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SUPPLEMENTARY MATERIALS

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Supplementary Text
Figs. S1 to S8
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Movie S1

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OIL BIODEGRADATION

Water droplets in oil are microhabitats for microbial life

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Anaerobic microbial degradation of hydrocarbons, typically occurring at the oil-water transition zone, influences the quality of oil reservoirs. In Pitch Lake, Trinidad and Tobago—the world's largest asphalt lake—we found that microorganisms are metabolically active in minuscule water droplets (1 to 3 microliters) entrapped in oil. Pyrotag sequencing of individual droplet microbiomes revealed complex methanogenic microbial communities actively degrading the oil into a diverse range of metabolites, as shown by nuclear magnetic resonance and Fourier transform ion cyclotron resonance mass spectrometry. High salinity and water-stable isotopes of the droplets indicate a deep subsurface origin. The 13.5% water content and the large surface area of the droplets represent an underestimated potential for biodegradation of oil away from the oil-water transition zone.

Petroleum hydrocarbons are excellent electron donors and carbon sources for microorganisms; therefore, they are readily degraded under oxic conditions. Albeit kinetically slower, anaerobic degradation of petroleum hydrocarbons also occurs with electron acceptors such as sulfate, nitrate, and ferric iron or under methanogenesis (*1*). Methanogenic degradation has been detected for oil reservoirs (*2*, *3*), and although microorganisms are found throughout entire reservoirs (*4*), it is currently understood that the bulk of biodegradation processes are taking place at the oil-water transition zone (*4*, *5*). Because oil wells usually produce pressurized water/oil suspensions containing disturbed microbial communities, such samples provide limited information on the habitat and processes in situ. We hypothesized that microbial life should be possible in the oil body itself, within water enclosures containing active microbial communities.

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We collected undisturbed oil samples from Pitch Lake, the world's largest natural asphalt lake, in La Brea, Trinidad and Tobago (*6*) (fig. S1A). When oil samples were spread on aluminum foil, small bubbles were visible beneath the oil surface. Many bubbles contained gas and collapsed upon puncturing, whereas some contained entrapped water droplets of 1 to 3 μl. Sampling and microscopic inspection of single water droplets showed that they indeed harbored microorganisms, some of which were actively motile under the microscope (fig. S1B). This observation substantiated that microbial life can exist in such microliter-scale water droplets entrained in oil, contrary to a previous hypothesis that the low water activity would impose water stress, making such environments too extreme for microbial life (*7*). However, the water activity in the droplets is much higher than in the surrounding oil. Furthermore, a dissolved ion analysis of bulk water showed that there were no obvious limitations for life caused by the lack of essential nutrients such as ammonia (95 mg/l) or phosphate (5 mg/l).

Bacterial communities of single water droplets and of bulk oil showed a diverse composition (Fig. 1). Pyrotags were dominated by members of the orders Burkholderiales and Enterobacteriales. Other prominent lineages present in the water droplets were the Bacteriodales, Rhodospirillales, Sphingomonadales, and to a lesser extent Thermotogales and Nitrosomonadales. The taxa identified in the water droplets were typical for oil samples (e.g., the Burkholderiales) and largely consistent with an earlier characterization of solid samples from the Trinidad Pitch Lake (*7*) and also from a similar type of oil seep in California (*8*). A recent metagenomic investigation of samples from 10 oil reservoirs also identified similar taxa to be predominant (*9*). The repeated detection of 16*S* ribosomal RNA gene sequences of presumably aerobic, hydrocarbon-degrading populations such as the Burkholderiales in anoxic oil reservoirs might indicate heretofore unrecognized physiological features of these taxa. The sequences clustering within the Enterobacteriales in our study were of mostly unclassified affiliation.

Archaea were detectable in 7 of 12 analyzed droplets and consisted almost exclusively of known methanogens within the Methanosarcinales and Methanomicrobiales. These taxa indicate that acetotrophic and hydrogenotrophic methanogenesis play a role in the biodegradation of the pitch. In addition to these methanogenic archaea, halotolerant or halophilic Halobacteriales were also present in all archaea-positive water droplets, albeit at lower relative abundance. These organisms indicate a high salt origin of the water droplets, presumably from the formation water of the reservoir. The presence of methanogens suggests that methanogenesis was an important terminal electron-accepting process in the water droplets. The emitted gas on Pitch Lake, however, was dominated by thermogenic methane, with only a minor contribution of biogenic origin, as indicated by stable isotope ratios of carbon [−46.6 ± 0.2 per mil (‰)] and hydrogen (−169.4 ± 4.6‰) of methane extracted from the oil and respective reports in the literature (*7*, *10*).

To determine the origin of the water droplets, we analyzed the dissolved ion composition as well as water-stable isotopes of bulk water samples from the oil. Both methods require water samples of >100 μl and were therefore possible for water droplets extracted from the oil but not from single droplets. The water exhibited a near-neutral pH of 7.2 with salt concentrations similar to sea water (500 mM Na^+, 536 mM Cl^-); these findings exclude rainfall or fresh surface water as a direct source of the droplets. Furthermore, surface water cannot disperse into the heavy oil phase without severe mechanical shearing. The elevated salt concentrations rather suggest a subsurface origin of the droplets with either seawater or brine influence, indicating that they must have been entrapped in the oil already in the reservoir or during the ascent.

A deep subsurface origin of the water droplets was also strongly supported by stable isotope analysis of the bulk water phases (Fig. 2). The $^{18}O/^{16}O$ and $^{2}D/^{1}H$ stable isotope ratios of the bulk water separated by gravitation and by

centrifugation were very similar and clearly deviated from the local meteoric water line (LMWL) in a dual isotope plot; these results exclude a direct origin from precipitation or seawater (Fig. 2). Because the enrichment of $\delta^{18}O$ was much larger than that of $\delta^{2}H$, this shift also cannot originate from evaporation of seawater or fresh water (such values can only develop along the local evaporation line; gray shaded area, Fig. 2). Rather, the pronounced enrichment of $\delta^{18}O$ indicates prolonged rock-water interactions of the water under high temperature (*11*), or water from deep sedimentary basins (*12*), resulting in enriched $\delta^{18}O$ values (+6.5 ± 0.1‰ to +7.0 ± 0.1‰) relative to the potential local sources of sea or precipitation water (−1 to 0‰). Thus, both the salinity and stable isotopes of the bulk water samples indicate a deep origin of the water droplets from ancient seawater or brines.

We also analyzed the metabolites of hydrocarbon degradation for bulk oil, droplet water, and water extracts to determine whether the microorganisms in the water droplets were actively degrading the oil (*13*). Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) revealed a rich suite of oxygenated and sulfonated molecules in the water droplets, often as carboxylic acids. Oxygen was the most abundant heteroatom also according to nuclear magnetic resonance (NMR) spectroscopy (Fig. 3 and fig. S6). Carboxylation and other functionalizations of aromatics were reflected in noticeable ^{1}H NMR downfield chemical shift displacements. Progressive oxidation, hydrogenation, and formation of CHOS compounds were deduced by FTICR-MS for more than 100 unsaturated compounds (fig. S7). The majority of these organic molecules reflected transformed oil constituents. Most were single and fused alicyclic rings with diversely branched aliphatic side chains. The overall proportion of unsaturated compounds was around 6%, similar to the oil composition (*14*). Linear aliphatic chains were absent (<1% overall contribution), consistent with a preferred microbial utilization of these more easily biodegradable compounds (*2*). Moreover, olefins (>2% of ^{1}H NMR integral) were present in water droplets but were noticeably absent in the oil itself. The abundance of olefinic protons was one-fourth of the sum of the aromatic protons present, suggesting partial de-aromatization of oil compounds during microbial transformation. This finding corresponds well with known anaerobic polycyclic aromatic hydrocarbon degradation pathways where aromatic ring reduction is a prerequisite for initiating ring cleavage (*13*, *15*, *16*).

Physical extraction of the oil using sterile water showed a fractionation of functionalized, low-molecular weight compounds into the aqueous phase (Fig. 3C and fig. S7). However, the molecular composition of the aqueous extract as observed by FTICR and NMR represented a mere subset of low-molecular mass oil constituents and was clearly distinct from the molecular composition of the droplet water. Hence, biodegradation must have been the major source of the functionalized organics in the water droplets,

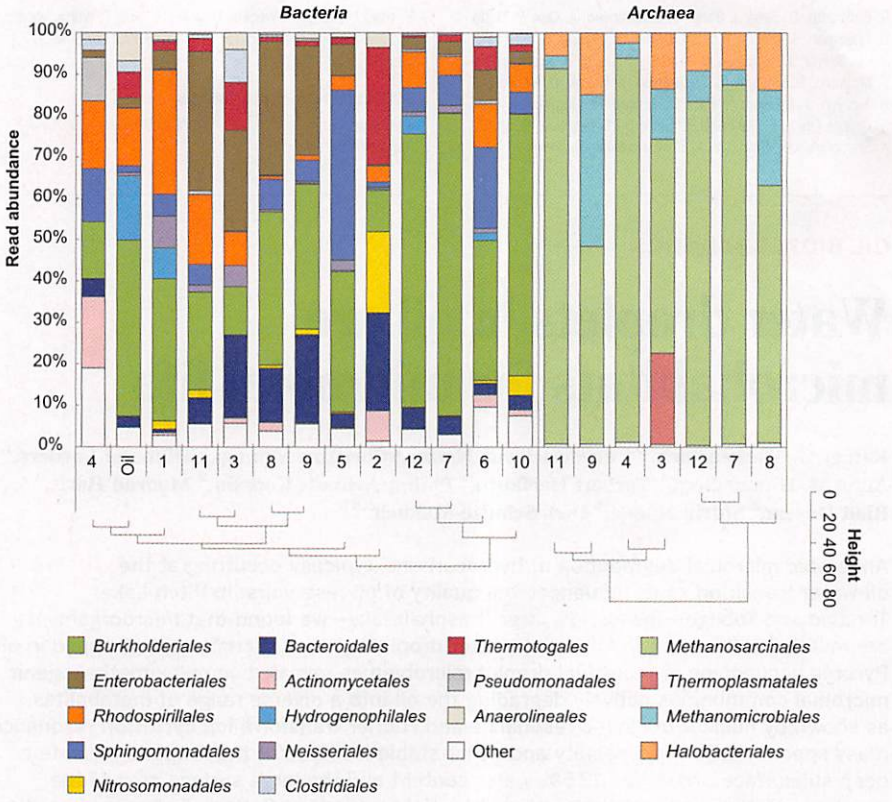

Fig. 1. Microbial community composition of bulk oil and single water droplets. Results of 12 single droplets and bulk-phase DNA extract sequencing of bacterial and archaeal pyrotags are arranged according to compositional clustering. Classified orders below a total abundance of 11% and unclassified taxa are summarized as "Other."

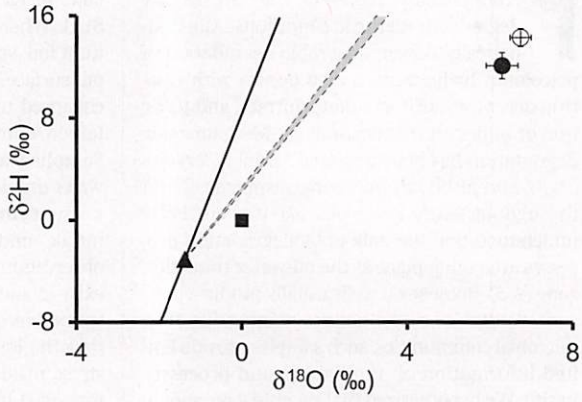

Fig. 2. Stable isotope composition of water droplets. $\delta^{18}O$ and $\delta^{2}H$ (±SD) of bulk water samples from oil separated by gravity (open circle) or by centrifugation (solid circle). Data are compared to seawater (black square), to the local meteoric water line (LMWL, solid line), and to its long-term precipitation average (black triangle) from the closest station of the Global Network of Isotopes in Precipitation (GNIP) database of the International Atomic Energy Agency (IAEA) in Barbados. The local evaporation line (LEL) is depicted by the gray shaded area.

indicating that the microorganisms were not only entrapped in the water droplets but were also actively degrading the engulfing oil.

Our observation that water droplets sustain an actively degrading microbial population in a hydrocarbon-saturated environment has implications for assessing biodegradation of oil reservoirs. In addition to near the oil-water transition zone, water is present not only as thin films surrounding the sediment grains, but also—and especially in poorly sorted facies of clastic reservoirs—in zones of high water saturation where capillary entry pressures are high. Oil-water contacts are also gradational, reflecting saturation heterogeneity in the reservoir that probably extends down to tens of micrometer-scale pore assemblages (*17*). The latter would certainly leave room for water droplets as observed in our study, although in a different geometry and imbedded in the sandstone matrix. Even if the droplets revealed in our study may have actually entered the bulk oil phase during the ascent to Pitch Lake, they still provide a proxy for processes in small water-filled cavities in the oil-saturated parts of the reservoir, distant from the oil-water transition zone. Assuming that most of the 13% water content could be dispersed in such small aqueous microcompartments rather

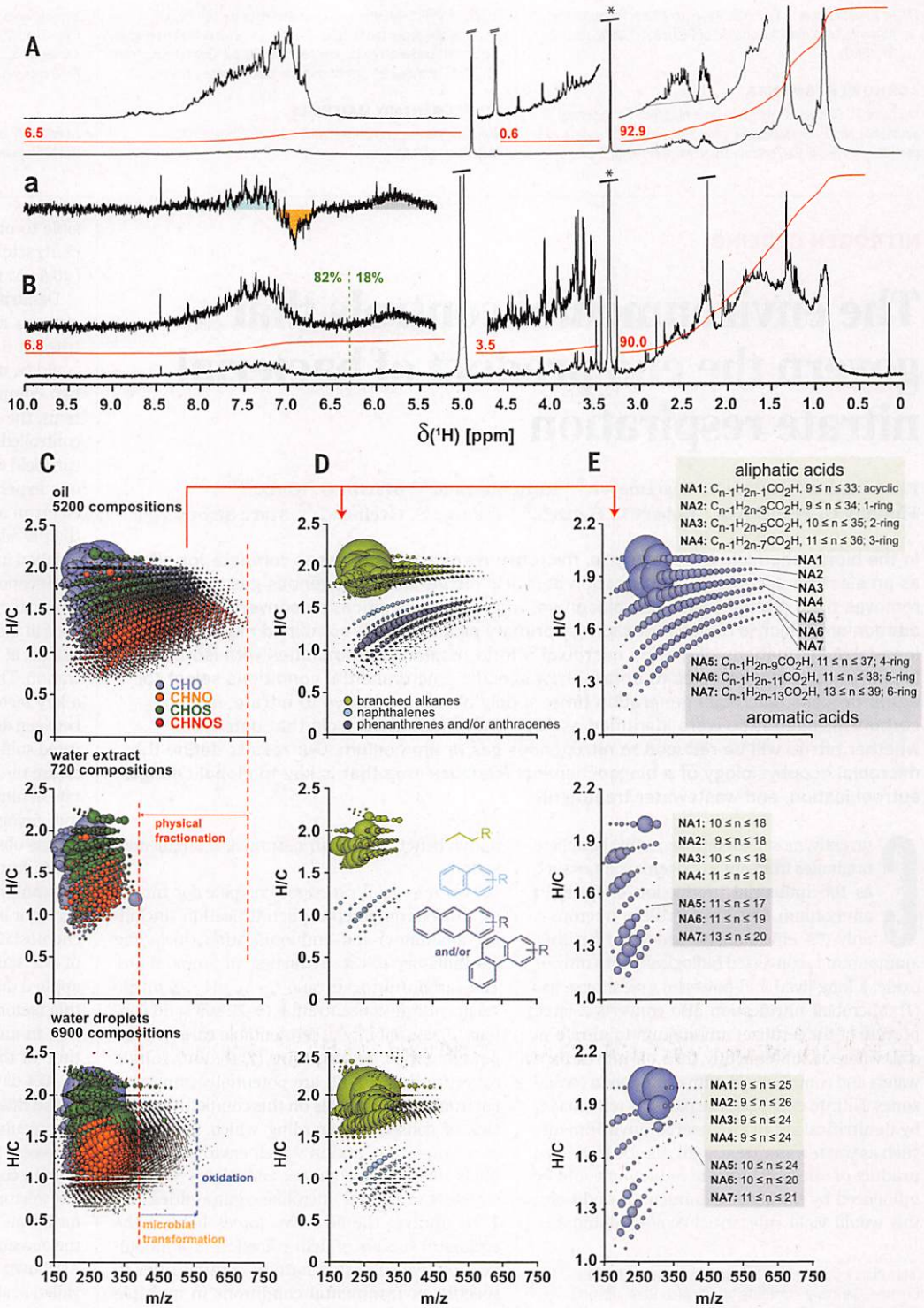

Fig. 3. Characterization of metabolites in water droplets. (**A** and **B**) ^{1}H NMR spectra of bulk oil (A) and enclosed water (B) (CD_3OD, 800 MHz) with vertical expansions. Section a: Difference NMR spectrum of HC_{sp2} units (water minus oil); digits represent ^{1}H NMR section integrals (see text). (**C** to **E**) Negative electrospray FTICR-MS data depicted as H/C versus mass diagrams of oil (top), aqueous extract (center), and water droplets (bottom), with circle areas indicating relative abundance. (C) All assigned molecular compositions are color-coded according to CHO (blue), CHOS (green), and CHNO (orange) molecular series. (D) Molecular series of substituted branched alkanes (H/C > 1.5, bright green) and alkylated polycyclic aromatic hydrocarbons (PAH, blue) with up to three condensed rings. (E) Molecular series of single carboxylic acids R-COOH decomposed into aliphatic (double bond equivalent (DBE <4) and aromatic (DBE >4) carboxylic acids, with members of these molecular series provided.

than dissolved in the actual oil, the biologically active volume could extend substantially into the oil reservoir, even if at lower activities relative to the oil-water transition zone.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
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Tables S1 to S5
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NITROGEN CYCLING

The environmental controls that govern the end product of bacterial nitrate respiration

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In the biogeochemical nitrogen cycle, microbial respiration processes compete for nitrate as an electron acceptor. Denitrification converts nitrate into nitrogenous gas and thus removes fixed nitrogen from the biosphere, whereas ammonification converts nitrate into ammonium, which is directly reusable by primary producers. We combined multiple parallel long-term incubations of marine microbial nitrate-respiring communities with isotope labeling and metagenomics to unravel how specific environmental conditions select for either process. Microbial generation time, supply of nitrite relative to nitrate, and the carbon/nitrogen ratio were identified as key environmental controls that determine whether nitrite will be reduced to nitrogenous gas or ammonium. Our results define the microbial ecophysiology of a biogeochemical feedback loop that is key to global change, eutrophication, and wastewater treatment.

Currently, most fixed nitrogen in the biosphere originates from anthropogenic sources such as the industrial production of fertilizer ammonium. Uptake of fertilizer by crops is only 17% efficient, and 1 to 5% of fertilizer ammonium is converted biologically into nitrous oxide, a long-lived and powerful greenhouse gas (*1*). Microbial nitrification also converts a large portion of the fertilizer ammonium to nitrate in soil, where it subsequently runs off into surface waters and contributes to eutrophication in coastal zones. Nitrate emissions are partially remediated by denitrification in engineered environments such as wastewater treatment plants. If the end product of microbial nitrate reduction could be influenced by tuning environmental conditions, this would yield substantial ecological and economic benefits for both natural and engineered systems.

Two microbial processes compete for nitrate as an electron acceptor: denitrification (including anammox) and ammonification (including dissimilatory nitrate reduction to ammonium). The carbon/nitrogen ratio (*2*–*5*), pH (*5*), nitrite versus nitrate concentration (*5*–*7*), soil sand content (*5*), availability of fermentable carbon compounds (*4*, *8*), temperature (*7*, *9*), and sulfide concentration (*10*–*12*) are potentially important environmental controls on this competition. The lack of consensus regarding which factors are most important, and in which environments, is likely due to the complex and highly variable structure of natural microbial communities.

To unravel the selective forces behind the ecological success of denitrification or ammonification, we subjected natural communities to specific environmental conditions in multiple parallel long-term incubations (*13*). The source community was from coastal, sandy tidal flat sediments that make substantial contributions to global denitrification and perform both denitrification and ammonification (*12*, *14*). We did not aim to reproduce the sediment community in the laboratory; we simply used the sediment as a highly microbially diverse inoculum to enable the selection of optimally adapted nitrate-reducing communities. We performed 15 parallel anoxic incubations (Fig. 1 and table S1) with continuous substrate supply, which made it possible to maintain the nitrate and/or nitrite concentrations in the in situ micromolar range (<0.5 μM for nitrite, <10 μM for nitrate).

Denitrification and ammonification have two electron acceptors in common: nitrate and nitrite. In theory, the outcome of the competition could be most easily explained by which of these two compounds is supplied. In natural ecosystems, the relative supply of nitrate and nitrite is controlled by nitrification, a two-step process that can yield either compound as the end product. In our experiments with nitrate as the terminal electron acceptor, ammonification emerged as the prevalent pathway, whereas supply of nitrite resulted in denitrification prevalence (Fig. 1). Denitrification was always observed as the prevalent respiratory pathway when nitrite was supplied, even in the presence of fermentable substrates and sulfide, at low pH or at a reduced copper concentration. Thus, the supply of nitrite or nitrate was a key factor in the outcome of the competition between denitrification and ammonification. If elevated sulfide concentrations or changes in pH decrease the rate of nitrite production relative to the rate of nitrate production (*15*, *16*), this would therefore favor ammonification over denitrification, as was observed in some previous studies (*5*, *12*).

We further investigated the apparent success of ammonification with nitrate as the electron acceptor in a 400-day chemostat incubation. In a chemostat, the growth rate (or generation time) of the cultivated bacteria is controlled by the applied dilution rate, enabling us to test whether this factor affected the outcome of the competition in any way. The average in situ generation time of the sampled community was estimated at ~0.4 days, a value derived from a metagenome of the tidal flat community (*17*). During the 400-day incubation, the generation time was varied between 1.0 and 3.4 days and the nitrite and nitrate concentrations always remained in the low micromolar range (<0.5 μM for nitrite, <10 μM for nitrate). The generation time strongly affected the outcome of the competition for nitrate (Fig. 2). As shown by mass balancing, denitrification prevailed at short generation times, whereas ammonification was most successful at long generation times, with a tipping point detected at a generation time of ~1.7 days (Fig. 2). After 185 days, we increased the generation time in the chemostat from 1.0 to 1.7 days and ammonification slowly became dominant; however, after day 230, denitrification regained prevalence. Ammonification only recovered after the generation time was further increased to 3.4 days after day 240.

To determine the mechanisms responsible for the observed selective effect of microbial generation time, we characterized the selected

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