Enhanced protein and carbohydrate hydrolyses in plume-associated deepwaters initially sampled during the early stages of the Deepwater Horizon oil spill

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ARTICLE INFO

Available online 9 September 2013

Keywords:
Extracellular enzyme activity
Deepwater Horizon oil spill
Hydrocarbon plume
Peptidase
Carbohydrate hydrolase

ABSTRACT

Oil spilled in the ocean can be biodegraded through a cascade of microbial processes, including direct degradation of petroleum-derived hydrocarbons, as well as subsequent degradation of transformation byproducts and exopolymeric substances (EPS) that are produced by microbes to emulsify hydrocarbons and facilitate access to oil. In the aftermath of the Deepwater Horizon oil spill, we measured enzymatic hydrolysis of carbohydrates and peptides in waters initially collected from within and outside of the deep hydrocarbon plume. The rationale is that the presence of EPS and other transformation byproducts in the deepwater plume may have enhanced heterotrophic bacterial metabolism in the cold deepwater environment. Our investigation targets carbohydrate and peptide hydrolase activities as indicators of the degradation of high molecular weight organic matter, including EPS substrates. Deepwater associated with the hydrocarbon plume revealed higher peptidase activity compared to non-plume deepwater samples. Enzymatic hydrolysis of carbohydrates, measured by the means of exo-acting enzyme activity (β-glucosidase), was also more rapid inside compared to outside the deepwater plume. Hydrolysis rates and patterns of endo-acting polysaccharide hydrolases, measured by means of distinct polysaccharide substrates in longer-term incubations, demonstrated more rapid plume-associated hydrolysis of two (laminarin and xylan) of the three substrates hydrolyzed in deepwaters. Our results indicate that microbial communities associated with the deepwater plume exhibited 'primed' responses to addition of specific substrates, which may structurally resemble components found in bacterial EPS and oil degradation byproducts. Bacterial transformation of oil-degradation byproducts thus likely contributed to microbial growth and respiration measured inside the deepwater plume.

1. Introduction

The Deepwater Horizon (DWH) oil spill in April, 2010, was the largest accident in the history of the oil and gas offshore industry (Atlas and Hazen, 2011). It was also the first accident of this magnitude that affected deep-sea marine environments, as massive quantities of gas and dissolved hydrocarbons were released from the broken riser pipe, forming plumes of dissolved hydrocarbons at depths between 1000 m and 1300 m (Diercks et al., 2010; Reddy et al., 2011). The formation of deepwater gas and oil plumes resulted in the development of a microbial community whose composition was quite distinct from that found in non-plume associated deepwaters (Hazen et al., 2010; Valentine et al., 2010, 2012; Redmond and Valentine, 2011; Bælum et al., 2012; Yang et al., in review).

The plume-associated bacterial community was quite active, sustaining high oxidation rates of ethane and propane (Valentine et al., 2010) and of petroleum-derived hydrocarbons (Hazen et al., 2010), consistent with local anomalies of oxygen (Valentine et al., 2012) and nutrients (Shiller and Joung, 2012) measured inside the plume. In general, microbial communities involved in oil degradation include a cascade of primary oil degraders and secondary consumers, which metabolize oil degradation products as well as other bacterial metabolites of primary oil degraders (Head et al., 2006). A major fraction of oil degradation products typically include protein- and carbohydrate-rich organic molecules (Hazen et al., 2010); metabolites include bacterial exopolymeric substances (EPS) that are produced in large amounts by oil degraders to emulsify crude oil (Hino et al., 1997). For example, EPS produced by *Halomonas* strains isolated from oil-contaminated DWH surface water were effective catalyst for the solubilization of aromatic hydrocarbons and emulsification of weathered surface oil collected from the DWH spill site (Gutierrez et al., 2013). Such EPS are rich in proteins and carbohydrates (Gutierrez et al., 2007), and can

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http://dx.doi.org/10.1016/j.dsr2.2013.09.003

fuel heterotrophic bacterial metabolism in the water column. We found evidence of such a degradation cascade in a roller table incubation with oil-contaminated surface water from the DWH spill site, in which the formation of oil aggregates and the activities of oil aggregate-associated bacteria stimulated the activities of natural bacterial communities not directly associated with primary oil degradation (Ziervogel et al., 2012).

The goal of this study was to compare the potential of the deepwater microbial community, initially sampled in the early stages of the DWH oil spill, to access proteins and carbohydrates as substrates inside and outside of the deepwater plume. We hypothesized that the presence of oil and EPS in the deep plume would enhance the activities of microbial extracellular enzymes that are required to access and degrade proteins and polysaccharides (Arnosti, 2011). Studies to date of the DWH oil spill and its aftermath have focused particularly on physical/chemical aspects of oil dynamics and time-dependent changes in microbial community composition; comparatively few studies (e.g. Valentine et al., 2010; Bælum et al., 2012; Gutierrez et al., 2013) have focused on the measurements of microbial activities. Here, we measured the activities of extracellular enzymes in water initially sampled inside and outside of the deepwater plume, and compared our results with information from other DWH surveys, as well as with the comparatively few other measurements of enzyme activities in the deep marine water column.

2. Material and methods

2.1. Water sampling, storage and processing

During a cruise of the R.V. *Walton Smith* (May 30–June 05, 2010), water from depths of 900 m to 1240 m was collected at six different sites inside and outside of the deepwater plume of petroleum-derived hydrocarbons using Niskin bottles mounted on a CTD rosette (see Table 1 for a detailed description of sampling sites). Water samples from separate casts were stored in 250-mL PET bottles at 4 °C and transported back to the home laboratory at the University of North Carolina, Chapel Hill, where storage continued for 4 weeks. The relatively long storage was the result of time and logistical constraints that were unavoidable in the course of our efforts to respond to this unforeseen and (at the time) still-evolving oil spill event. The water collected from this cruise was used for two sets of experiments: (1) to assess enzymatic capabilities of microbial communities in deepwaters near the wellhead (present work), and (2) to investigate the formation of oil-rich marine snow in oil-contaminated deepwaters (Passow et al., 2012). Since these experiments required sufficient sample volumes, we had to combine deepwater samples from different casts that had an elevated fluorescence signal indicative of the hydrocarbon plume. We also combined deepwater samples that showed no fluorescence or only background levels of fluorescence (Table 1). Combination of the individually collected samples was necessary in order to carry out the full suite of experimental measurements. The alternative would have been to carry out a minimal set of measurements that would not have provided new insight into heterotrophic activities in the aftermath of the formation of the deepwater plume. The two sample types are hereafter referred to as deepwater plume and non-plume deepwater, and should be considered as water representing an averaged response of the combined samples. The corresponding deepwater CTD plots show the two sample types as SUB II and PLUME II (Fig. S1 in Passow et al., 2012). All analyses described below were conducted therefore on two bulk samples: non-plume deepwater and deepwater plume (Table 1). The bulk samples were subsampled for the analyses described below.

2.2. Bacterial enzymatic activity

Enzyme activities were measured using two experimental approaches that provide different information about the timescales of microbial responses to substrates, as well as the specific types of active enzymes. The first approach uses small substrate proxies to measure the activities of exo-acting enzymes cleaving terminal units from polymers. These activities can be measured with short-term (several hour) incubations, and are generally considered to reflect the enzymatic response of the microbial community present in the sample at the time the substrate is added. We used L-leucine-4-methylcoumarinyl-7-amide (MCA) hydrochloride (final concentration: 1200 μM) and MUF-β-D-glucopyranoside (final concentration: 1000 μM; both substrates from Sigma-Aldrich) at enzyme-saturating levels as substrate proxies to measure leucine–aminopeptidase (hereafter referred to as peptidase) and β-glucosidase activity according to Hoppe (1983). For these experiments, deepwater plume ($n=6$) and non-plume samples ($n=3$) as well as killed controls (0.1-μm filtered and autoclaved deepwater; $n=3$) were incubated for 22 h at 4 °C in the dark. Initial tests revealed that these incubation times were sufficient to yield detectable changes in fluorescence. Two point fluorescence measurements were made using a Turner Biosystems TBS-380 fluorometer, with excitation/emission channels set to "UV" (365 nm excitation, 440–470 nm emission). Changes in fluorescence over time measured in live treatments were corrected for the readings in killed controls. Fluorescence changes were calibrated using MUF and MCA standards, and used to calculate potential hydrolysis rates.

The second experimental approach measured the activities of specific endo-acting enzymes that cleave target polysaccharides mid-chain. These experiments require comparatively long incubation times and therefore integrate microbial enzymatic induction

Table 1
Sample location and depth as well as their relative degree of oil contamination given as CDOM fluorescence. See text for details on sample storage and processing.

Sample ID	Station ID	Sampling date	Lat (°N)	Long (°W)	Sample depth (m)	Sample description/ oil contamination	CDOM fluorescence (mg m^3)[a]	Distance to wellhead (km)
Deep non-plume	WS67A	June 4, 2010	28.74	88.43	1110	No plume	0	4.2
Deep non-plume	WS79A	June 5, 2010	28.85	88.49	900	No plume	0	13.3
Deep plume	WS16B	June 5, 2010	28.70	88.56	1025	No plume	0	17.3
					1100	No plume	0	
	WS47A	May 30, 2010	28.72	88.39	1000	Upper part of plume	1	1.8
					1105	Upper part of plume	4	
					1180	Plume max[b]	27	
Deep plume	WS46A	May 30, 2010	28.69	88.43	1170	Inside plume, above max	11	6.6
					1210	Plume max	12	
	WS58A	June 1, 2010	28.73	88.38	1240	Plume max	19	0.9

[a] Fluorescence signal corrected for background.
[b] Highest CDOM fluorescence of the cast.

and growth responses (including possible changes in community composition) to substrate addition. Endo-acting enzyme activities were measured with fluorescently labeled (FLA) pullulan, laminarin, xylan, fucoidan, arabinogalactan, and chondroitin sulfate (Fluka or Sigma). Labeling of all polysaccharides with fluoresceinamine was conducted as described in Arnosti (2000). The six polysaccharides used here differ in monomer composition and linkage position; many are components of marine algae (Painter, 1983), and are therefore present in considerable quantities in the ocean (Alderkamp et al., 2007). Moreover, enzymes that specifically hydrolyze these substrates have been identified in a variety of marine environments (Arnosti et al., 2011) as well as in the genomes of recently sequenced marine bacterial isolates (Bauer et al., 2006; Glöckner et al., 2003; Weiner et al., 2008).

Polysaccharide hydrolysis experiments were conducted as described in Arnosti (2003). In brief, this method measures the potential of microbial communities to hydrolyze specific polysaccharides by determining the change in molecular weight with time of the total added polysaccharide. Since the amount of time required for a polysaccharide pool to be hydrolyzed to lower molecular weights is not known *a priori*, incubations are conducted as time series. The timepoint at which hydrolysis is first detected, as well as the rate of hydrolysis, therefore provides information about microbial community capabilities. Hydrolysis initially detected after longer incubation times indicates that the specific enzyme activities are uncommon among members of a microbial community, or require induction or cell growth for substantial hydrolysis to occur. Conversely, enzyme activities that are detectable at early timepoints in an incubation series indicate that these enzymes are widespread among the members of a community, or are quickly produced in response to substrate addition.

For these experiments, single FLA-polysaccharide substrates were incubated at a final concentration of 3.5 μM monomer equivalent in duplicate 15 ml deepwater non-plume and plume samples in the dark at 4 °C for a total of 14 days. Throughout the incubation, 1 mL subsamples were taken after 0, 1, 3, 7, and 14 days. All subsamples were stored frozen until analysis. Samples were analyzed using a gel-permeation chromatography system with fluorescence detection, as described in detail in Arnosti (2003). Substrates incubated with killed control waters showed no significant changes in molecular weights over time.

The rates reported here represent potential hydrolysis rates, since added substrate competes with naturally occurring substrates for enzyme active sites. Given the level of substrate addition, however, hydrolysis rates are likely zero-order with respect to substrate and represent maximum potential rates. DOC concentrations in non-plume deepwater and deepwater plume at the time of sampling were 70–130 μM and 200–300 μM, respectively (S.B. Joye, personal communication).

2.3. Microbial cell abundance

Concurrent with initiation of enzyme activity measurements, microbial cell abundance was measured according to Porter and Feig (1980), using 4',6-diamidino-2-phenylindole (DAPI). Samples were filtered through a 0.2 μm GTBP isopore filter (Millipore, Billerica, MA) and duplicate filters per sample were examined under an epifluorescence microscope (Olympus, magnification × 1000) equipped with a digital camera (Olympus TH4–100). Cells from 10 randomly chosen frames per filter were counted.

2.4. Statistical analysis

Enzyme activities measured by the means of small substrate proxies are given as their statistical mean ($n \geq 3$) ± standard deviation. We compared mean values of deepwater plume and non-plume associated hydrolysis rates (per cell and on a volume basis) using the Student's *t*-test; differences were considered significant when *p*-values were < 0.05. Due to the limited sample volumes available, polysaccharide hydrolysis experiments were carried out in duplicate, and rates are reported as average values of $n = 2 \pm$ range of duplicates.

3. Results and discussion

The temporal succession of organisms inside the plume, and the contrast in community composition inside and outside of the plume, points at metabolically highly active communities within the plume that successively transformed oil-derived components (Redmond and Valentine, 2011; Bælum et al., 2012; Yang et al., in review). Analysis of samples collected from the deepwater plume revealed the presence of microbial 'flocs' that were enriched in carbohydrates, proteins, and oil degradation products (Hazen et al., 2010), representing a spatial and temporal enrichment of substrates and organisms. Greatly enhanced levels of peptidase activities in the deep plume relative to the non-plume deepwaters (Fig. 1A) are consistent with the presence in the original plume waters of EPS and microbial 'flocs', and may particularly be associated with the presence of oil-degrading bacteria of the genus *Colwellia* that were enriched in oil-derived flocs in the deepwater plume (Bælum et al., 2012). *Colwellia psychrerythraea* first drew attention in the literature due to its distinctive cold-active aminopeptidase (Huston et al., 2000, 2004) and due to its capability to produce cryoprotectant EPS in cold conditions (Marx et al., 2009). Since *Colwellia* could be consistently enriched in the aftermath of the DWH oil spill from contaminated water samples (Redmond and Valentine, 2011; Bælum et al., 2012; Gutierrez et al., 2013, in press), it may well contribute to aminopeptidase activities in situ. Although the community composition of our samples at the times of measurement is not known, cell-free cold-active peptidases have been reported in the deep ocean (Baltar et al., 2010b) and have been shown to retain activity in seawater on timescales of days to weeks (Steen and Arnosti, 2011). They therefore could also contribute to total hydrolytic activity even after the organisms producing them are no longer highly active.

Our data extend the database of enzymatic activity measurements in the deep marine water column. Although peptidase activities can be measured in most marine waters, comparatively few measurements have been made in deep ocean waters. The

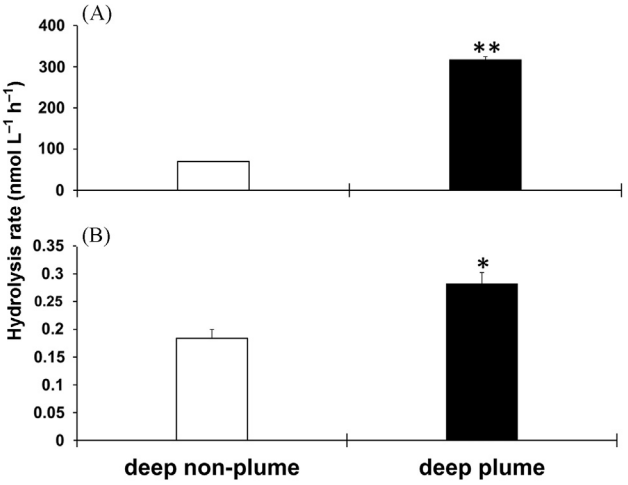

Fig. 1. Peptidase (A) and β-glucosidase (B) activity in deepwater plume and non-plume samples. Error bars indicate standard deviations of $n = 6$ (deepwater plume) and $n = 3$ (deepwater non-plume). Note the different scales on the *y*-axis. $^{*}p < 0.01$; $^{**}p < 0.001$ (Student's *t*-test).

rates measured within the plume were one to two orders of magnitude higher than previously reported deepwater activities (Hoppe and Ullrich, 1999; Misic and Fabiano, 2006; Tamburini et al., 2002, 2009; Baltar et al., 2009, 2010; Zaccone et al., 2012).

After accounting for the higher cell numbers in the deep plume (Table 2) relative to non-plume deepwaters, cell-specific peptide hydrolysis rates in plume waters were still more than double those of non-plume deepwaters (Table 2). Cell-specific peptidase activity inside the plume was comparable to rates measured in uncontaminated surface waters collected just outside the DWH spill site, stored in the same manner as the deepwater samples, and incubated at 25 °C (Ziervogel et al., 2012). Plume associated cell-specific peptidase activity was also higher than most previous deepwater reports (Baltar et al., 2009, 2010); only in deepwaters of a coastal upwelling zone of the Indian Ocean higher cell-specific peptidase activities have been reported (Hoppe and Ullrich, 1999).

In our deepwater plume sample, β-glucosidase activity was on an average only slightly elevated inside the plume compared to outside the plume (Fig. 1B). These rates are still enhanced compared to most of the (few) other locations in which β-glucosidase activity has been measured in deep ocean waters (Hoppe and Ullrich, 1999; Misic and Fabiano, 2006; Baltar et al., 2009, 2010). Cell-specific β-glucosidase activity inside and outside the plume (Table 2) was in the same range as previously reported rates in the deep Atlantic (Baltar et al., 2009, 2010) and Indian Ocean (Hoppe and Ullrich, 1999).

Our polysaccharide hydrolysis experiments probe the structural specificity of specific endo-acting enzymes, revealing aspects of carbohydrate metabolism not evident through the use of small substrate proxies. Three of the six polysaccharides were hydrolyzed in deepwater (plume and non-plume), a pattern consistent with a study of polysaccharide hydrolase activities in the water column carried out at a nearby site 3 years prior to the DWH oil spill (Steen et al., 2012). The presence of the plume therefore did not fundamentally change the types of polysaccharide hydrolases active in the deepwater column. The time course of laminarin and xylan hydrolyses, however, differed conspicuously between plume- and non-plume-associated samples: in the plume-associated samples, laminarin hydrolysis was measurable after just 1 day of incubation, and xylan hydrolysis was measurable after 3 days of incubation (Fig. 2A). In the non-plume samples, in contrast, laminarin hydrolysis began at a much lower level, and xylan hydrolysis was only measurable at low levels after 7 days' incubation (Fig. 2B). Conditions in the plume had already induced hydrolytic activities to a substantial degree, and reduced or eliminated the lag times that are otherwise typical for laminarin and xylan hydrolyses. The lag-times seen for laminarin and xylan hydrolyses in the non-plume samples, as well as the general range of hydrolysis rates, are consistent with measurements in 2007 of polysaccharide hydrolysis at a depth of 905 m at a nearby site on the Gulf of Mexico continental slope (Mississippi Canyon 118, 10 nautical miles northwest of the Macondo wellhead). For those samples, laminarin and xylan hydrolyses were measurable only after longer periods (6 and 11 days, respectively) of incubation (Fig. 2C; Steen et al., 2012). The accelerated commencement of laminarin and xylan hydrolyses inside compared to outside the plume is also consistent with a comparison of gene function in plume-associated and non-plume associated deepwater communities, carried out with samples collected at nearly the same time (late May/early June 2010) as our samples. GeoChip analysis of DNA demonstrated that a range of genes associated with degradation of high molecular weight carbohydrates, including xylan and starch, were more abundant in plume-associated than in non-plume associated deepwaters (Lu et al., 2012).

In contrast to the patterns of laminarin and xylan hydrolyses, chondroitin hydrolysis was not enhanced in the plume relative to the non-plume deepwaters. Chondroitin hydrolysis was first evident in the 7 day samples, and was considerably higher in the non-plume than in the plume-associated deepwater samples (Fig. 2A and B). Lower rates of chondroitin hydrolysis, accompanied by selective enhancement of other specific enzyme activities

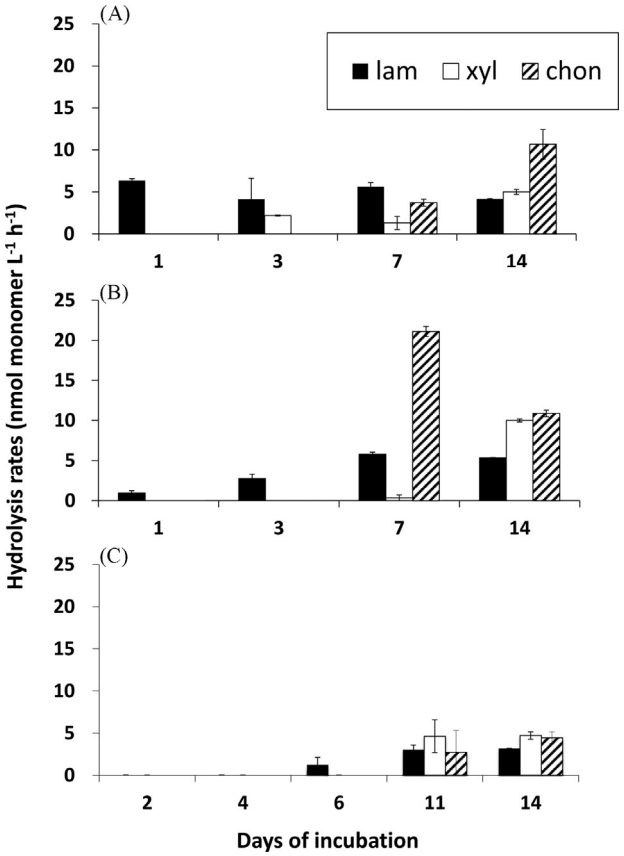

Fig. 2. Hydrolysis rates of laminarin (lam), xylan (xyl) and chondroitin (chon) during 14 days' incubation of deepwater plume (A) and non-plume (B). Panel (C) shows deepwater (905 m) hydrolysis rates from MC118, located 10 nautical miles from the DWH spill site, measured in 2007 (data replotted from Steen et al., 2012). Fucoidan, arabinogalactan and pullulan remained unhydrolyzed in all three incubations. Error bars in (A) and (B) indicate the range of duplicate measurements; error bars in (C) are standard deviations of $n=3$ measurements.

Table 2
Average cell-specific enzymatic activities (amol cell^{-1} h^{-1}) and bacterial cell abundance (cell numbers × 105 mL^{-1}) in deepwater plume and non-plume samples with *p*-values (Student's *t*-test).

Parameter	Deep plume (s.d.; *n*)	Deep non-plume (s.d.; *n*)	*p*-values
Peptidase	574.5 (13.5; 6)	270.1 (0.6; 3)	3.8E−08
β-glucosidase	0.5 (0.04; 6)	0.7 (0.06; 3)	0.01
Bacterial cells	5.5 (1.3; 20[a])	2.6 (0.7; 20[a])	5.1E−08

[a] Bacterial cells were counted in 10 microscopic fields on duplicate filters.

(including laminarin and xylan), were also observed in a comparison of surface water samples with and without the DWH-derived oil slick (Arnosti et al., submitted for publication), and may be a general feature of microbial communities exposed to oil from the DWH spill. This structural selectivity—in particular, enhancement of selected polysaccharide hydrolases, and lower/delayed production of others—could be related to the specific structure of bacterial EPS produced during the course of microbial oil degradation (Gutierrez et al., 2013).

In summary, our results of enhanced polysaccharide hydrolysis and peptidase activities in deepwater microbial communities initially dominated by oil-degrading bacteria indicate that plume-associated microbial communities were 'primed', presumably by the presence of EPS and oil degradation byproducts, to degrade a selected range of specific high molecular weight substrates. The spectrum of substrates hydrolyzed was similar to that hydrolyzed by microbial communities outside of the deepwater plume, and to deepwater microbial communities sampled nearby several years prior to the DWH oil spill. Microbial transformation of complex organic matter derived from oil-degradation byproducts, including EPS, may have played an important role in heterotrophic microbial growth and respiration in oil contaminated deepwaters during the early stages of the spill (Valentine et al., 2010; Hazen et al., 2010). The reproducible patterns of polysaccharide hydrolysis in deepwaters of the northern Gulf of Mexico prior to and after the spill indicate that the hydrolytic capabilities of these communities were sped up, but not greatly changed in nature, by the presence of oil degradation byproducts and EPS.

Acknowledgments

We thank the captain and crew of the RV *Walton Smith* as well as chief scientist S.B. Joye (University of Georgia) for assistance during sampling. We thank Tingting Yang, and Lisa Nigro (all UNC) for collecting the samples aboard ship. At UNC, Benjamin Rhodes helped measure enzyme activities, and Avery Bullock counted bacterial cells. We also thank Sherif Ghobrial (UNC) for assistance with the gel permeation chromatography system to measure polysaccharide hydrolysis. This work was funded by NSF RAPID Grant OCE-1045115 to Andreas Teske, and NSF Grant OCE-0848703 to Carol Arnosti. Funding was also provided by a grant from BP/the Gulf of Mexico Research Initiative to support consortium research entitled "Ecosystem Impacts of Oil and Gas Inputs to the Gulf (ECOGIG)" administered by the University of Mississippi. The GRIIDC dataset IDs for this paper are "R1.x132.135:0001 R/V *Walton Smith* May–July 2010 Microbiological Genetics" and "R1. x132.135:0002 R/V *Walton Smith* May–July 2010 Physiological Activity".

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