Deep-sea bacteria enriched by oil and dispersant from the Deepwater Horizon spill

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Running title: Enrichment of oil degraders from Gulf of Mexico

Summary

The Deepwater Horizon oil spill resulted in a massive influx of hydrocarbons into the Gulf of Mexico (the Gulf). To better understand the fate of the oil, we enriched and isolated indigenous hydrocarbon-degrading bacteria from deep, uncontaminated waters from the Gulf with oil (Macondo MC252) and dispersant used during the spill (COREXIT 9500). During 20 days of incubation at 5ºC, CO_2 evolution, hydrocarbon concentrations, and the microbial community composition were determined. Approximately 60% to 25% of the dissolved oil with or without COREXIT, respectively, was degraded, in addition to some hydrocarbons in the COREXIT. $FeCl_2$ addition initially increased respiration rates, but not the total amount of hydrocarbons degraded. 16S rRNA gene sequencing revealed a succession in the microbial community over time, with an increase in abundance of *Colwellia* and *Oceanospirillales* during the incubations. Flocs formed during incubations with oil and/or COREXIT in the absence of $FeCl_2$. Synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy revealed that the flocs were comprised of oil, carbohydrates and biomass. *Colwellia* were the dominant bacteria in the flocs. *Colwellia* sp. strain RC25, was isolated from one of the enrichments and confirmed to rapidly degrade high amounts (approximately 75%) of the MC252 oil at 5ºC. Together these data highlight several features that provide *Colwellia* with the capacity to degrade oil in cold, deep marine habitats, including aggregation together with oil droplets into flocs and hydrocarbon degradation ability.

Introduction

On April 20, 2010, high-pressure oil and gas caused the Deepwater Horizon drilling rig in the Gulf of Mexico to explode resulting in the second largest marine oil spill in the history of petroleum industry. It has been estimated that ~4.1 million barrels of light crude oil and natural gas leaked into the Gulf from the Macondo well (MC252) during the ~3 months before the well head was capped (OSAT, 2010). Primarily in an attempt to improve safety for surface operating vehicles, ~1.8 million gallons (~37,500 barrels) of the chemical dispersant COREXIT (mainly 9500, but also 9527 formulation) was applied to the Gulf surface as well as directly into the wellhead at 1500 meters below surface level (mbsl; 9500 only). Very little is known about the effects and persistence of COREXIT in the environment, including its impact on indigenous microbes in the Gulf (Judson*, et al.*, 2010).

During the spill, hydrocarbon concentrations varied from 100% at the source to undetectable with increasing distance from the wellhead. During exploratory and surveillance cruises conducted for the US Coast Guard, National Oceanic and Atmospheric Administration (NOAA), and the US Environmental Protection Agency (EPA), a plume or cloud of dispersed MC252 crude oil was detected at 1100-1220 mbsl at distances up to 35 km from the wellhead (Camilli*, et al.*, 2010). The plume is thought to have formed due to application of COREXIT at the wellhead and a combination of physical and chemical properties at 1100 mbsl depth (e.g. pressure, temperature (~5ºC), and salinity (Adcroft*, et al.*, 2010, Dasanayaka & Yapa, 2009, Yapa*, et al.*, 2008), that resulted in the oil attaining neutral buoyancy at this depth. During the ongoing release of

oil several research groups reported the presence of the plume, but after the wellhead was capped the plume was no longer detectable (OSAT, 2010).

Several reports have provided strong evidence that microbial processes were a key factor for degradation and removal of oil from the plume (OSAT, 2010). For example, Hazen *et al.* (2010) reported a shift in the composition of the microbial community in the deep-sea plume within one month of the spill (May 2010). They found that sequences corresponding to Oceanospirillales dominated in the oil plume, but that they were rare in uncontaminated water at the same depth. Later sampling of the plume in June, 2010 reported that sequences representative of *Cycloclasticus* and *Colwellia* were dominant, together accounting for more than 95% of the sequence data (Valentine et al., 2010). Recently, Redmond & Valentine (2011) reported that *Colwellia* sequences increased in abundance during enrichment on crude oil at cold temperatures (4 °C) compared to warmer (20 °C) incubation conditions and suggested that temperature was a major determinant in selection of this group of microorganisms.

Here we aimed to study the succession of the indigenous microbial community and the formation of microbial flocs in deep-sea water from the Gulf of Mexico during laboratory enrichments at cold temperature (5°C) and with high concentrations of MC252 oil and COREXIT. We also aimed to select and isolate specific members of the microbial community having the capability to degrade oil in the presence or absence of dispersant. The source of inoculum was uncontaminated seawater collected from the Gulf of Mexico during the Deepwater Horizon oil spill at the depth of the reported hydrocarbon plume (1100 mbsl). We also looked at the impact of Fe^{2+} on the degradation rates because iron

was reported to be a potentially limiting nutrient for microbial growth in the water column (Hazen*, et al.*, 2010, Lu*, et al.*, 2011, OSAT, 2010).

During the enrichments the community composition was monitored by 454 pyrotag sequencing of 16S rRNA genes. We also monitored respiration and degradation of oil and COREXIT during the incubations. These experiments enabled us to gain a better understanding of the pattern of microbial succession, and the response of specific microbial populations when exposed to high concentrations of hydrocarbons such as occurred during the Deepwater Horizon oil spill or that naturally occur from oil seeps on the ocean floor.

Results

Respiration during enrichments

Microbial populations in water collected from the deep-sea in the Gulf of Mexico were enriched to grow on and degrade high concentrations of Macondo MC252 oil, in the presence or absence of the dispersant, COREXIT 9500 at low temperature (5°C). Additional treatments included Fe amendment and sterile controls. As a dissolved oxygen concentration of 6.07 mg/L was measured on site (Hazen, *et al.*, 2010), our experiments were done under aerobic conditions. Dissolved oxygen concentrations measured on 0, 5, and 20 days of incubation were 8.4, 7.5, and 6.1 mg/L, respectively.

During the enrichments we observed significant differences (t-test, $P<0.05$) in CO_2 evolution patterns between the different treatments (Fig. 1a). There was a substantial increase in CO_2 evolution in the enrichments containing oil and/or COREXIT compared to sterile controls. In the enrichments with oil alone and oil+Fe, the cumulative CO_2

evolution during the 20-day incubation period was in the range of 2.8-3.0 mg CO_2, and in those with COREXIT in the range 4.4-4.9 mg CO_2, regardless of the presence of oil (Fig. 1a). In the presence of $FeCl_2$ there was an immediate onset of CO_2 evolution, while in the enrichments without $FeCl_2$, there was a lag phase of ~6 days before CO_2 evolution commenced. However, regardless of the addition of $FeCl_2$, the total amount of CO_2 that accumulated after 20 days was similar.

Degradation of Macondo crude oil

During the incubations we determined the concentration of dissolved crude oil (measured as total petroleum hydrocarbons, TPH) by extracting aliquots of the seawater and analyzing by GC-FID as described below. In the treatments with oil and no COREXIT, oil adhered to the sides of the bottles, reducing the amount in the seawater to approximately 30% of the initial concentration of 100 ppm. In samples with COREXIT more of the oil was dissolved; ~70% of the initial concentration (Fig. 1b), as expected because COREXIT is a dispersant. After 5 days of incubation 25% of the dissolved oil was degraded in samples with oil alone and 40% in samples with oil and COREXIT. After 20 days no additional oil was degraded in the samples with oil alone, but 60% had been degraded in samples with oil and COREXIT (for mass balances see supplemental information Fig. S2). While the oil degradation was reported as TPH, the majority of compounds lost were straight chain alkanes, which were predominant components of the MC 252 source oil.

COREXIT 9500 degradation

The concentrations of COREXIT components were also measured during the incubations. COREXIT 9500 is a water soluble mixture containing hydrocarbons (50%), glycols (40%) and dioctylsulfosuccinate (DOSS) (10%) and these different classes of compounds were degraded at different rates (Fig. 1c). While degradation of the hydrocarbon compounds was initially fast, slowing down in the latter phase, the glycol compounds and DOSS were degraded at a linear rate. Although the largest portion of degraded mass originated from the hydrocarbon fraction, still a significant amount of the DOSS was degraded, while the glycols were more recalcitrant.

Cell counts

There was an increase in microbial cell density in all of the enrichments with oil and/or COREXIT added (Fig. 1d). The bacterial counts for the source water before incubation was in the range $2\text{-}4\times10^4$ cells ml^{-1}. Over time we observed a significant (t-test, $P<0.05$) increase in the cell density in the oil and/or COREXIT amended enrichments. The highest densities of $1\text{-}2\times10^7$ cells ml^{-1} were obtained in enrichments with both oil and COREXIT, corresponding to the highest respiration rates also observed in those treatments. In enrichments with oil alone the growth was slower with $\sim1\times10^5$ cells ml^{-1} on day 5 of incubation and $\sim2\times10^6$ cells ml^{-1} on day 20 (Fig. 1d).

Microbial community analysis

One of our main goals was to determine which specific members of the indigenous microbiota in the deep-sea of the Gulf of Mexico were enriched in the presence of high concentrations of oil. Therefore, DNA was extracted during the

incubations and 16S rRNA genes were sequenced using a 454 pyrotag sequencing approach. A total of 148,276 quality-filtered reads were clustered in OTUs with 97% similarity enabling identification of OTUs down to genus level, but no differentiation on the species level was possible. During the enrichments there was a clear succession in the bacterial community composition (Fig. 2). In particular, Colwelliaceae increased from barely detectable in the source water to relative abundances of 15 to 30% in all of the enrichments, similar to the recent findings reported by Redmond and Valentine (2011). Also bacteria within the Oceanospirillales, including *Oleispira* spp. that was previously found to be abundant in the deep-sea oil plume during the Deepwater Horizon oil spill (Hazen*, et al.*, 2010), increased from being rare (i.e.<1% relative abundance) to relative abundances of 5-10% as the hydrocarbons were degraded. As these values are all relative, the overall microbial abundance increased substantially, such that even those groups that declined in relative abundance may have increased in total numbers.

Microbial floc formation

Large flocs (Fig. 3a and 3c) were observed in all of the enrichments with oil and/or COREXIT and without $FeCl_2$, while in enrichments with $FeCl_2$ there was no floc formation (Fig. 3b). Typically, there was one large floc suspended in the water after 20 days of incubation, but already after 2 days cell aggregates were detectable by light microscopy (Fig. 3a). After 20 days of incubation a typical floc had a size of 1-4 cm, was white in color, and suspended in the water.

SR-FTIR spectromicroscopy at the LBNL Advanced Light Source (ALS) was used to monitor the composition of the flocs during their formation. The SR-FTIR spectra

revealed intense absorption peaks (Fig. 4) between 3100 and 2800 cm^{-1}, strong absorption peaks at ~1730 and ~1545 cm^{-1}, and absorption profiles between 1200 and 900 cm^{-1} associated with the flocs (Fig. 4). These absorption features are well described for the hydrocarbon (C–H) vibration modes of the MC252 oil, the carbonyl (C=O) vibrational modes of oil degradation products, amide II (N–H) vibrational modes of proteins, and ring vibrations of diverse polysaccharide and exopolysaccharide (EPS) groups (Hazen*, et al.*, 2010, Naumann, 2000). A time course analysis of image and intensity of absorption features revealed that the chemical and biochemical composition of the flocs evolved over time (Fig. 5). The intensity of the absorption bands corresponding to MC252 oil components increased in intensity from 0.056 relative absorbance units (a.u.) to 1.11 a.u. during the initial 20 days of incubation and subsequently declined to 0.98 a.u. for the remaining 20-40 day incubation period. This suggests that the oil tended to initially concentrate in the floc material, but was subsequently degraded. Meanwhile, the absorption intensity of the protein amide II signal increased from 0 (at t = 0 days) to 0.123 a.u. (t = 40 days) throughout the incubation period indicating an increasing abundance of biological material. The absorption intensity and the absorption profile complexity throughout the infrared fingerprint region (~1500 to 700 cm^{-1}) also increased during the incubation period (Fig. 4) and exhibited the spectral features typical of marine mucilages containing polysaccarides (Berto*, et al.*, 2005) or marine snow (Mecozzi & Pietrantonio, 2006).

The bacterial community composition in the flocs was determined by 16S rRNA gene 454 pyrotag sequencing. The sequencing data revealed that Colwelliaceae were the dominant taxa in the flocs at relative levels of 70% at all time points (Fig 2). The

microbial diversity was also considerably lower in the flocs compared to the bulk seawater (Fig. 2). Another group that was detected in the flocs corresponded to Methylococcaceae that increased in relative abundance from less than 1% at 10 days of incubation to 16% after 40 days of incubation. All of the bacteria that were detected in the flocs were also detected in the bulk water, but their relative amounts differed in the two sample types, suggesting that only certain members of the community were associated with the flocs and their formation.

Isolation of hydrocarbon degrading Colwellia.

Because of the dominance of Colwelliaceae in the enrichments and in the flocs, a representative strain was isolated in order to determine whether it was capable of oil degradation. Subsamples were collected from one of the enrichments on oil and COREXIT at the conclusion of the 20-day incubation period and streaked onto Marine Broth agar plates. After incubation at 5°C for one week, individual, smooth, beige colonies appeared on the plates. SEM imaging revealed that the cells were slightly curved rods (Fig. 6). The cells were approximately 1.5-2 μm long and approximately 0.25-0.4 μm wide. 16S rRNA gene sequencing revealed that the isolate was a *Colwellia* species (strain RC25) with 98.6% sequence homology to the most abundant *Colwellia* spp. observed by 16S pyrosequencing in the original enrichments. The 16S rRNA gene sequence of *Colwellia* sp. RC25 had 96% sequence similarity to the type strain, *Colwellia psychretryhraea* 34H, isolated by Methé *et al*. (2005). Cells of strain RC25 were gram negative, non-spore forming, appeared motile and grew at 5°C.

The isolate was transferred to liquid medium containing 100 ppm MC252 oil and 60 ppm COREXIT. After 10 days of incubation, approximately 75% of the initial amount of MC252 oil was degraded by *Colwellia* strain RC25 (Fig. 7), demonstrating that this organism is a likely candidate for the observed hydrocarbon degradation in the enrichments.

Discussion

As a result of millions of years of natural oil seeps from the seafloor, oil residues in the Gulf of Mexico are a common phenomenon (OSAT, 2010) and hence it is not surprising that the microbial potential for biodegradation of oil compounds is present. Several studies have reported degradation of oil in surface seawater (Gertler*, et al.*, 2009, Kasai*, et al.*, 2002, Zahed*, et al.*, 2010), but to our knowledge no investigations of oil degradation potentials in deep sea environments were reported prior to the Deepwater Horizon oil spill. Although the deep sea is notable due to its low ambient temperature and high pressure, the microbial responses to oil determined from our experiments are similar to those reported for several other environments (Atlas & Hazen, 2011).

Different hydrocarbon fractions of Macondo (MC252) oil in the deepwater plume resulting from the Deepwater Horizon oil spill were previously found to be largely removed by a combination of dispersion and microbial degradation by the indigenous microbes in the deep sea (Atlas & Hazen, 2011, Hazen*, et al.*, 2010, Lu*, et al.*, 2011, Valentine, *et al.,* 2010, Kessler, *et al.,* 2011). Here enrichments were performed with high concentrations of MC252 oil to identify specific members of the indigenous community that could respond to high inputs of hydrocarbons. Uncontaminated seawater collected

from a depth of 1100 mbsl in the Gulf of Mexico during the time of the Deepwater Horizon oil spill was used as a source of inoculum. The oil served as a carbon substrate and induced a substantial microbial bloom over a short 20-day incubation period. When COREXIT 9500 was added, more of the oil was in solution and approximately twice as much was potentially available for degradation by bacteria in the seawater. The hydrocarbon components of the dispersant, COREXIT 9500, were also mineralized and served as a carbon source for microbial growth.

Amendment with limiting nutrients, nitrogen and phosphorous, has sometimes been used to increase the rate of degradation in previous oil spills (Bragg*, et al.*, 1994). However, the Gulf samples were replete in N and P, although low in Fe (Hazen*, et al.*, 2010). A positive impact of iron addition on biodegradation rates of oil has been reported previously in habitats with iron limiting conditions (Teralmoto*, et al.*, 2009, Wang*, et al.*, 2010). When we added $FeCl_2$ to the enrichments, we observed an initial increase in degradation and an increase in microbial growth rates, but after 20 days of incubation no difference in terms of amount of oil degraded was detected. Another effect of the addition of $FeCl_2$ was the absence of floc formation. Therefore, although Fe concentrations were low the natural microbial community still had the capacity to grow and degrade the hydrocarbons without Fe supplementation.

In agreement with this enrichment study, flocs were also observed in The Gulf of Mexico *in situ* after substantial amounts of oil had been degraded following the Deepwater Horizon oil spill (Hazen *et al.*, 2010). To our knowledge no such floc formation has previously been reported in association with oil degradation. However, marine flocs, also known as marine snow, are otherwise a common phenomenon in marine ecosystems (Azam & Malfatti, 2007), where they represent hot-spots for nutrients and thereby microbial activity (Azam & Long, 2001). We found that cells began to

aggregate immediately after addition of oil to the water (Fig. 3a) and our SR-FTIR data suggest that the initial floc formation occurred on the surface of oil droplets (Fig. 5c). Multiple aggregates formed within days, but with time they merged together into one large floc. Eventually, the flocs were comprised of a complex structure of EPS, protein, oil, and oil degradation products and bacteria. A model of proposed steps in the floc formation based on our cumulative data is shown in figure 5.

Interestingly, the flocs were primarily comprised of a select group of bacteria that were enriched from the original source water, dominated by an OTU with closest match to *Colwellia.* Members of the *Colwelliaceae* have previously been shown to produce EPS under extreme conditions (i.e. low temperature, high pressure, and low salinity) (Marx*, et al.*, 2009), and some species contain genes for enhanced survival in cold environments (Methé *et al.*, 2005). Species of *Methylococcaceae* and *Rhodobacteriaceae* were also found in the flocs and these have representatives that have previously been associated with degradation of hydrocarbons (Redmond*, et al.*, 2010, Brakstad & Lodeng, 2005, Coulon*, et al.*, 2007, McKew*, et al.*, 2007). We propose that the ability of some members of the community, to produce EPS and aggregate into flocs was a key physiological mechanism for the oil degrading community to aggregate together with oil droplets and to conserve nutrients.

The same *Colwellia* sequence that we found in the flocs was also enriched in the water phase during the incubations. Valentine *et al*. (2010) also reported that *Colwellia* sequences dominated near-well plume samples during the Deepwater Horizon spill, and Redmond & Valentine (2011) showed that *Colwellia* sequences were particularly enriched at low temperature (4 °C) compared to a higher temperature (20 °C). Using

stable isotope probing (SIP) *Colwellia* sequences were found to be the most abundant sequences in heavy DNA from incubations with ethane, propane and benzene, suggesting that they had the capability to oxidize these compounds (Redmond & Valentine, 2011). An OTU with closest match to *Oleispira*, a member of the Oceanospirillales previously suggested to be involved in hydrocarbon degradation (Yakimov et al., 2003), was also initially enriched in the presence of oil. Subsequently, *Colwellia* sequences increased in relative amounts, similar to the succession pattern observed *in situ* during the Deepwater Horizon oil spill (Hazen et al., 2010, Valentine et al., 2010, Redmond & Valentine, 2011). One hypothesis is that there was a natural succession of the indigenous deep-sea microbiota over time in the deep-sea oil plume, depending on the hydrocarbon fractions available to the microbial community, as also recently suggested by Redmond & Valentine (2011). Collectively, these findings suggest that representatives of the Collwelliaceae and Oceanospiralleles play a predominant role in hydrocarbon degradation in the deep sea. In particular, *Colwellia* could have a competitive advantage in the presence of high concentrations of oil and dispersant due to their ability to produce EPS and form flocs.

In order to better understand the degradation capability and physiology of *Colwellia* in the enrichments, we successfully obtained an isolate (*Colwellia* sp. strain RC25) and demonstrated that it is able to degrade hydrocarbons in crude oil at low temperature. A psychrotrophic lifestyle is apparently a key feature of the *Colwellia* genus. For example, *Colwellia* sequences have also been detected in oil contaminated sea ice (Brakstad*, et al.*, 2008) and the type species of the genus (*C. psychrerythraea* 34H) was isolated from arctic marine sediments (Huston et al., 2000). *C. psychrerythraea* 34H

was genome sequenced, revealing several features that may contribute towards a psychrophilic lifestyle (Methé *et al.*, 2005). Interestingly, the *C. psychretryhraea* 34H genome included genes for production of EPS that were postulated to be important for biofilm formation. In our study, SR-FTIR spectromicroscopy revealed that EPS were abundant in the flocs that were dominated by *Colwellia*. Therefore, it would be interesting to determine if the genes for this process are similarly found in strain RC25 in future studies. In addition the sequenced genome of *C. psychretryhraea* 34H suggest the presence of putative dioxygenases and monooxygenases critical to ring cleavage and aliphatic compound degradation (Methé *et al.*, 2005). Together with our results, this suggests that *Colwellia* have potential for degradation of hydrocarbons in cold marine habitats.

Here we also provide the first data regarding COREXIT 9500 degradation by indigenous microbes from the Gulf, although other laboratories (Garcia*, et al.*, 2009) have done studies on biodegradability of comparable dispersant agents. *In situ* evidence suggests a slow degradation of DOSS at plume depth after the Deepwater Horizon oil spill (Kujawinski*, et al.*, 2011). We found that both DOSS and the glycol compounds were degraded more slowly than the hydrocarbon fraction in COREXIT 9500. Although none of the glycol compounds were detected *in-situ* in the Gulf during surveillance cruises (OSAT, 2010), they are designed to be easily soluble in water and could have become so diluted that their concentrations were below the detection limit. One serious concern for the massive use of COREXIT 9500 during the MC252 oil spill was its unknown toxic effects, but in our enrichments we found no negative effects of high amounts of COREXIT on growth of indigenous microorganisms from the site.

In conclusion we demonstrated a high potential for microbial degradation of oil in the deep sea of the Gulf of Mexico. The bacterial communities adapted rapidly to the introduction of hydrocarbons with sequences representative of first *Oleispira*, then *Colwellia* becoming dominant. These data help to explain how the indigenous microbial community in the Gulf evolves when exposed to high inputs of hydrocarbons. The combination of 16S sequencing and the SR-FTIR approach that we used enabled us to monitor the evolution of flocs during exposure to high concentrations of oil for the first time; the ecological significance of which remains to be investigated.

Experimental procedures

Water, oil, and COREXIT 9500 collection

Uncontaminated water was collected from the Gulf of Mexico on June 6, 2010 during the active phase of the Deepwater Horizon oil spill on a cruise aboard the R/V Ocean Veritas as described in Hazen et al. (2010). Water collected for this study was sampled along with the general monitoring done by the ship using a CTD sampling rosette (Sea-Bird Electronics Inc., Bellevue, WA). The exact position for the sampling was 28.6746ºN, 88.3298ºW at 1100 mbsl. No oil was detected at this position *in situ* using a dual colored dissolved organic matter (CDOM) WETstar fluorometer (WET Labs, Philomath, OR). Temperature at the depth of the sampling site was 4.8°C, dissolved oxygen (DO) was 6.07 mg/L, and Fe was below the detection limit (<50 ppb). The water was stored and shipped in the dark at 4°C to Lawrence Berkeley National Laboratory (LBNL, CA, USA) in the dark.

Macondo (MC252) oil was sampled on May 22, 2010 directly from the Discovery Enterprise drillship located above the wellhead during the Deepwater Horizon oil spill. A detailed analysis of the oil composition is given in Hazen *et al.* (2010). COREXIT 9500 (Nalco, Sugar Land, TX), the dispersant used during the oil spill, was kindly provided by Thomas Azwell (UC Berkeley).

Enrichment setup

Enrichments were established in 125 ml serum bottles with 100 ml of uncontaminated water collected from the Gulf as described above, together with combinations of 100 mg/L MC252 oil, 60 mg/L COREXIT 9500, and 0.1 mM $FeCl_2$. Five different conditions were investigated; oil, oil+COREXIT, COREXIT, oil+$FeCl_2$, and oil+COREXIT+$FeCl_2$. Controls included water alone, water sterilized with 10 mM NaN_3 and water sterilized with 10 mM NaN_3 and supplemented with 100 mg/L oil and 0.1 mM $FeCl_2$. Additional enrichments were set up with 100 ml uncontaminated water from the Gulf and 100 mg/L oil in order to study floc formation. Bottles were sealed with Teflon coated rubber stoppers and attached to a Micro-oxymax respirometer (Columbus Instruments, Ohio, USA) to measure CO_2 evolution. The respirometer attachment had a closed loop that cycled the headspace with periodic measurement of CO_2 evolution every second hour. The total headspace in the bottles was 150 ml, including the bottle headspace and headspace in the tubing of the respirometer, enough to maintain aerobic conditions throughout the experiment. As CO_2 is present in seawater as carbonate, the numbers obtained from the respirometer were corrected by multiplying the measured concentrations by 2.3. This multiplication factor was determined in a separate experiment

where we investigated the CO_2 uptake of sterile seawater at 5°C and found that the ratio between CO_2 dissolved in seawater (including total inorganic carbon) and CO_2 in the headspace was 2.3 (For further information see supporting information, Figure S1).

Because of the need to destructively sacrifice samples for hydrocarbon and DNA extraction, as described below, each enrichment was set up with three replicates (n=3) for sampling points 0, 5, and 20 days and after 2, 5, 10, 20, and 50 days of incubation for the set of enrichments used to study floc formation. In addition, at each sampling point cells were enumerated using a standard acridine orange staining protocol (Francisc*, et al.*, 1973) and imaged with a FITC filter on a Zeiss Axioskop (Carl Zeiss, Inc., Germany). The enrichments were incubated at 5ºC in the dark. To verify that aerobic conditions were present throughout the incubation period, bottles were opened in an anaerobic chamber and dissolved oxygen was measured using the AccuVac colorimetric method (HACH, Loveland, USA).

Microbial Isolation and Identification

Subsamples collected from one of the enrichments with oil and COREXIT after 20 days incubation were streaked onto Marine Broth agar plates. After incubation at 5°C for one week, individual colonies appeared on the plates. Colonies were subsequently individually transferred to modified minimal marine medium (Coates et al., 1995), supplemented with 100 ppm MC252 oil, 60 ppm COREXIT and 1g/L bactopeptone. The ability of the cultures to degrade hydrocarbons was determined as described below.

For 16S rRNA based identification of the isolate, DNA was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio Inc, Carlsbad,CA). PCR amplification was carried out using universal bacterial 16S rRNA gene primers 1492R and 27F in 50ul reactions. Verified 16S amplicons were purified using the procedure provided in the MoBio Ultraclean PCR Clean-up kit (MoBio, Carlsbad, CA). Samples were submitted to the UC Berkeley DNA Sequencing Facility for 16S rRNA sequencing using the Applied Biosystems BigDye Terminator V3.1 Cycle Sequencing protocol and ABI Sequencing Analysis Software Version 5.1.

Scanning Electron Microscopy (SEM) was performed on 0.2 micron pore size Millipore filters (Millipore, Billerica, MA 01821, USA). A 1.0 ml aliquot of the suspended sample solution was pushed through the Millipore filters using a syringe. The filters were then washed several times with 0.1M sodium cacodylate buffer (pH 7.4) to remove excess material followed by on-ice fixation with 2% glutaraldehyde for 1 hour, and on-ice post-fixation with 1% OsO_4 for 1 hour. Fixation was followed by dehydration with a graded ethanol series (20%, 40%, 50%, 70%, 90%, 100%, 100%), critical point drying using a Tousimis AutoSamdri 815 Critical Point Dryer (Tousimis, Rockville, MD 20851, USA), and sputter coating with gold-palladium using a Tousimis Sputter Coater (Tousimis, Rockville, MD 20851, USA). Images were collected using a Hitachi S5000 Scanning Electron Microscope (Hitachi High Technologies America Inc, Pleasanton, CA 94588, USA).

Hydrocarbon measurements

Samples were taken from the enrichments for hydrocarbon measurements after 0, 1, 5, and 20 days of incubation. To determine hydrocarbon concentrations derived from the presence of oil in the samples, 500 µL of chloroform was added to a 5 ml water sample and mixed by vortexing. The chloroform fraction was removed and the water re-extracted 2 additional times and the extracts combined. The extract was dried over anhydrous sodium sulfate and analyzed without further concentration. Hydrocarbon concentrations were measured using a GC-FID (Agilent, Santa Clara, USA) enabling individual quantification of the majority of the crude oil constituents as previously described (Hazen*, et al.*, 2010). Selected samples were run on a GC/MSD (Agilent, Santa Clara, USA) for compound identification. Results are reported as Total Petroleum Hydrocarbons (TPH) and quantification was accomplished by comparison to known concentrations of diluted MC252 oil standards. In the samples containing the COREXIT dispersant, 26 compounds were grouped into hydrocarbon, glycol, and DOSS fractions and assessed.

To test the hydrocarbon degradation potential of *Colwellia* sp. strain RC25, a 10% inoculum of washed cells was incubated at 5°C in serum bottles containing 50 ml of modified minimal marine medium with 100 ppm MC252 oil and 60 ppm COREXIT. To ensure aerobic conditions, 5 ml of air were injected into the serum bottles at regular intervals during the incubation period. At 3, 6, 10, 14, 21, 28 and 37 days of incubation, triplicate samples were harvested for hydrocarbon analysis as detailed above, with the following modifications; (1) the entire 50 ml volume was sacrificed for each analysis and

(2) the bottle was washed three times with chloroform and these washes were added to the analyses.

DNA extraction and sequencing of enrichments and flocs

Subsamples were taken from the enrichments before the start of the experiment (Day 0), and after 5 and 20 days of incubation. 80 ml water (including visible flocs when present) were split into two 50 ml tubes and centrifuged at 18,000 ×g for 15 min. The supernatant was filtered through a 0.22 µm Sterivex filter (MilliPore, Billerica, USA). Filters and pellets were stored at -80°C until extracted. When sampling floc material alone, intact flocs were carefully removed from the water with a pipette. The flocs were carefully rinsed three times in sterile filtered milliQ water before transferring to a microcentrifuge tube for DNA extraction.

DNA was extracted as described in Hazen *et al.* (2010) with the following modifications: Cell pellets were dissolved in extraction buffer and transferred along with the entire filter to a 1.5 ml PULSE tube (Pressure BioSciences, South Easton, USA). The PULSE tube was placed in a Barocycler NEP 3229 (Pressure BioSciences, South Easton, USA) and the cells were lysed using pressure cycling with 20 cycles of 20 sec at 35,000 psi and 10 sec at atmospheric pressure. After pressure lysis the entire solution and filter were transferred to the Lysing Matrix E tube (MP Biomedicals, Solon, USA).

16S rRNA gene sequences were amplified from the DNA extracts using the primer pair 926f/1392r as described in Engelbrektson *et al.* (2010), with an additional wobble added to the 926F primer to improve coverage of the archaea (5’ - cct atc ccc tgt gtg cct tgg cag tct cag aaa ctY aaa Kga att gRc gg – 3’, including titanium adapter

sequence). The reverse primer included a 5 bp barcode for multiplexing of samples during sequencing. Sequencing of the PCR amplicons was performed at DOE's Joint Genome Institute (JGI) or at the GeneChip™ Microarray Core (San Diego, USA) using Roche 454 GS FLX Titanium technology, with the exception that the final dilution was $1e^{-8}$ (Allgaier, *et al.*, 2010). The raw sequence reads and quality files were deposited into the NCBI sequencing read archive under project number SRA049463. The 16S rRNA gene sequence of *Colwellia* strain RC25 has been deposited to NCBI GenBank with accession number JQ627834.

Community Profiling

Pyrotag sequences were analyzed using the QIIME pipeline (Caporaso, *et al.*, 2010). Briefly, 16S rRNA gene sequences were clustered with uclust (Edgar, 2010) and assigned to operational taxonomic units (OTUs) with 97% similarity. Representative sequences from each OTU were aligned with Pynast (Caporaso, *et al.*, 2010) using the Greengenes core set. Taxonomy was assigned using the Ribosomal Database Project's Naïve Bayesian classifier (Wang, *et al.*, 2007) with a confidence of 80%.

SR-FTIR spectromicroscopy of flocs

Synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy in the mid infrared region (~4000–650 cm^{-1} wavenumber) was used to detect petroleum products, petroleum degradation products, as well as macromolecules of biological origin as previously described (Hazen, *et al.*, 2010, Holman, *et al.*, 2010).

The advantage of this approach is that it has a signal-to-noise ratio 100-1000 times better than conventional FTIR approaches (Holman*, et al.*, 2010). For the present study, SR-FTIR analyses were conducted on fresh flocs, which were gently rinsed with sterile filtered, deionized water in order to minimize interference from molecules in the seawater. The floc was placed onto an infrared transparent ZnSe disc prior to imaging. For each SR-FTIR imaging measurement, the entire view-field of the floc was divided into equal-sized 5-μm×5-μm squares before scanning. Photons were focused through the floc using a Nicolet Nic-Plan IR microscope (with a numerical aperture objective of 0.65), which was coupled to a Nicolet Magna 760 FTIR bench (Thermo Scientific Inc., MA, USA). The SR-FTIR transmission spectra at each position were collected using a single-element MCT (mercury-cadmium-telluride) detector at a spectral resolution of 4 cm^{-1} with 8 co-added scans and a peak position accuracy of 1/100 cm^{-1}. Background spectra were acquired from locations without any floc material and were used as reference spectra for both samples and standards to remove background H_2O and CO_2 absorptions. A data cube of position-associated infrared spectra was obtained following each SR-FTIR data acquisition experiment. This data cube was then subjected to an array of data processing calculations using both Matlab and Thermo Electron's Omnic version 7.3, which included the computational conversion of each transmission to absorption spectrum and baseline removal. Spectral absorption peaks, which could be linked to target molecules in the flocs, were integrated. The relative concentration of a particular chemical component is presented as absorbance units (a.u.).

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Supporting information available

Detailed information on the CO_2 dissociation experiment is available in supporting information. Furthermore two figures (Figure S1 and S2) are available showing CO_2 dissolution/dissociation in seawater and chemical mass balances.

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Figure 1. Enrichments of deep-sea bacteria collected from the Gulf of Mexico during the Deepwater Horizon oil spill to high concentrations of Macondo (MC252) crude oil (100 ppm) and the dispersant, COREXIT 9500 (60 ppm). (a) Measurement of CO_2 evolution (microbial respiration); (b) degradation of MC252 oil; (c) degradation of components in COREXIT 9500; (d) microbial cell counts. Treatments were as follows: –○– seawater control, –□– sterile seawater control, –△– sterile seawater with oil and $FeCl_2$ added, –■– oil alone, –▲– oil+COREXIT, –▼– COREXIT, –◆– Oil+Fe, –★– Oil+COREXIT+Fe, --▼-- hydrocarbon fraction in COREXIT 9500, ···●··· glycol fraction in COREXIT 9500, and -·■··- dioctylsulfocussinate (DOSS) fraction in COREXIT 9500. ⬇ indicates the time points that triplicate enrichments were sacrificed during the incubations. Error bars represent standard error of three replicate enrichments.

Figure 2. Relative abundance of different phylogenetic groups based on operational taxonomic units (OTUs) obtained by pyrotag sequencing of amplified 16S rRNA genes. Each bar represents the mean of 3 replicate enrichments. C = COREXIT.

Figure 3. Microscopic images of cell aggregation and floc formation. Panels (a) and (b) are microscopic images of cells collected on 0.2 μm filters and stained with acridine orange: (a) cells that are aggregating in the presence of oil alone after 2 days incubation, (b) lack of cell aggregation in the presence of oil and $FeCl_2$ after 2 days of incubation. (c) A microscopic image of a portion of a floc collected from an enrichment after 40 days of incubation with oil.

Figure 4. SR-FTIR spectra of floc material collected after 0 (no floc material), 5, 10, 20, and 40 days of incubation with 100 mg L^{-1} MC252 oil. Tentative band assignment of the protein amide I and II vibration modes at ~1648 and ~1542 cm^{-1}, of the carbohydrate vibration modes at ~1000 cm^{-1}, of alkane C-H vibration modes in oil from MC252, and of carbonyl (C=O) vibration modes at ~1730 cm^{-1} in oil oxidation products.

Figure 5. SR-FTIR images of the formation and growth of a floc during enrichments with 100 mg L^{-1} MC252 oil at five different time points**.** (a) An illustrative model of the evolution of floc formation over time. Colors in the illustration correspond to the following: yellow, oil; blue, EPS, with associated microbial cells. The scale for the first 3 time points is in tens of μm and for the last 2 time points in hundreds of μm. (b) Microscopic images of a floc at t = 0, 5, 10, 20, and 40 days. (c) Distribution heat map of alkane C-H vibration modes in MC252 oil, of protein amide II vibration modes at ~1542 cm^{-1}, of the carbohydrate/EPS vibration modes at ~1000 cm^{-1}, and of the carbonyl (C=O) vibration modes at ~1730 cm^{-1} in oil oxidation products. The corresponding maximum absorbance intensity (the value given in white) is given in absorbance units (a.u.).

Figure 6. Scanning Electron Microscopic (SEM) image of a single *Colwellia* sp. RC25 cell. The cell is approximately 1.5-2 μm long and approximately 0.25-0.4 μm wide.

Figure 7. Total hydrocarbons recovered from *Colwellia* sp. RC25 incubations with Macondo (MC252) crude oil (100 mg/L) and the dispersant, COREXIT 9500 (60 mg/L)

after aerobic incubation at 5C for 37 days. The data represent the mean of triplicate samples.

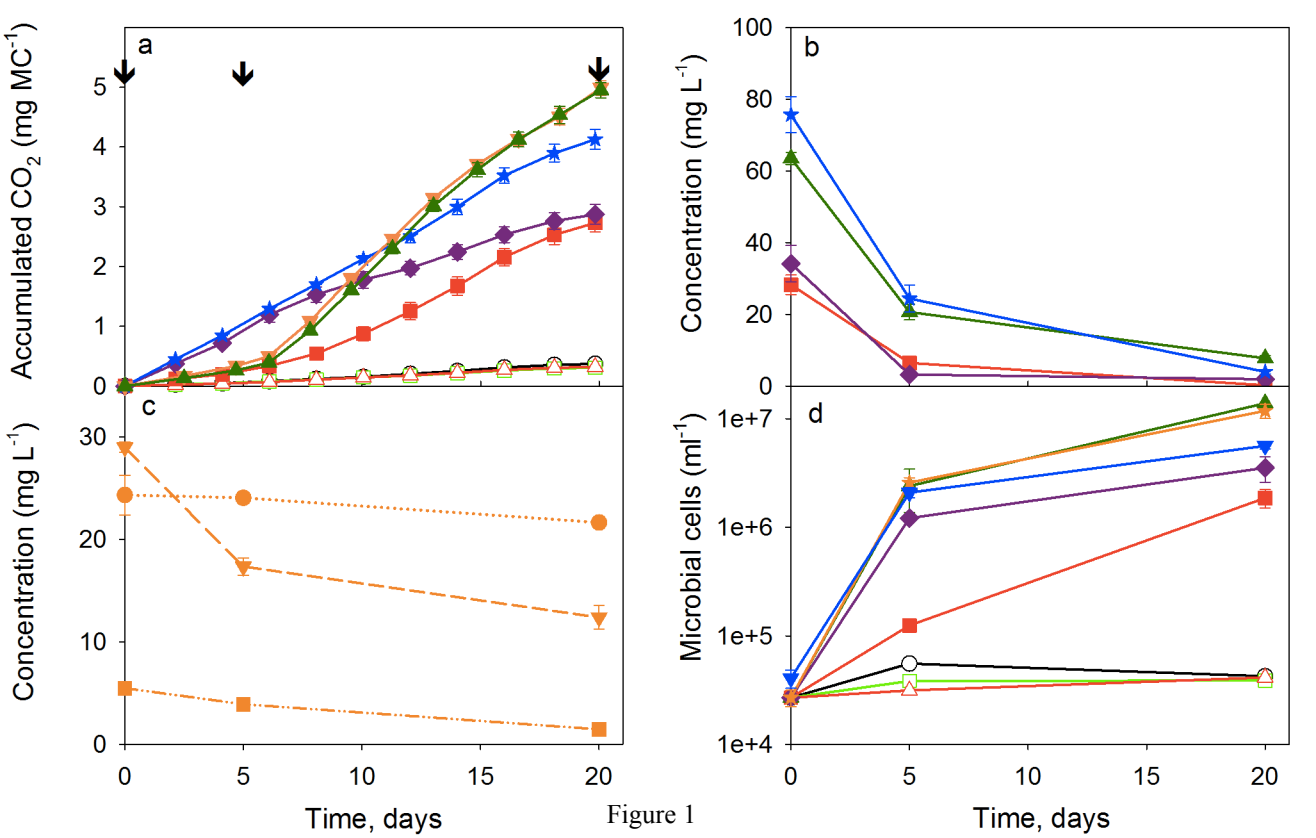
a
b
c
d
Accumulated CO_2 (mg MC^{-1})
Concentration (mg L^{-1})
Concentration (mg L^{-1})
Microbial cells (ml^{-1})
Time, days
Time, days

Figure 1

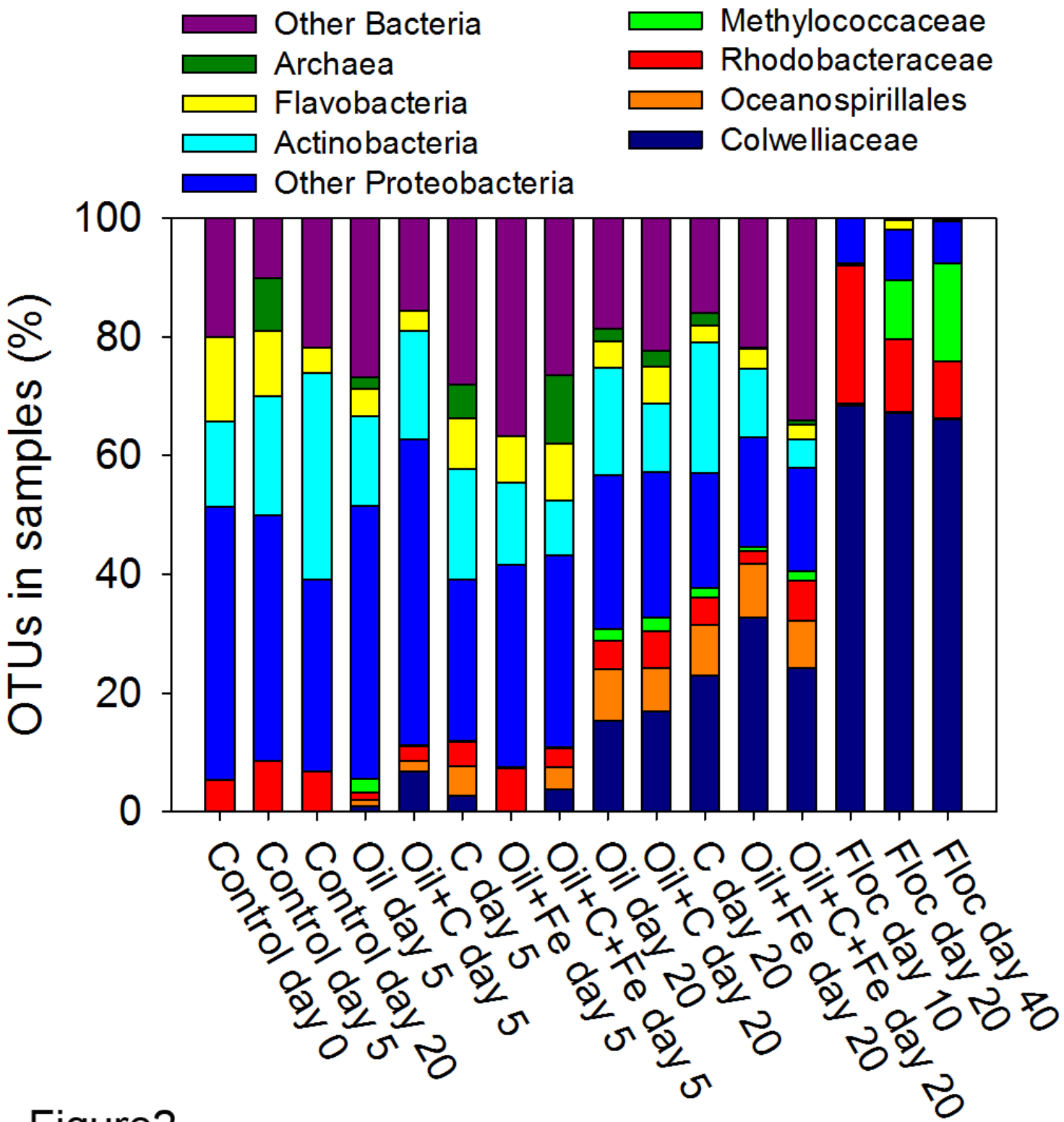
Other Bacteria
Archaea
Flavobacteria
Actinobacteria
Other Proteobacteria
Methylococcaceae
Rhodobacteraceae
Oceanospirillales
Colwelliaceae
OTUs in samples (%)
100
80
60
40
20
0
Control day 0
Control day 5
Control day 20
Oil day 5
Oil+C day 5
C day 5
Oil+Fe day 5
Oil+C+Fe day 5
Oil day 20
Oil+C day 20
C day 20
Oil+Fe day 20
Oil+C+Fe day 20
Floc day 10
Floc day 20
Floc day 40

Figure2

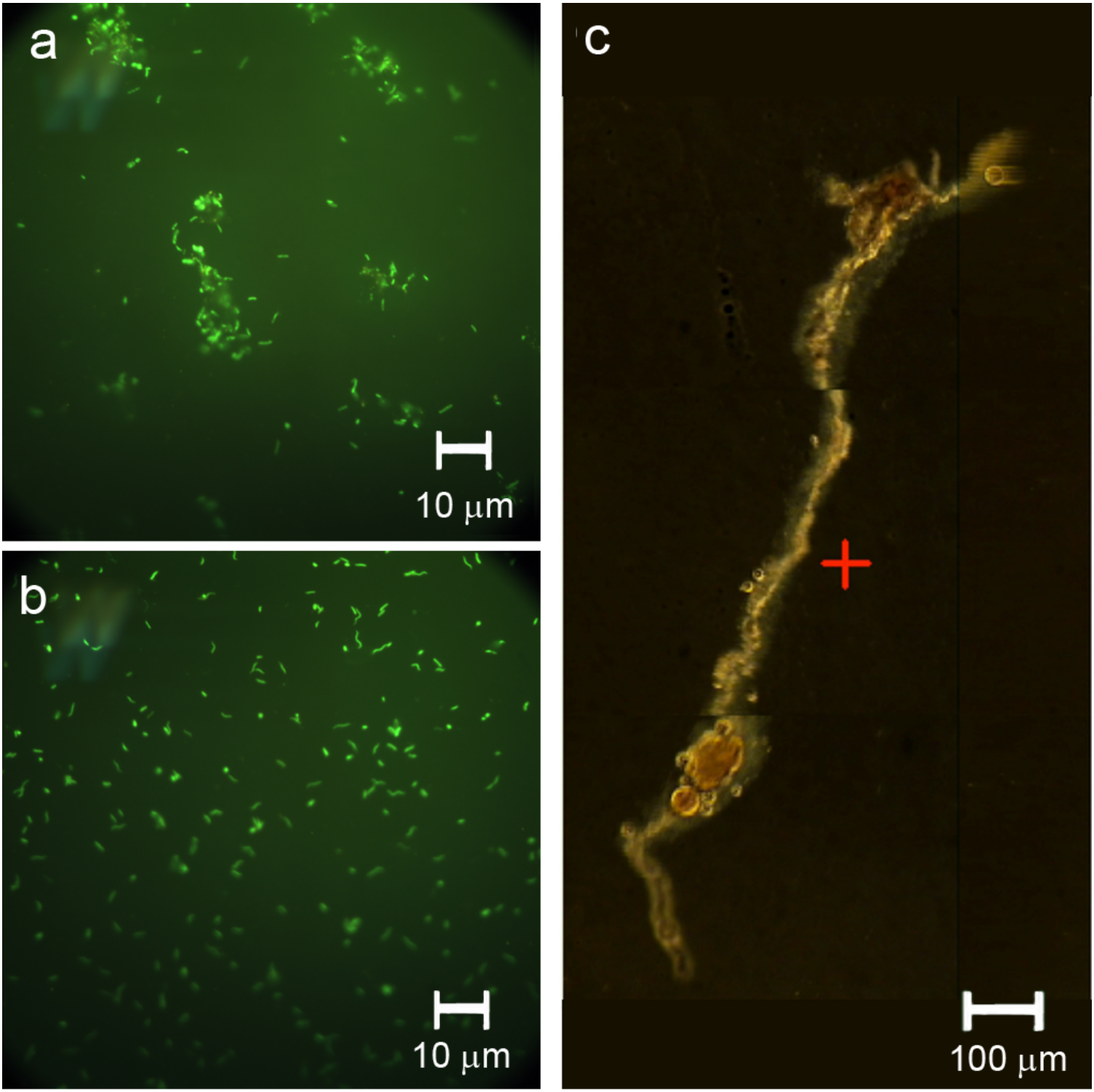

Figure 3

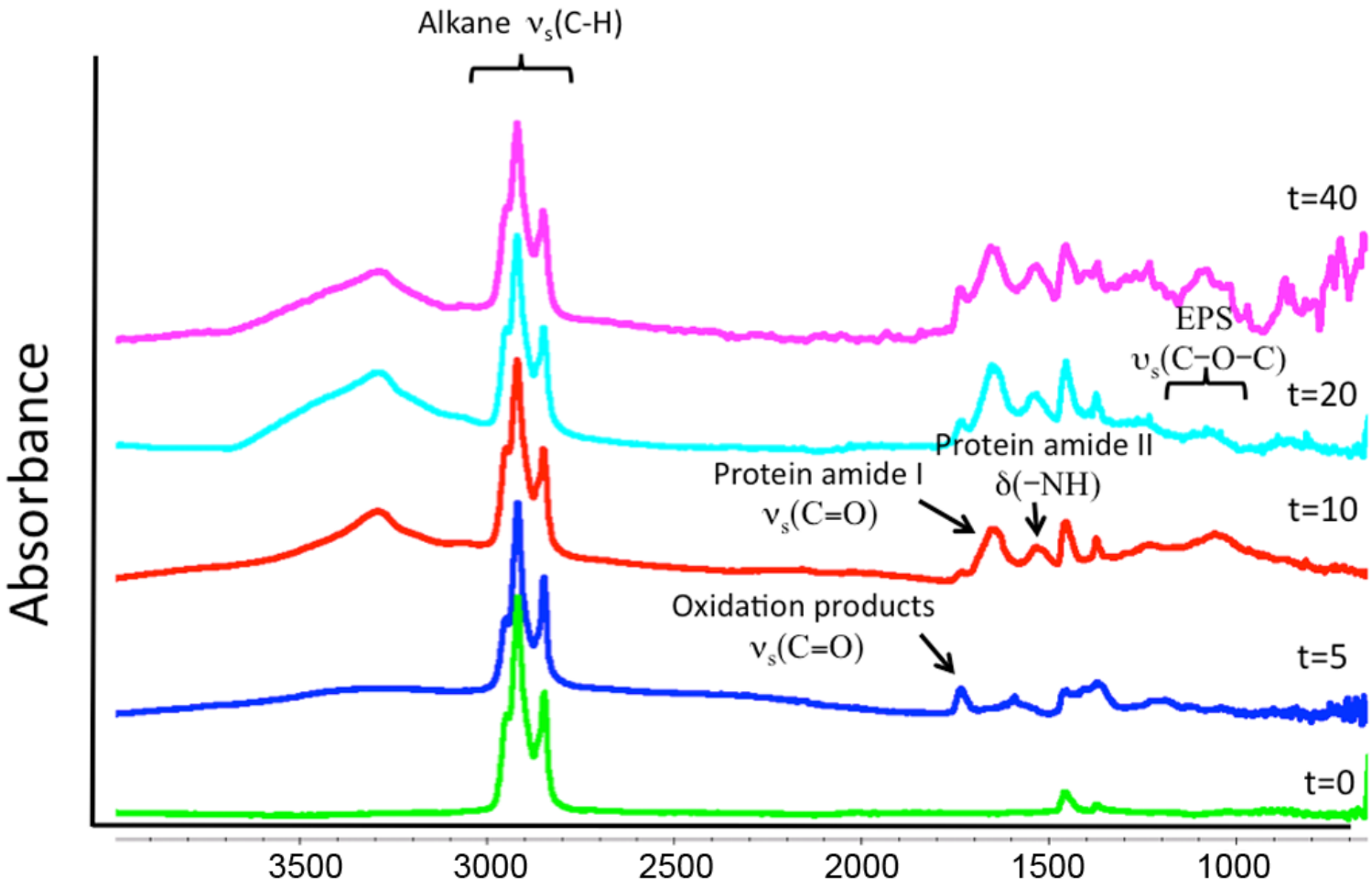
Alkane ν_s(C-H)
t=40
EPS
υ_s(C–O–C)
t=20
Protein amide II
δ(–NH)
Protein amide I
ν_s(C=O)
t=10
Oxidation products
ν_s(C=O)
t=5
t=0
Absorbance
3500
3000
2500
2000
1500
1000
Wavenumber (cm^{-1})

Figure 4

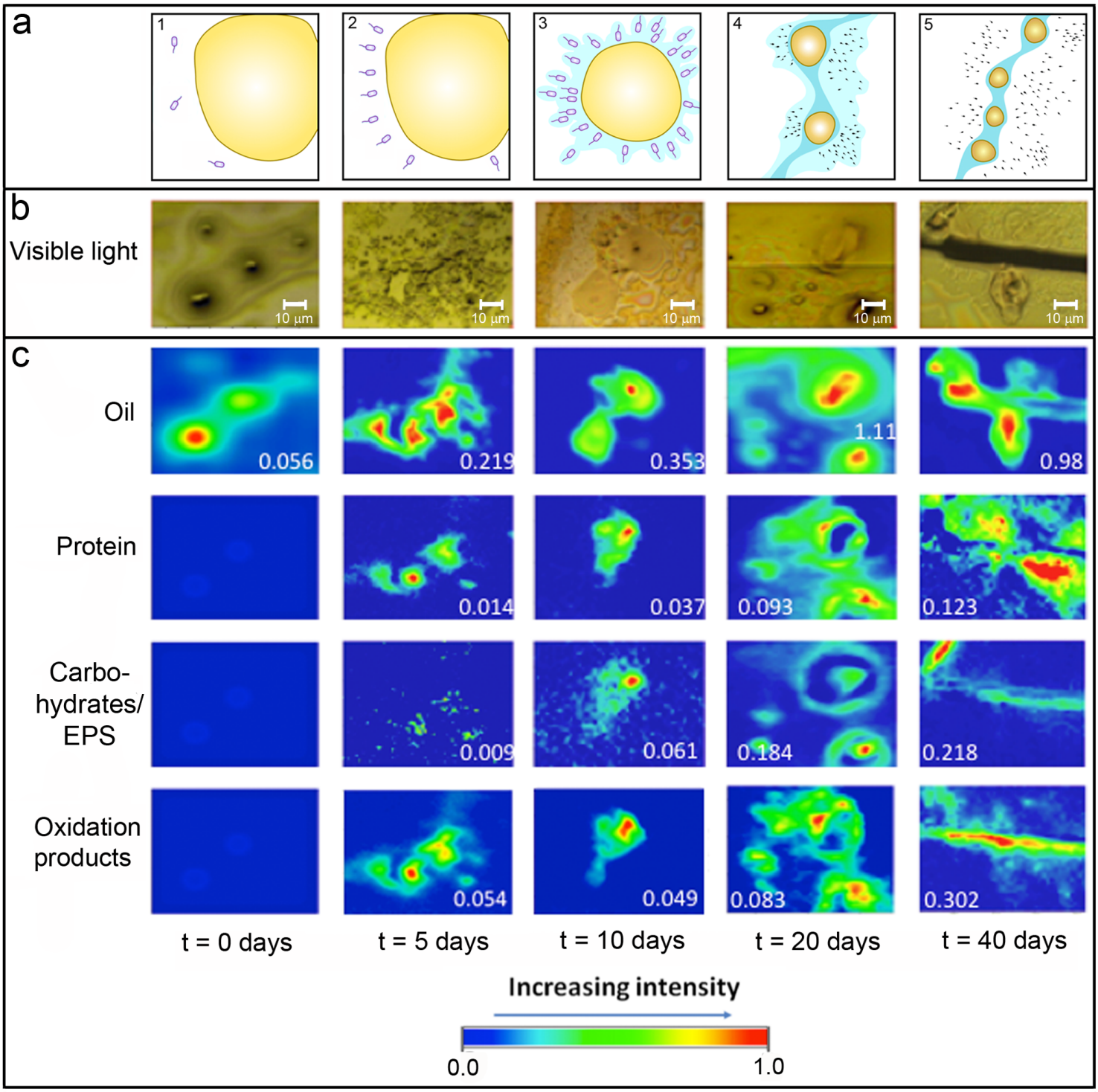
a
1
2
3
4
5
b
Visible light
10 µm
c
Oil
0.056
0.219
0.353
1.11
0.98
Protein
0.014
0.037
0.093
0.123
Carbo-
hydrates/
EPS
0.009
0.061
0.184
0.218
Oxidation
products
0.054
0.049
0.083
0.302
t = 0 days
t = 5 days
t = 10 days
t = 20 days
t = 40 days
Increasing intensity
0.0
1.0

Figure 5

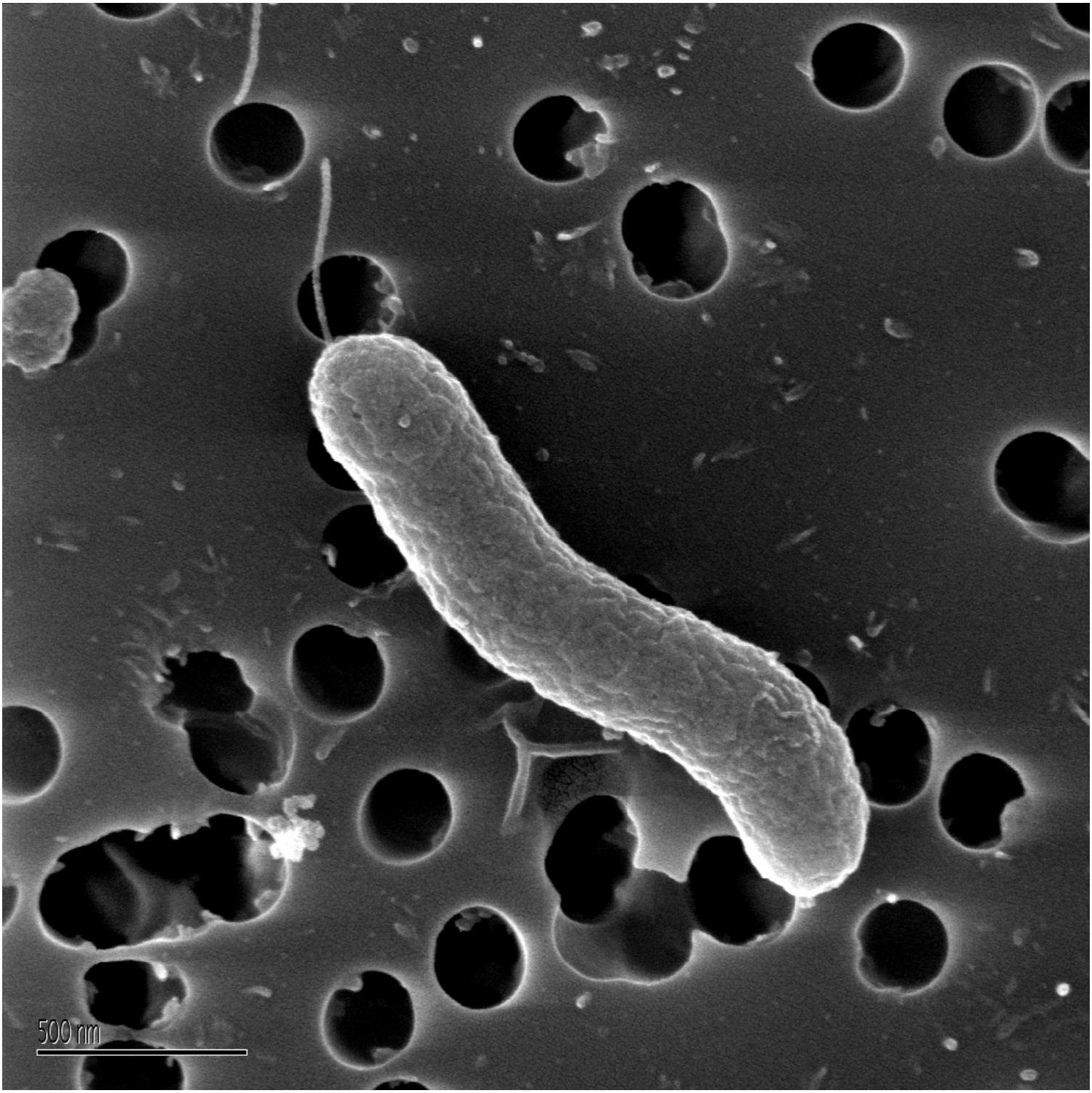

Figure 6

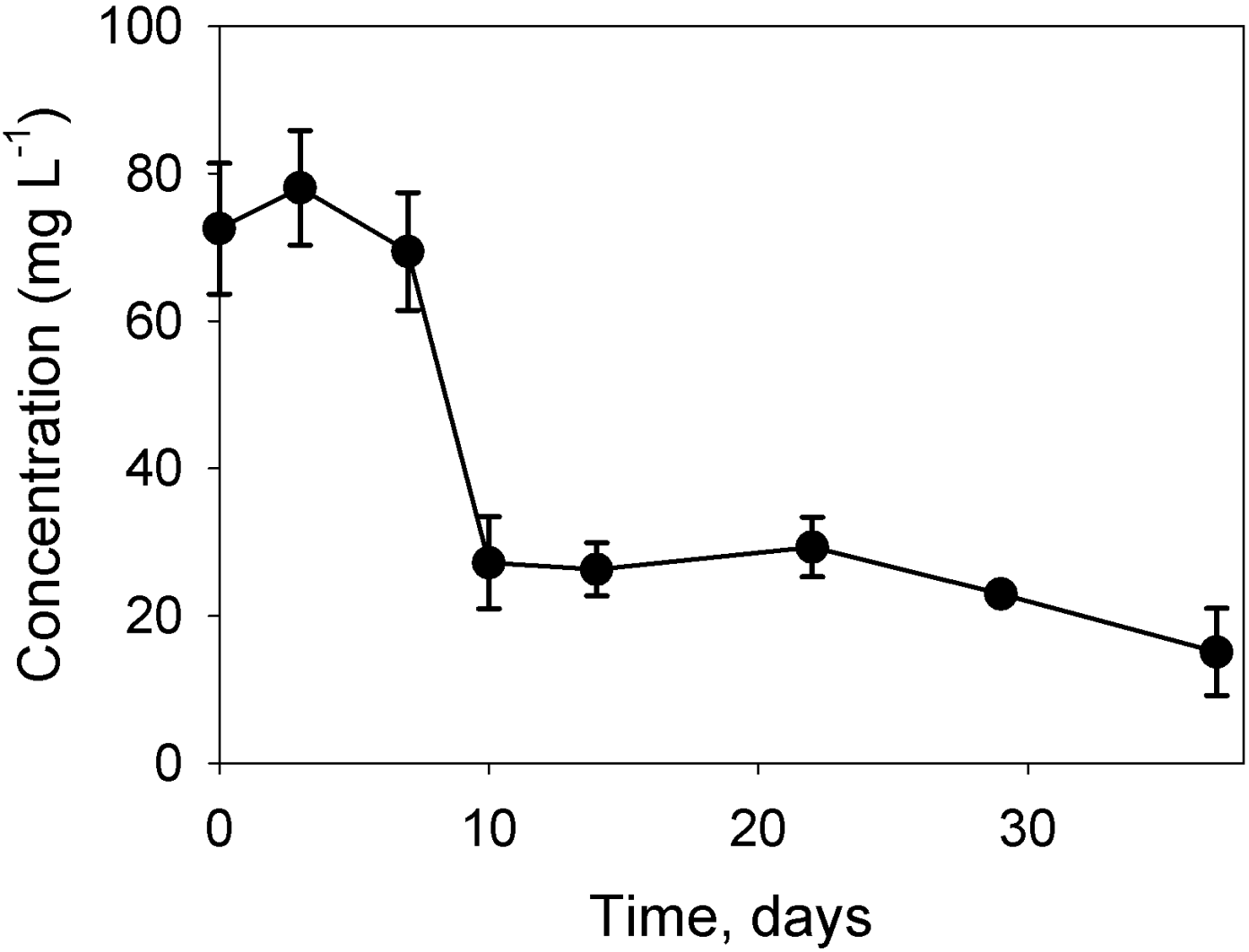

Figure 7