 *Pseudomonas* when preyed upon by *Heteromita*. Protist stimulatory effects have been
411 attributed to such processes as physical aeration of sediments, release of nutrients as a result
412 of grazing, alterations in community structure that reduce competition among bacterial
413 degraders, and maintenance of a rapidly growing, and therefore active, bacterial population
414 (Tso and Taghong, and references therein). There are several possible explanations for the
415 different results obtained here and in other studies. It could be that protist grazing affects

bacterial populations involved in degradation of different hydrocarbons differently, and/or that impacts of grazing are variable depending on the origin of the sediments and its starting hydrocarbon composition. These are questions that require additional studies to address. Additionally, the impact of different protist inhibitors, including those used in this study, on the whole bacterial community (not just known degraders) as well as on protozoa needs to be further examined. For example, it is possible that inhibitors such as cytochalasin B, which temporarily inhibit feeding for a period of time, may not eliminate feeding in all protozoa. Although Tso and Taghon (2006) tested its effectiveness on a culture of the ciliate *Euplotes*, its effectiveness on other taxa is unknown. Some protozoa are known to be involved in hydrocarbon degradation (e.g., Raikar et al., 2001), and inhibition of feeding may not be enough to eliminate their participation in hydrocarbon degradation. Conducting studies in the future under oxic conditions with protist inhibitors for much longer periods than 30 days may help to determine if temporary pulses of nutrients from killed protists are temporarily stimulating hydrocarbon degradation.

4.2. *Bacterial community composition*

Concomitant with shifts in the protistan community structure, changes in the bacterial community were observed in response to our various microcosm treatment conditions. Proteobacteria, particularly Gammaproteobacteria, were quantitatively the most important group in all three of our bacterial clone libraries. The T_0 library produced OTUs affiliating with known hydrocarbon degraders including members of the Oceanospirillaceae and Piscirickettsiaceae. Among the Gammaproteobacteria, members of the Alteromonadales

439 order were most abundant. The Alteromonadales are a metabolically diverse group that has
440 previously been detected in oil-fouled marine environments (Redmond and Valentine, 2011).
441 We detected sequences affiliating with Piscirickettsiaceae and specifically to *Cycloclasticus*
442 (data not shown), a genus of polycyclic aromatic hydrocarbon (PAH) degraders that have
443 previously been observed in Gulf of Mexico sediments (Geiselbrecht et al., 1998). Due to
444 their cyclic structure, PAHs are more difficult to biodegrade than straight-chain alkanes and
445 typically linger following the hydrocarbon-degrader bloom.
446 Incubation with added nutrients (OxicN treatment) resulted in an increase in the
447 clone library representation of Alphaproteobacteria and Epsilonproteobacteria but in an
448 overall loss of bacterial diversity compared to the T_0 sample. This is in agreement with
449 previous studies, both laboratory and field-based, that have noted the rapid increase and
450 dominance of Gammaproteobacteria and to a lesser extent Alphaproteobacteria as a result of
451 an increase in carbon levels due to the introduction of petroleum (Roling et al., 2002; Kostka
452 et al., 2011; Röling et al., 2004b). Among the Proteobacteria, known hydrocarbon-degrading
453 genera such as *Alcanivorax* have been widely observed in oil-contaminated ecosystems and
454 are thought to be particularly adept at utilizing straight-chain alkanes during the early stages
455 of hydrocarbon degradation. Members of the Alcanivoracaceae were not detected at T_0 but
456 did emerge following hydrocarbon addition after 30 days. Alphaproteobacteria, particularly
457 members of the Rhodobacteraceae, have been detected in weathered, alkane-depleted,
458 hydrocarbon-contaminated, marine sediments (Kostka et al., 2011; Kasai et al., 2001)
459 suggesting that they may be degrading persistent hydrocarbons such as PAHs during the
460 later stages of hydrocarbon degradation. The occurrence of OTUs affiliating with
461 Alcanivoracaceae and Rhodobacteraceae in our OxicN microcosms following a 30-day

462 incubation may indicate that the microbial population is shifting back from an alkane-rich to
463 an alkane-depleted sediment community, although such correlations between specific
464 microbial taxa and specific hydrocarbon shifts over our 30 day incubations was not
465 investigated in this project.

466 Incubation with a eukaryotic metabolic inhibitor (OxicNI) resulted in reduced protist
467 diversity and an increase in hydrocarbon degradation. Effects of the inhibitors we used on
468 bacterial populations in these sediments are unknown, and bacterial activities may have been
469 suppressed to an unknown degree. We assume that hydrocarbon degradation was principally
470 a result of bacterial activity, although we cannot rule out inputs from archaea and protists to
471 these processes. Our results may have been different had we used sediments from a site
472 where microbial communities have not been previously exposed to hydrocarbons.

473 Previous continued exposure to hydrocarbons may have selected for a community
474 better able to tolerate/metabolize hydrocarbons. The effects of protist grazers on
475 hydrocarbon degrading bacterial communities should be examined in a wider range of
476 sediment types with and without previous hydrocarbon exposure before it can be concluded
477 whether or not protists suppress hydrocarbon degradation.

478

479 4.3 *Eukaryotic community composition*

480

481 The Mississippi Canyon 118 area is an active Gulf of Mexico gas hydrate vent site
482 discovered in 2002. Gas (C1-C5 hydrocarbons) vents freely from the seafloor in this area,
483 which is cratered and covered by mounds of carbonate rock (Sassen et al., 2006).
484 Chemosynthetic communities form conspicuous bacterial mats (*Beggiatoa* spp.), and

485 mussels, bivalves, and tubeworms are common. The bacterial community directly associated
486 with an MC118 mat has previously been described and was dominated by
487 Deltaproteobacteria (Lloyd et al., 2010); however little is known of the protistan populations
488 found at the site.

489 The eukaryotic 18S rRNA gene clone library from the T_0 sample contains members
490 of multiple taxonomic groups that reflect the starting population of protists in our
491 experimental microcosms. These included Stramenopiles, Rhizaria, and Fungi; however
492 alveolates, particularly the ciliate subphylum Intramacronucleata, dominated. The
493 Intramacronucleata are a diverse group that includes many common predatory marine
494 ciliates including *Euplotes*. The activity of ciliates in oil-contaminated seawater mesocosms
495 has been well documented (Gertler et al., 2010). Gertler et al. noted changes in the ciliate
496 population over the course of their incubations that they attributed to changes in prey size
497 caused by development of microcolonies and biofilms of OHCB (Gertler et al., 2010).

498 We similarly detected a change in the eukaryotic community clone library
499 composition in incubations without protist inhibitors (OxicN). Although this change resulted
500 in an overall increase in alveolate diversity, the quantity of alveolate (particularly ciliate)
501 OTUs recovered decreased after 30 days following the addition of the diesel oil spike.
502 Conversely, we observed an increase in stramenopile OTUs. The largest increase within
503 stramenopile OTUs occurred for members of the family Coscinodiscophyceae, commonly
504 known as centric diatoms. This is in contrast to previous studies that have noted a significant
505 decrease in the abundance of diatoms in oil amended seawater microcosms (Jung et al.,
506 2010). It is possible that the toxic effects of hydrocarbons on sediment diatoms was
507 minimized in this study due to the inclusion of inorganic nutrients, or that the community in

these Rudyville hydrocarbon seep sediments was pre-adapted to hydrocarbon exposure. Due to changes in environmental conditions typical of microcosm experiments away from those found *in situ*, and in this case, a 10°C temperature increase, changes in community composition are expected, and it is not possible to tease apart the relative influences on final microbial community composition of temperature shifts, nutrient additions, and hydrocarbon spike in this study. Although our microcosms were all covered with foil to exclude light, we can't rule out the possibility that enough light was available to stimulate this diatom growth. Alternatively, they may have the ability to survive for some period of time heterotrophically, similar to recently described abilities within cyanobacteria (Muñoz-Marín et al. 2013). A follow-up investigation into the apparent ability of these diatoms to sustain growth under dark conditions was outside the scope of this project. Clearly, it is premature to generalize impacts of hydrocarbons on eukaryotic communities without examining a wider range of sediment types (including those previously and not previously exposed to hydrocarbons, tracking community changes that may occur without any additions, and testing similar sediments at *in situ* temperatures.

All of the eukaryotic OTUs recovered following aerobic incubations with addition of protist inhibitors (OxicNI) affiliated with the phylum Cercozoa and specifically, to Silicofilosea. This suggests that, at the dosage used here, the eukaryotic inhibitors inhibited most eukaryotes, but did not effectively knock out all protists. The use of mutagens for studying grazing in microbial communities is widespread, however, the target specificity of such inhibitors may be variable (Sanders and Porter, 1986; Chakraborty et al., 2003). Furthermore, these protist inhibitors may potentially have detrimental effects on some prokaryotes as well. For this reason we elected to use concentrations of inhibitors that we

determined a priori using microscope observations to be just high enough to eliminate most protists (data not shown). Nonetheless, these concentrations may have suppressed prokaryotic activities to an unknown extent in our experiments.

Members of the Cercomonadida are small, heterotrophic flagellates found abundantly in water and soil environments globally (Myl'nikov and Karpov, 2004). They are known bacteriovores that have previously been shown to affect bacterial community dynamics (Murase et al., 2006) and have been detected in oil-polluted habitats (Dalby et al., 2008). The increase in degraded hydrocarbons observed in our OxicNI treatment may have been due in part to stimulatory effects of continued grazing by members of Cercomonadida (Silicofilosea) that survived addition of the protist inhibitors. Cercomonads may be active grazers in Gulf of Mexico sediments and their activities may have a stimulatory effect on the breakdown of *n*-alkanes by the bacterial community. Alternatively, or in addition, removal of most protist predators may have allowed hydrocarbon-degrading members of the bacterial community to increase in numbers, diversity, and/or activity. Since addition of inhibitors resulted in an increase in phylotypes related to the Alteromonadales, it is likely that most stimulation of hydrocarbon degradation is due to reduced grazing pressure.

5. Conclusions

The current study used microcosms to investigate the impact of protists on hydrocarbon degradation and taxonomic composition of bacterial communities in a Gulf of Mexico hydrocarbon seep sedimentary microbial community following exposure to a spike of diesel fuel oil under varying conditions. Our results suggest that grazing pressure by protists

554 negatively impacts the hydrocarbon degradative capacity of the community in these
555 sediments. Unlike previous studies however, the addition of nutrients did little to stimulate
556 biodegradation. Although bacterial hydrocarbon degrading community structure is heavily
557 studied, our results underline the complex nature of marine hydrocarbon consuming
558 microbial consortia and suggest that additional insight, including field studies, into bacteria-
559 protist interactions in different oil contaminated sedimentary environments are needed.
560
561
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563
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Table and Figure Legends

Table 1. Microcosm treatments used in this study.

Figure 1. GCxGC-FID biodegradation data for microcosm treatments showing average phytane/ *n*-C_{18} ratios for different treatment groups and time points. The dashed line represents the phytane/*n*-C_{18} ratio value for the WP 681 USEPA Standard Oil.

Figure 2. GCxGC-FID degradation data for microcosm treatments showing average pristane/ *n*-C_{17} ratios for different treatment groups and time points. The dashed line represents the pristane/*n*-C_{17} ratio value for the WP 681 USEPA Standard Oil.

Figure 3. GCxGC-FID chromatogram showing example of the loss of *n*-C_{17} and *n*-C_{18} peaks due to microbial activity at T_0 (a) versus an oxic microcosm with added nutrients and inhibitor (b).

Figure 4. Percent representation in each library of taxonomic diversity of (a) Bacteria and (b) proteobacteria recovered from selected microcosm clone libraries (operational taxonomic units clustered at 97% sequence similarity).

Figure 5. Percent representation in each library of taxonomic diversity of eukaryotic operational taxonomic units (OTUs) from selected clone libraries clustered at 97% sequence similarity.

Supplementary Table 1. GCxGC-FID biodegradation data for microcosm treatments. Note: not all replicates were measured for all treatment groups.

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Table 1

Treatment	Sediment	Oil	Nutrients	Inhibitor	Oxygen
Oxic Control T_0 (+nut)	sterile slurry	+	+	−	+
Oxic Control T_0	sterile slurry	+	−	−	+
Oxic Control T_F (+nut)	sterile slurry	+	+	−	+
Oxic Control T_F	sterile slurry	+	−	−	+
Anoxic Control T_F (+nut)	sterile slurry	+	+	−	−
Anoxic Control T_F	sterile slurry	+	−	−	−
Oxic T_F (+ nut)[a]	slurry	+	+	−	+
Oxic T_F (+inhib)	slurry	+	−	+	+
Oxic T_F (+nut +inhib)[a]	slurry	+	+	+	+
Oxic T_F	slurry	+	−	−	+
Anoxic T_F (+nut)	slurry	+	+	−	−
Anoxic T_F (+inhib)	slurry	+	−	+	−
Anoxic T_F (+nut +inhib)	slurry	+	+	+	−
Anoxic T_F	slurry	+	−	−	−

a. Treatments for which 18S rRNA gene clone libraries were analyzed in addition to a T_0 sample.

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Supplementary
Table 1

Treatment (amendments, replicate #)	oxygen	pristane/*n*-C_{17}	phytane/*n*-C_{18}	RSD
Oxic (+nut +inhib, rep 1)	+	na*	na	
Oxic (+nut +inhib, rep 2)	+	0.88	1.26	
Oxic (+nut +inhib, rep 3)	+	0.88	1.43	
Oxic +nut+inhib treatment average		0.88	1.35	0/0.12
Oxic (+nut, rep 1)	+	1.03	1.66	
Oxic (+nut, rep 2)	+	0.44	0.76	
Oxic (+nut, rep 3)	+	0.45	0.76	
Oxic +nut treatment average		0.64	1.06	0.33/0.51
Oxic (+inhib, rep 1)	+	1.16	1.8	
Oxic (+inhib, rep 2)	+	1.49	2.08	
Oxic (+inhib, rep 3)	+	0.89	1.39	
Oxic +inhib treatment average		1.18	1.75	0.30/0.34
Oxic (rep, 1)	+	0.58	0.97	
Oxic (rep, 2)	+	0.48	0.84	
Oxic (rep, 3)	+	1.25	1.72	
Oxic treatment average		0.77	1.17	0.41/0.47
Anoxic (+nut, rep1)	-	0.36	0.62	
Anoxic (+nut +inhib, rep 1)	-	0.34	0.58	

Anoxic (rep 1)	-	0.42	0.68	
Anoxic (+inhib, rep 1)	-	0.33	0.55	
Anoxic treatment average		0.36	0.61	0.04/0.05
Oxic Control T_0 (+ nut, rep 1)*	+	0.37	0.63	
Oxic Control T_F (+nut, rep 1)*	+	0.42	0.68	
Oxic Control T_0 (rep 1)*	+	0.32	0.63	
Oxic Control T_F (rep 1)*	+	0.37	0.59	
Oxic Control average		0.37	0.37	0.04/0.03
Anoxic Control T_F (+nut, rep 1)*	-	0.33	0.56	
Anoxic Control T_F (rep 1)*	-	0.32	0.57	
Anoxic Control average		0.33	0.57	0.007/0.007

RSD= relative standard deviation
*= sterile slurry

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Figure 1

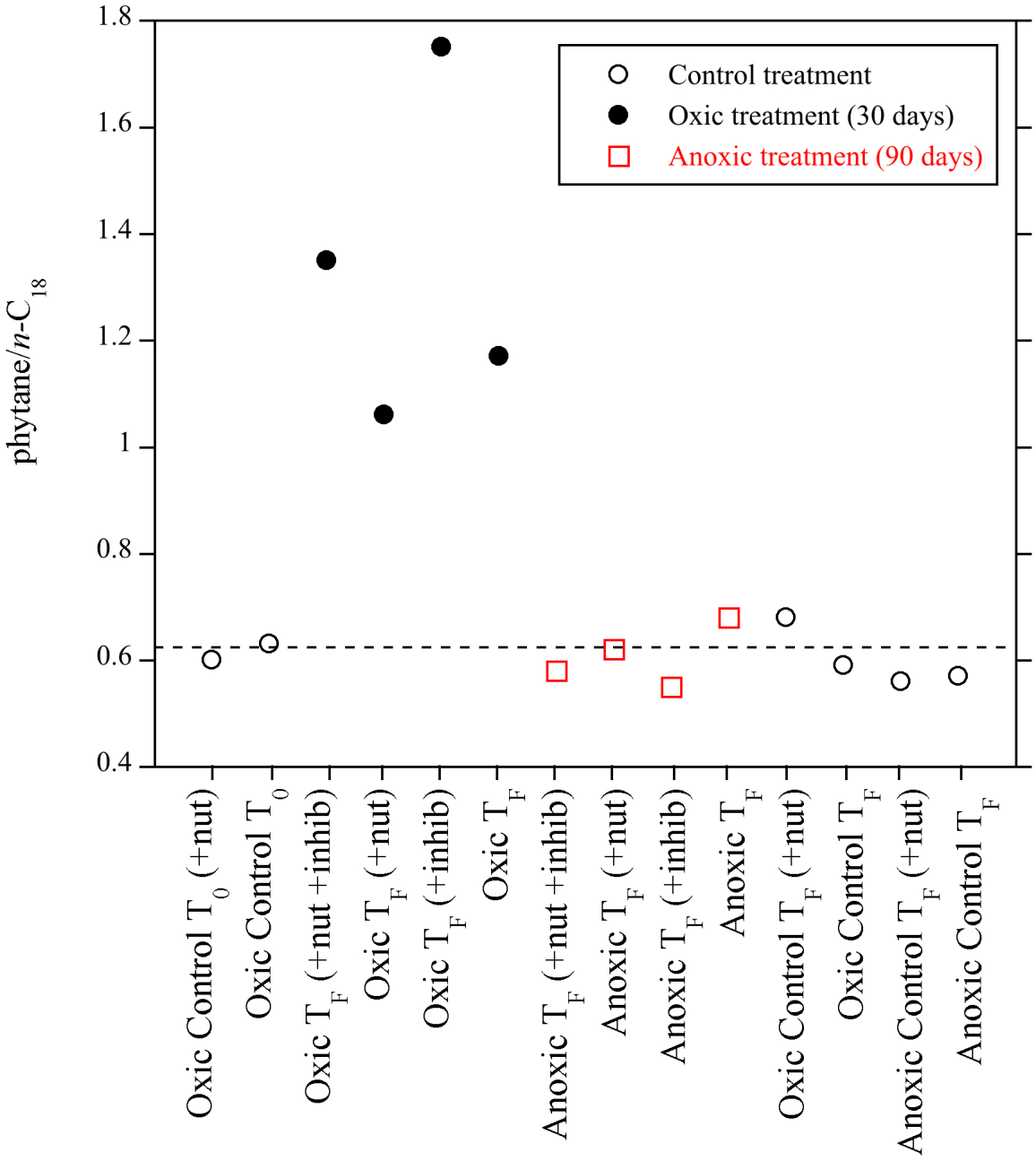

phytane/n-C18
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
Control treatment
Oxic treatment (30 days)
Anoxic treatment (90 days)
Oxic Control T0 (+nut)
Oxic Control T0
Oxic TF (+nut +inhib)
Oxic TF (+nut)
Oxic TF (+inhib)
Oxic TF
Anoxic TF (+nut +inhib)
Anoxic TF (+nut)
Anoxic TF (+inhib)
Anoxic TF
Oxic Control TF (+nut)
Oxic Control TF
Anoxic Control TF (+nut)
Anoxic Control TF

Figure 2

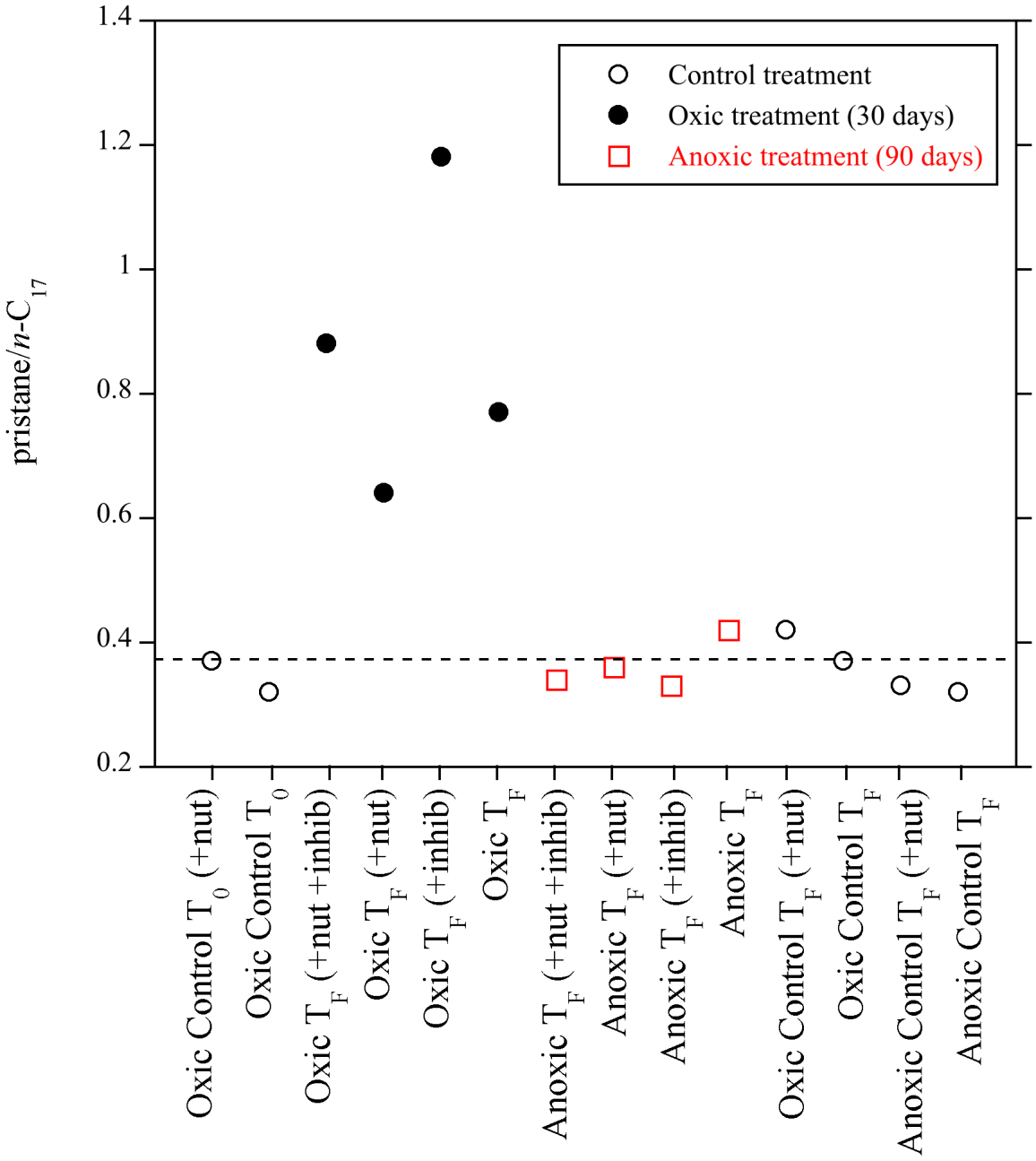

pristane/n-C17
1.4
1.2
1
0.8
0.6
0.4
0.2
Control treatment
Oxic treatment (30 days)
Anoxic treatment (90 days)
Oxic Control T0 (+nut)
Oxic Control T0
Oxic TF (+nut +inhib)
Oxic TF (+nut)
Oxic TF (+inhib)
Oxic TF
Anoxic TF (+nut +inhib)
Anoxic TF (+nut)
Anoxic TF (+inhib)
Anoxic TF
Oxic Control TF (+nut)
Oxic Control TF
Anoxic Control TF (+nut)
Anoxic Control TF

Figure 3

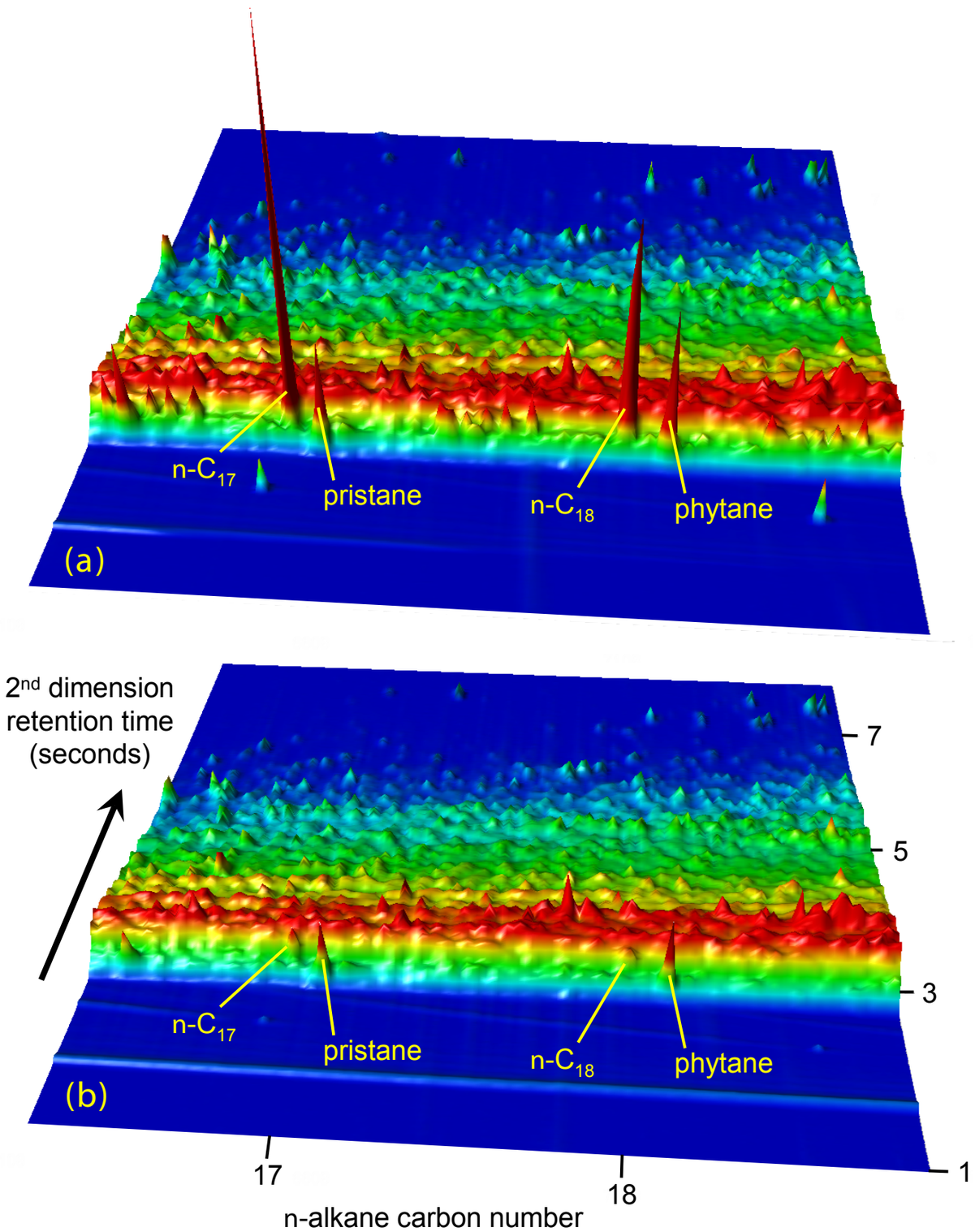

n-C17
pristane
n-C18
phytane
(a)
2nd dimension
retention time
(seconds)
7
5
3
1
n-C17
pristane
n-C18
phytane
(b)
17
18
n-alkane carbon number

Figure 4

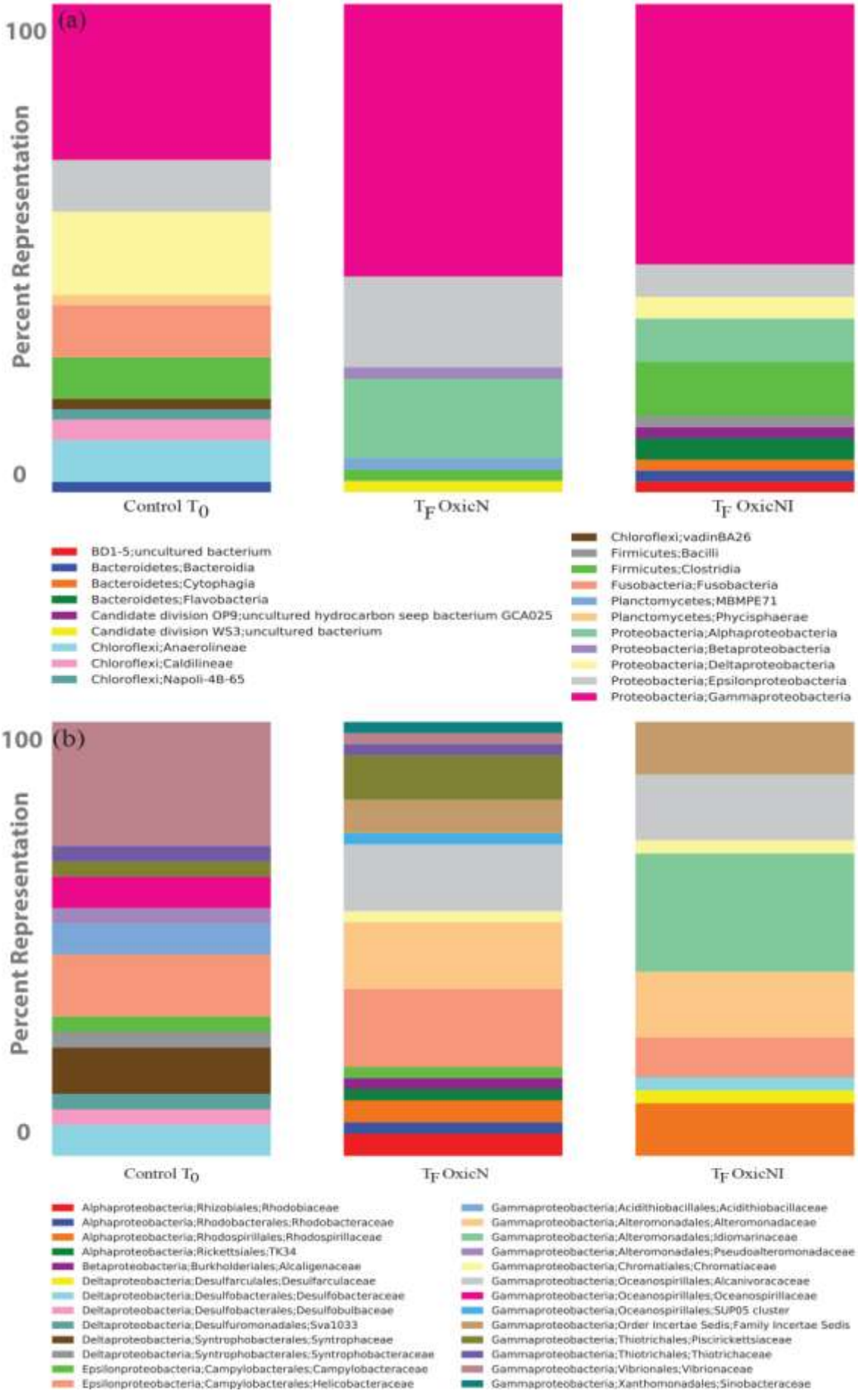
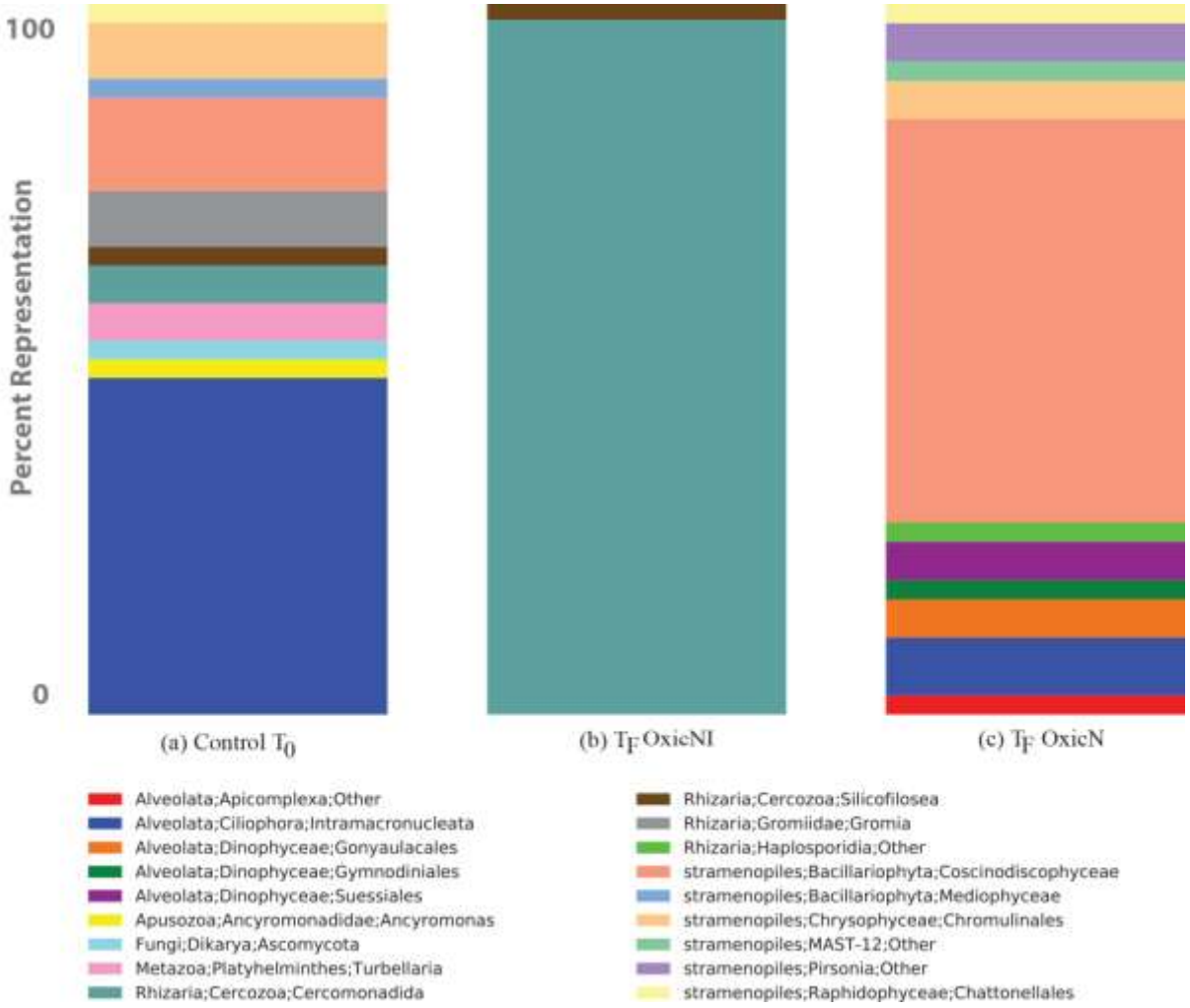

(a)
100
Percent Representation
0
Control T_0
T_F OxicN
T_F OxicNI
BD1-5;uncultured bacterium
Bacteroidetes;Bacteroidia
Bacteroidetes;Cytophagia
Bacteroidetes;Flavobacteria
Candidate division OP9;uncultured hydrocarbon seep bacterium GCA025
Candidate division WS3;uncultured bacterium
Chloroflexi;Anaerolineae
Chloroflexi;Caldilineae
Chloroflexi;Napoli-4B-65
Chloroflexi;vadinBA26
Firmicutes;Bacilli
Firmicutes;Clostridia
Fusobacteria;Fusobacteria
Planctomycetes;MBMPE71
Planctomycetes;Phycisphaerae
Proteobacteria;Alphaproteobacteria
Proteobacteria;Betaproteobacteria
Proteobacteria;Deltaproteobacteria
Proteobacteria;Epsilonproteobacteria
Proteobacteria;Gammaproteobacteria
(b)
100
Percent Representation
0
Control T_0
T_F OxicN
T_F OxicNI
Alphaproteobacteria;Rhizobiales;Rhodobiaceae
Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae
Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae
Alphaproteobacteria;Rickettsiales;TK34
Betaproteobacteria;Burkholderiales;Alcaligenaceae
Deltaproteobacteria;Desulfarculales;Desulfarculaceae
Deltaproteobacteria;Desulfobacterales;Desulfobacteraceae
Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae
Deltaproteobacteria;Desulfuromonadales;Sva1033
Deltaproteobacteria;Syntrophobacterales;Syntrophaceae
Deltaproteobacteria;Syntrophobacterales;Syntrophobacteraceae
Epsilonproteobacteria;Campylobacterales;Campylobacteraceae
Epsilonproteobacteria;Campylobacterales;Helicobacteraceae
Gammaproteobacteria;Acidithiobacillales;Acidithiobacillaceae
Gammaproteobacteria;Alteromonadales;Alteromonadaceae
Gammaproteobacteria;Alteromonadales;Idiomarinaceae
Gammaproteobacteria;Alteromonadales;Pseudoalteromonadaceae
Gammaproteobacteria;Chromatiales;Chromatiaceae
Gammaproteobacteria;Oceanospirillales;Alcanivoracaceae
Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae
Gammaproteobacteria;Oceanospirillales;SUP05 cluster
Gammaproteobacteria;Order Incertae Sedis;Family Incertae Sedis
Gammaproteobacteria;Thiotrichales;Piscirickettsiaceae
Gammaproteobacteria;Thiotrichales;Thiotrichaceae
Gammaproteobacteria;Vibrionales;Vibrionaceae
Gammaproteobacteria;Xanthomonadales;Sinobacteraceae

Figure 5


100
Percent Representation
0
(a) Control T_0
(b) T_F OxicNI
(c) T_F OxicN
Alveolata;Apicomplexa;Other
Alveolata;Ciliophora;Intramacronucleata
Alveolata;Dinophyceae;Gonyaulacales
Alveolata;Dinophyceae;Gymnodiniales
Alveolata;Dinophyceae;Suessiales
Apusozoa;Ancyromonadidae;Ancyromonas
Fungi;Dikarya;Ascomycota
Metazoa;Platyhelminthes;Turbellaria
Rhizaria;Cercozoa;Cercomonadida
Rhizaria;Cercozoa;Silicofilosea
Rhizaria;Gromiidae;Gromia
Rhizaria;Haplosporidia;Other
stramenopiles;Bacillariophyta;Coscinodiscophyceae
stramenopiles;Bacillariophyta;Mediophyceae
stramenopiles;Chrysophyceae;Chromulinales
stramenopiles;MAST-12;Other
stramenopiles;Pirsonia;Other
stramenopiles;Raphidophyceae;Chattonellales