ACS ES&T Water

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Article

Rhamnolipid Biosurfactants Enhance Microbial Oil Biodegradation in Surface Seawater from the North Sea

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Cite This: *ACS EST Water* 2023, 3, 2255−2266

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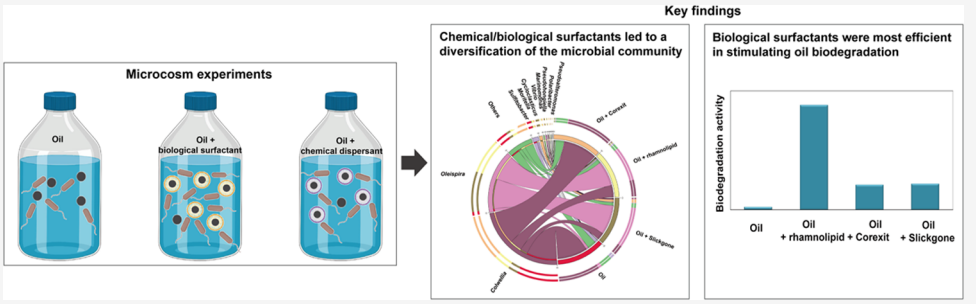

ABSTRACT: Biosurfactants are promising alternatives to chemical dispersants for combating marine oil spills; however, the impacts of biosurfactants on microbial community composition and oil biodegradation activities remain largely unknown. Here, we conducted a time-course microcosm experiment mimicking oil spill scenarios with surface seawater from the North Sea, amended with either the biosurfactant rhamnolipid or a dispersant (Corexit 9500 or Slickgone NS). Radioactive tracer assays to track hexadecane and naphthalene oxidation as well as bacterial production revealed the highest hydrocarbon oxidation rates and general microbial activities in the rhamnolipid-amended oil microcosms, followed by oil microcosms with Slickgone and Corexit. Impacts on the microbial community composition differed among treatments, and growth of oil-degrading *Colwellia* was stimulated remarkably in Corexit-amended oil and oil-only microcosms, while potential oil-degrading *Oleispira* were highly enriched in the presence of oil in combination with rhamnolipid or Slickgone. Furthermore, increased abundances of *Colwellia* and *Oleispira*, and stimulated bacterial production in microcosms with only rhamnolipid, Corexit, or Slickgone, indicated their involvement in biosurfactant/dispersant biodegradation. Our findings highlight varying microbial impacts resulting from rhamnolipid and chemical dispersants and suggest great promise for the application of biosurfactants in future marine oil spills.

KEYWORDS: *biosurfactants, chemical dispersants, oil biodegradation, oil-degrading microorganisms, radiotracer assay, microbial activity, marine oil spill*

INTRODUCTION

Increased oil exploration and transportation activities worldwide make the oceans more vulnerable to oil spills that often impart long-term negative impacts on marine ecosystems.[1,2] Various physicochemical technologies have been developed to clean up spilled oil, but hydrocarbons are ultimately biodegraded by microorganisms.[3] Commercial chemical dispersants (*e.g.*, Corexit 9500, Slickgone NS, or Finasol OSR 52) are routinely applied as emergency response tools to manage marine oil spills. These dispersants enhance the water solubility of oil by dispersing oil into small droplets (<70 μm), with the aim of effectively increasing the bioavailability of oil and facilitating oil biodegradation by microorganisms.[4] However, the impact of chemical dispersants on oil biodegradation remains controversial. Although numerous studies have demonstrated enhancement of oil biodegradation in the presence of dispersants,[5−8] inhibitory effects of dispersants on oil biodegradation have also been demonstrated clearly.[9−11]

Impacts of dispersants on microbial communities and on oil biodegradation are likely related to the oil properties, the type of dispersant, as well as the physicochemical and microbial characteristics of the seawater. For example, the addition of Corexit 9500 to Macondo crude oil stimulated the growth of *Colwellia* and inhibited the oil biodegradation rate, probably by suppressing the activity of natural hydrocarbon-degrading *Marinobacter* in the deep seawater from the Gulf of Mexico.[9] In another study, Corexit 9500 favored the proliferation of *Thalassolituus* and enhanced the oil degradation rates in nutrient-amended seawater from the eastern Canadian

Received: January 30, 2023
Revised: June 26, 2023
Accepted: June 26, 2023
Published: July 19, 2023

Coast.[12] The toxicity of some chemical dispersant components to microorganisms,[13] the potential persistence of dispersant components (such as dioctyl sodium sulfosuccinate; DOSS),[14] and the increased environmental awareness raise the need to replace chemical dispersants with more environmentally friendly alternatives. Recently discussed potential alternatives are biosurfactants produced by several types of microorganisms.[15] Biosurfactants have lower toxicity, higher biodegradability, and higher biocompatibility in comparison to chemical surfactants.[15,16] Among the well-known biosurfactants, the rhamnolipids produced by *Pseudomonas aeruginosa* hold great potential for enhancing hydrocarbon bioavailability and biodegradation.[17,18] However, most previous investigations of rhamnolipids were conducted using enrichment cultures,[19,20] where communities are less diverse compared to natural microbial communities in real oil spill scenarios.

The impact of rhamnolipids on marine microbial communities is much less explored than that of chemical dispersants.[21] A recent study assessing the impacts of rhamnolipids and the chemical dispersant Finasol OSR 52 on cold and nutrient-limited surface seawater from the northeast Atlantic revealed an enrichment of distinct hydrocarbon-degrading bacteria and lower oil biodegradation in rhamnolipid-amended treatments.[22] Another two studies discussed the addition of biosurfactants selected for different microbial groups compared to chemical dispersants, such as Finasol OSR 52, Ultrasperse II, Slickgone NS, and Superdispersant 25, and that biosurfactants had no significant effect on the oil biodegradation rate.[23,24] While the efficacy of biosurfactants and chemical dispersants has been studied in terms of microbial community dynamics and bulk crude-oil degradation, direct comparisons of their impacts on the activities of hydrocarbon-degrading microorganisms are currently lacking.

Our study site, the German Bight, is a shallow, densely navigated basin[25] and has a substantial risk for oil spills caused by accidents involving tankers, pipelines, or drilling rigs. However, microbial responses to crude oil and its fate in the presence or absence of biosurfactants and chemical dispersants in seawater of this region are unexplored. In the current study, we aimed to identify the impacts of a biosurfactant (*i.e.*, rhamnolipid) and chemical dispersants (*i.e.*, Corexit 9500 and Slickgone NS) on the abundance, composition, and activity (tracked as hydrocarbon oxidation rates) of the microbial community in North Sea seawater.

MATERIALS AND METHODS

Seawater Sampling Procedure. Seawater samples (120 L) were collected from 1 m depth near the Helgoland Island in the German Bight, North Sea (54°11′3.3″N, 7°53′45.9″E), on April 11, 2017 (Figure S1). The sampling site is approximately 60 km from the Mittelplate oil field, which is Germany's largest oil field and located off the North Sea coast on the southern edge of the Schleswig−Holstein Wadden Sea. Seawater samples were obtained by filling six 20 L, sterile acid-washed plastic buckets. After sampling, seawater samples were stored on ice packs in the field (∼4 °C) and transported to the Tuebingen University laboratory, where the experiments were started within 24 h. The seawater samples were stored at 4 °C for 2 days prior to the experimental setup during the preparation of the water-accommodated fraction (WAF). WAF is a laboratory-prepared medium derived from low-energy mixing of crude oil with sterile seawater and is essentially free of oil slicks.[26] Various water-soluble compounds in crude oil are captured in WAF. In this study, WAFs were applied to assure reproducibility of data among laboratories.[26]

Microcosm Setup. Natural seawater was used to set up the microcosms. Eight treatments were prepared: (1) seawater only with no additions (Biotic); (2) crude oil alone, supplied as a WAF; (3) oil dispersed with rhamnolipid (>90%, purified from *Pseudomonas aeruginosa*, Sigma-Aldrich), supplied as a biosurfactant-enhanced WAF (Rha-BEWAF); (4−5) oil dispersed with Corexit 9500 (Nalco, Sugar Land, TX) or Slickgone NS (Dasic International, U.K.), supplied as chemically enhanced WAFs (Cor-CEWAF and Sli-CEWAF); and (6−8) rhamnolipid, Corexit 9500, and Slickgone NS alone (Rha-only, Cor-only, and Sli-only). In addition, an abiotic control comprising 0.22 μm-filtered and pasteurized (3 h at 65 °C) seawater with the same amount of WAF aliquots was set up. Crude MIPL-Pipelineblend oil (from Mittelplate oil field) with a density of 897.4−973.3 kg/m^3 (15 °C) was used. All WAFs were prepared as described previously.[9] Briefly, WAF, Rha-BEWAF, Cor-CEWAF, Sli-CEWAF, Rha-only, Cor-only", and Sli-only were prepared by mixing pasteurized seawater with crude oil or/and a dispersant/biosurfactant for 48 h at room temperature and subsequently by subsampling the WAFs, BEWAF, and CEWAFs, excluding contamination by oil or dispersants/biosurfactant phases. Seawater sampling of the experiments was carried out after incubation for 0, 3, 7, and 28 days. Microcosms were constructed in triplicate for the time points on days 0, 3, and 28, and one microcosm for each treatment was constructed for the time point on day 7.

To make the different treatments comparable, an experiment was designed to expose microbial communities to the same amount of dissolved organic carbon (DOC) (Figure S2) while varying the composition of the added DOC. Previous findings showed that, on average, a twofold increase of the DOC content was observed in oil-contaminated seawater.[27] We therefore quantified the DOC concentration of the seawater and of the different WAFs/CEWAFs and added aliquots to obtain a comparable final DOC concentration (∼500 μM DOC) across the treatments. This concentration of DOC was twice as high as the DOC concentrations of the *in situ* seawater (∼250 μM DOC). Each microcosm contained a final volume of 900 mL in a 1 L Schott bottle closed with a Teflon cap. The biotic control contained unamended seawater. The bottles were incubated at 7−8 °C to resemble the *in situ* seawater temperature (7 °C) and illuminated (broad-spectrum 36 W fluorescent light) with a 12:12 light/dark cycle. At each sampling time point, 400 mL of seawater per microcosm was filtered through 0.2 μm filter membranes (Millipore), and filters were stored at −80 °C for molecular biological analysis. Furthermore, 300 mL of seawater was stored at −20 °C for hydrocarbon analysis and additional chemical analyses were carried out immediately (pH, dissolved oxygen) or on stored samples (DOC). Subsamples for radioisotope activity assays were collected after the other samples, and assays were performed immediately after subsampling.

Hydrocarbon Extraction and Analysis. Hydrocarbons in each microcosm were solvent−solvent extracted on days 0, 3, 7, and 28 using dichloromethane as described previously[9] (see the Supporting Information). *n*-Alkane (C7-C40) and 16 U.S. Environmental Protection Agency (EPA)-listed priority polycyclic aromatic hydrocarbons (PAHs) were quantified by gas chromatography-mass spectrometry (GC-MS) installed

https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266

with an automatic injector (Agilent 7890-5977MS, Santa Clara, CA). The temperature programs for *n*-alkanes and PAHs have been described previously.[28] Quantitative analysis was performed using an eight-point external standard consisting of *n*-alkane (C8-C40, Sigma-Aldrich) or 16 PAHs (Sigma-Aldrich). The diluted concentrations were 0, 1, 2, 5, 10, 25, 50, and 100 mg/L for both standards. Compound identification was based on individual mass spectra and retention times in comparison to library data and to external standards that were injected and analyzed under the same conditions.

Microbial Biomass Production. Microbial biomass production was estimated from ^{3}H-leucine incorporation to provide a general index of microbial activity or growth rates.[29] The ^{3}H-leucine incorporation assay was conducted at 8 °C for each microcosm in triplicate. For each incubation, 1.5 mL subsamples were amended with 3.5 nM ^{3}H-leucine (activity: 0.47 μCi) and then incubated for 2 h at 8 °C. The incubations were terminated by adding 100% trichloroacetic acid (TCA). The cells in these samples were washed with 5% TCA and 80% ethanol. Afterward, 1.75 mL of Scintillation Cocktail (Ultima Gold, PerkinElmer) was added to the tubes, and the radioactivity was quantified using a Beckman LS-6500 liquid scintillation counter (Beckman, Fullerton, CA). The rate of bacterial production was calculated as described previously.[29]

^{14}C-Hydrocarbon Oxidation Rates. ^{14}C-Hexadecane and ^{14}C-naphthalene radiotracer assays were used to monitor the biodegradation rate of these hydrocarbons in seawater samples.[30] For each sample, 8 mL of seawater subsample was transferred to a headspace-free scintillation vial and amended with ^{14}C-hexadecane or ^{14}C-naphthalene (American Radiolabel Chemicals; ARC). The unit of radioactivity per 8 mL sample was 1.76 nCi. After incubation for 24 h, the incubation was halted by adding 2 mL of 2 M NaOH solution. Afterward, 1 g of activated carbon (Sigma-Aldrich) was added to the seawater to absorb the remaining ^{14}C-hexadecane/naphthalene substrate. The seawater was then transferred to a 250 mL flask. 5 mL of H_3PO_4 (≥80 wt %) was added to convert the ^{14}C-dissovoled inorganic carbon (DIC) to ^{14}C-CO_2, which was trapped using Carbo-Sorb (PerkinElmer). Total radioactivity (hydrocarbon substrate plus ^{14}C-CO_2) and ^{14}C-CO_2 were quantified using a Beckman LS-6500 liquid scintillation counter (Beckman, Fullerton, CA) following addition of a Scintillation Cocktail (Permafluor E+, PerkinElmer). The rate of ^{14}C-hexadecane/naphthalene oxidation was calculated as described previously.[30]

DNA Extraction, Cell Counts, and Real-Time Quantitative PCR (qPCR). Total DNA was extracted from filters using a FastDNA spin kit (Qbiogene, Irvine, CA) according to the instructions of the manufacturer. The DNA was stored at −80 °C until further analyses.

Seawater samples for cell counts were fixed with 3.7% formaldehyde for 1 h at room temperature. The volume filtered for each filter was optimized for each treatment and time point, resulting in 30−150 free-living cells per counting grid at 100× magnification. Free-living cell counts were performed with an epifluorescence microscope (Olympus BX40) after staining with 4′,6-diamidin-2-phenylindol (DAPI, 1 μg/mL). For each sample, a minimum of 10 counting grids or 1000 free-living cells were randomly selected and counted.

Since in addition to free-living cells, aggregated cells were observed under a microscope that could not be counted (Figure S3), the abundance of total microorganisms was assessed by quantifying 16S rRNA gene abundances on a CFX9Z optical Read-time Detection System (Bio-Rad Laboratories Inc., Hercules, CA) using primers 515f (GTGYCAGCMGCCGCGGTAA) and 806r (GGACTACNVGGGTWTCTAAT).[31] qPCR was performed in a reaction mixture containing 1× iTaq Universal SYBR Green (BioRad, Hercules, CA), 0.5 mM of each primer, and approximately 5 ng of DNA template. Blanks were run with water as a template instead of a DNA extract. The amplification was started by denaturing at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 74 °C, and plate read at 85 °C. Amplification efficiencies of 98−103% were obtained with R^2 values of 0.993−0.999.

Illumina MiSeq Sequencing of 16S rRNA Gene Amplicons and Data Analysis. The composition of the microbial communities was analyzed using Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene. The 16S rRNA gene fragment was amplified using primers 515f and 806r targeting the V4 region.[31,32] Library preparation steps (Nextera, Illumina) and 250 bp paired-end sequencing with MiSeq (Illumina, San Diego, CA) using v2 chemistry were performed by Microsynth AG (Switzerland). Primers were trimmed, and untrimmed sequences were discarded with Cutadapt,[33] and wrapped in containerized trimgalore v0.4.5 (https://github.com/FelixKrueger/TrimGalore) used with singularity v2.4.1.[34] Adapter and primer-free sequences were processed with QIIME2 software (Quantitative Insights Into Microbial Ecology, v2) v2018-2 with singularity v2.4.1.[35] Within QIIME2, DADA2 was employed to eliminate PhiX contamination, trim reads (forward reads were trimmed at 180 bp and reverse reads at 120 bp), correct errors, merge read pairs, and remove polymerase chain reaction (PCR) chimeras.[36] Ultimately, 5154 amplicon sequencing variants (ASVs) were obtained across all samples. ASVs were taxonomically assigned using QIIME2's "q2-feature-classifier" trained on the Silva database (nr_v132), and ASVs designated as chloroplasts were removed (totaling up to 0.02−31.2% of sequences per sample).

Statistical Analyses. Multiple comparisons using one-way ANOVA analysis followed by LSD-Duncan were applied to check the quantitative variance between different treatments and time points. SPSS version 20.0 was used for these statistical analyses (SPSS Inc., Chicago, IL). A *P*-value significance threshold of 0.05 was employed. Comparisons of bacterial community compositions in different treatments were performed by one-way analysis of similarities (ANOSIM) using PRIMER 6 (Plymouth Routines in Multivariate Ecological Research). Representative ASV sequences were used to construct a phylogenetic tree as described in the Supporting Information. The absolute abundances of the genera were obtained by using the 16S rRNA gene amplicon sequencing data of these specific taxa multiplied by the 16S rRNA gene abundances quantified by qPCR.

Accession Number of Nucleotide Sequences. The sequencing data were deposited at the National Center for Biotechnology Information (NCBI) in the Sequence Read Archive (SRA) under BioProject accession number PRJNA902952.

RESULTS

Biosurfactants Stimulate Microbial Growth. The microbial abundance (assessed by 16S rRNA gene abundance) increased significantly during incubation in all treatments

https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266

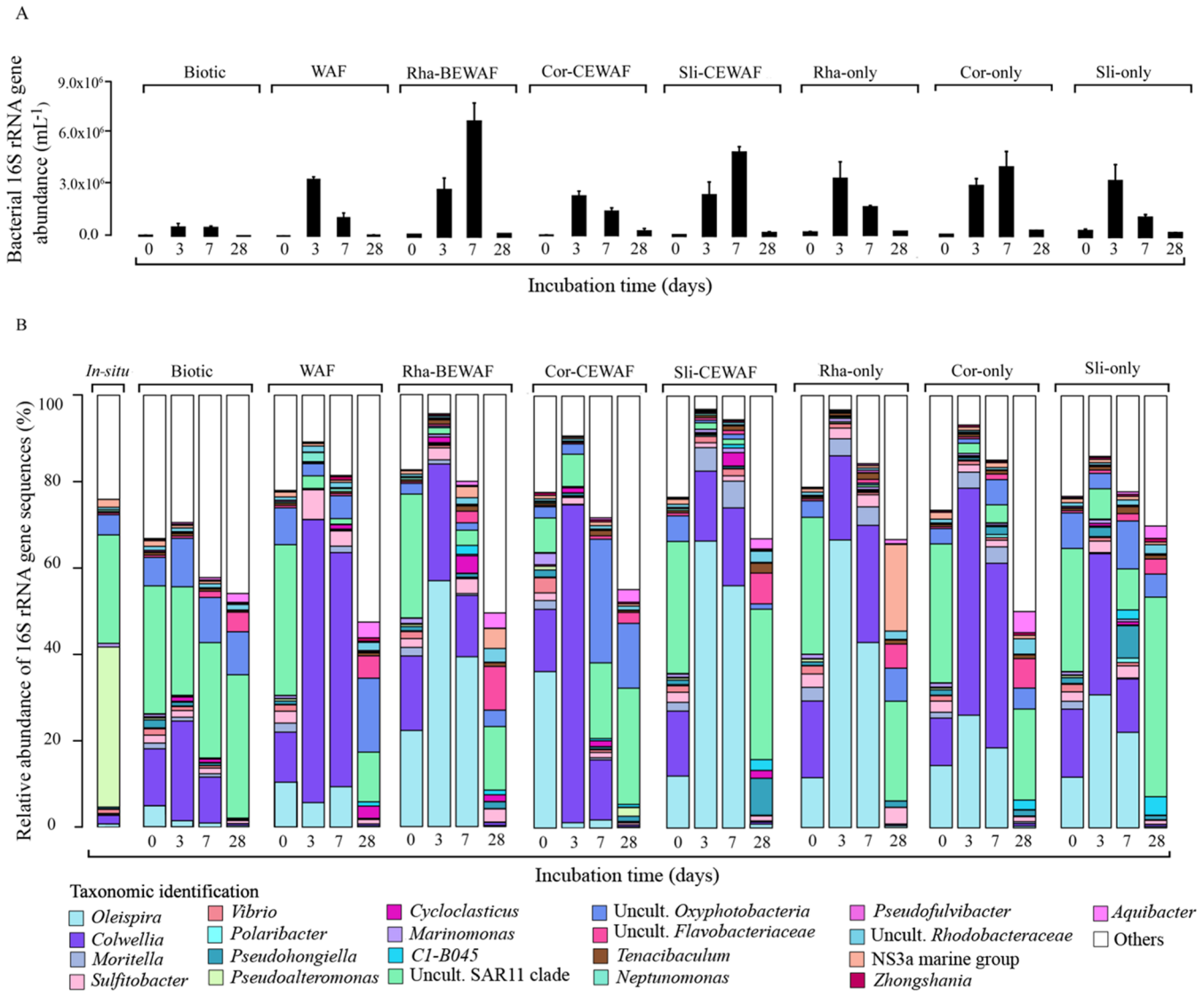

Figure 1. Dispersants and biosurfactants affect the abundance and composition of microbial communities. (A) Changes in bacterial 16S rRNA gene abundances in the microcosms of different treatments after incubation for 0, 3, 7, and 28 days. Error bars represent the standard errors of the mean of triplicate samples. (B) Relative abundance of bacterial groups in *in situ* seawater and microcosm samples (average of biological triplicate microcosms on days 0, 3, and 28, respectively, and of technical triplicates of microcosms on day 7). Cor-CEWAF, Sli-CEWAF, and Rha-BEWAF represent the seawater microcosms incubated with oil in the presence of Corexit 9500, Slickgone NS, or rhamnolipids. Cor-only, Sli-only, and Rha-only represent the seawater microcosms amended only with Corexit 9500, Slickgone NS, or rhamnolipid.

amended with biosurfactants and chemical dispersants, in both the presence and absence of oil ($P < 0.05$) (Figure 1A). The highest microbial abundance was detected in the rhamnolipid-dispersed oil treatment on day 7, representing an 8.6-fold higher 16S rRNA gene abundance compared to that on day 0. The second highest 16S rRNA gene abundance was detected in the CEWAF treatment with Slickgone on day 7. In the WAF treatment and the CEWAF treatment with Corexit, the 16S rRNA gene abundance reached their maximum on day 3. The 16S rRNA gene abundances decreased sharply in all treatments after incubation for 28 days. The 16S rRNA gene abundances in rhamnolipid-only or dispersant-only treatments increased notably as well after incubation for 3 and 7 days. In agreement with the 16S rRNA gene abundances, the free-living direct cell counts mostly resembled the qPCR findings and differences were likely attributed to the uncountable microbial cell aggregates that formed during the incubation (Figures S3 and S4).

Different Treatments Exhibit Varying Microbial Community Patterns. The microbial community diversity and composition varied across treatments and incubation time (PERMANOVA: $p = 0.005$) (Figures 1B, S5, and S6). All treatments showed growth of *Oleispira* and/or *Colwellia* as key microbial players, but these taxa differed in relative abundances (Figures 1B and S7). The dominant microbial responder to the additions of rhamnolipid-dispersed oil and CEWAF with Slickgone was *Oleispira*, whose relative abundance increased from 22.8 and 12.8% to 57.1 and 66.8% on day 3, respectively, representing 2.5- and 5.2-fold enrichments. In contrast, the relative abundance of *Oleispira* decreased sharply from 11.4 and 37.2% on day 0 to 5.84 and 1.27% on day 3 on treatments with WAF and CEWAF with Corexit, representing 1.95- and 29.3-fold decreases.

On day 7, the relative abundances of *Oleispira* were still as high as 40 and 56.8% on treatments with rhamnolipid-dispersed oil and CEWAF with Slickgone, respectively. In

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ACS EST Water 2023, 3, 2255−2266

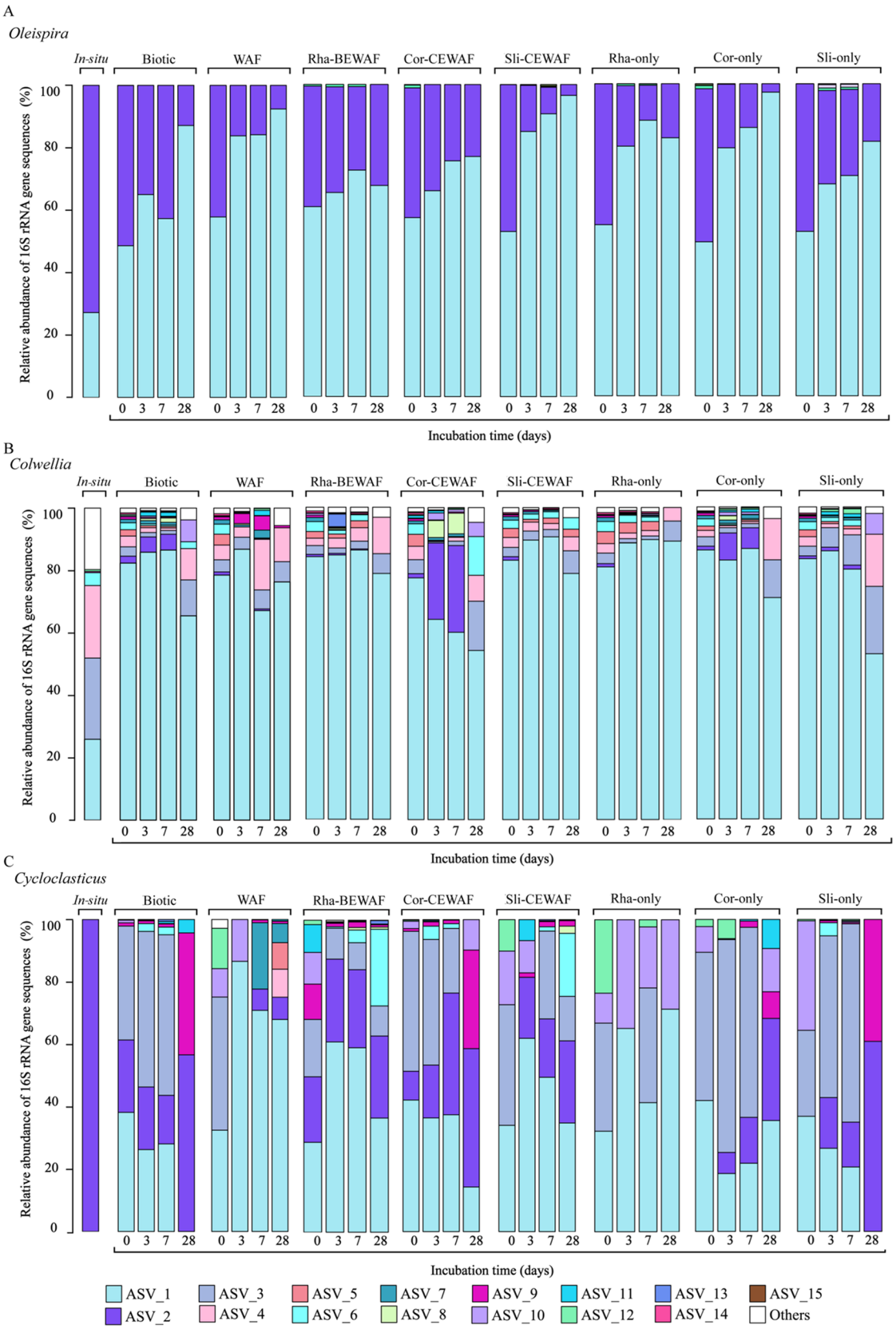

Figure 2. Different microbial ASVs of *Oleispira* (A), *Colwellia* (B), and *Cycloclasticus* (C) that responded to oil and/or dispersants/biosurfactants. The designations of the treatments are the same as those in Figure 1.

addition, significantly higher absolute abundances of *Oleispira* in the rhamnolipid-dispersed oil and CEWAF with Slickgone treatments compared to WAF and CEWAF with Corexit treatments were observed ($P < 0.05$) (Figure S7A). The enrichment of *Oleispira* was also observed for rhamnolipid-only and Slickgone-only treatments. Examination of closely related

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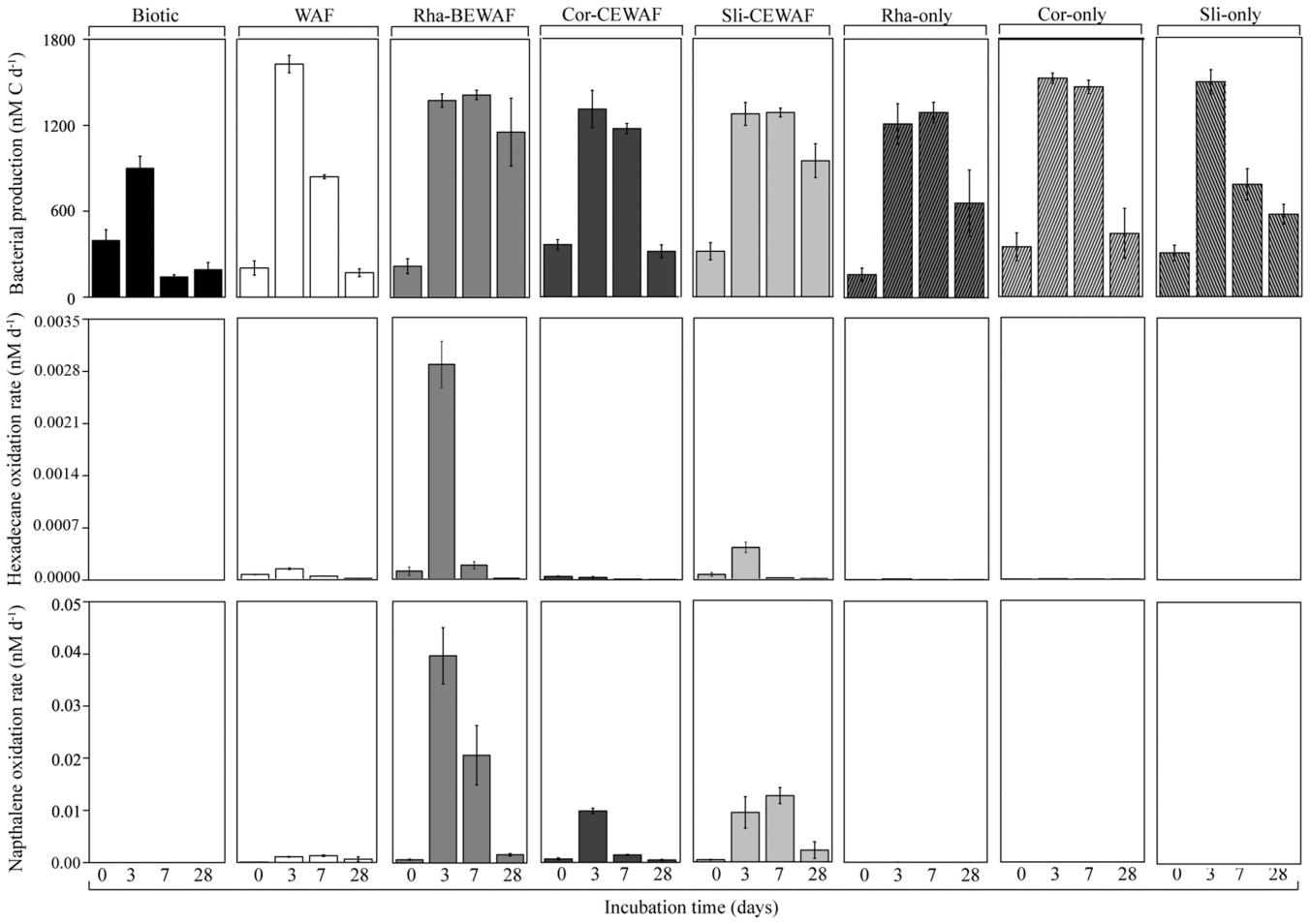

Figure 3. Changes in the bacterial production as well as ^{14}C-hexadecane and ^{14}C-naphthalene oxidation rates in different treatments over the incubation course of 28 days. (A) Rates of bacterial production increased significantly in all treatments compared to the biotic control. (B) Oxidation rates of ^{14}C-hexadecane and ^{14}C-naphthalene were used as model compounds for indicating the degradation of alkanes and PAHs, respectively. Standard errors of triplicate samples are shown. The designations are the same as those in Figure 1.

Oleispira taxa showed that the same *Oleispira* ASVs occurred across these treatments, dominated by *Oleispira* ASV_1 and ASV_2 (Figure 2A). *Oleispira* ASV_1, which was detected in *in situ* seawater samples, was significantly enriched in all oil-amended treatments ($P < 0.05$).

Colwellia, the second most abundant microbial responder to the additions of rhamnolipid-dispersed oil and CEWAF with Slickgone, only increased slightly by 1.0- to 1.5-fold during the incubation period. This increase was much less than the relative and absolute enrichment observed in WAF and CEWAF with Corexit treatments (Figures 1B and S7B). *Colwellia* dominated the microbial community in WAF, CEWAF with Corexit, and Corexit-only treatments by day 7. In these microcosms, the relative abundance of *Colwellia* increased substantially from 13, 15, and 11% to 68, 75, and 53% on day 3, respectively. An increased relative abundance of *Colwellia* was also detected in the Slickgone-only and rhamnolipid-only treatments. Notably, *Colwellia* ASV patterns varied among different treatments. For example, the relative abundances of *Colwellia* ASV_2, ASV_8, and ASV_10 increased in CEWAF with Corexit and Corexit-only treatments, whereas ASV_1, ASV_4, and ASV_9 increased in the WAF treatment (Figure 2B).

Oil addition in the presence of rhamnolipid and dispersants also enriched some additional genera (Figure 1B). For example, the relative abundance of the clade *C1-B045* in the rhamnolipid-dispersed oil treatment (2.10%) at day 7 was 129 times higher than that in the WAF treatment (0.02%). Marked enrichments of *Moritella* on days 3 and day 7 and of *Pseudohongiella* on day 28 were detected in the CEWAF with Slickgone treatment compared to all other treatments. An increased abundance of *Moritella* was moreover observed in rhamnolipid-only and Corexit-only treatments. In addition, the relative abundance of *Pseudoalteromonas* in the CEWAF treatment with Corexit (2.38%) was 83.4 times higher than that in the WAF treatment (0.03%) on day 28. In the WAF treatment, a significant enrichment of *Neptunomonas* and *Zhongshania* across the incubation was detected when compared with all other treatments.

The microbial response to rhamnolipid and dispersants with and without oil followed successional patterns. By day 7 or day 28, the early blooming genera *Oleispira* and *Colwellia* were substantially decreased in abundance. By this time, the initial dominant genera were overgrown by *Cycloclasticus*, *Tenacibaculum*, *Aquibacter*, uncultured SAR11 clade, and uncultured *Oxyphotobacteria* in all oil-amended treatments. For example, in the WAF treatment, *Cycloclasticus* increased over the entire incubation period and reached its maximum (3.40%) on day 28, representing a 34.5-fold relative increase compared to day 0. Slight increases in relative abundance of *Cycloclasticus* were

https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266

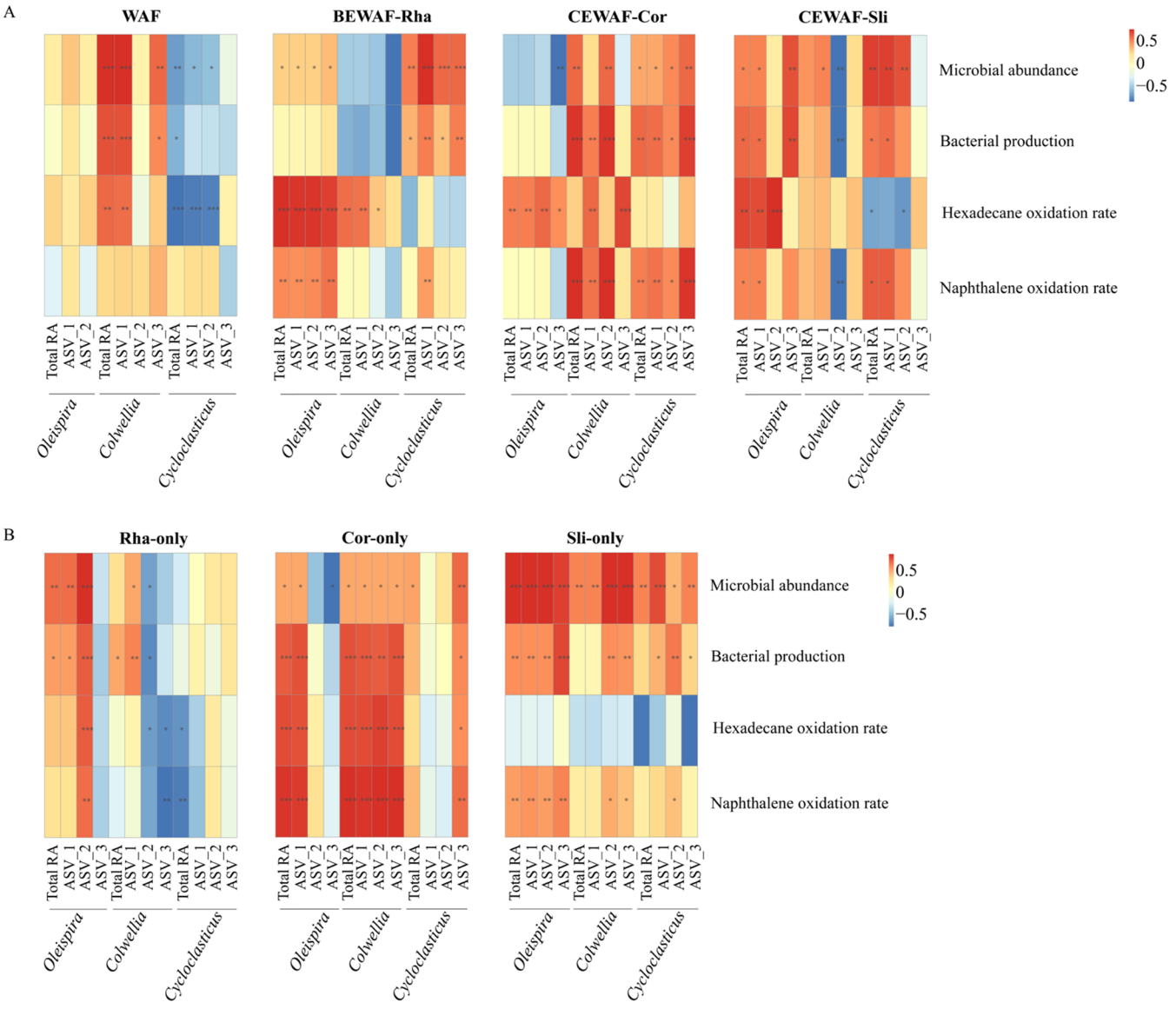

Figure 4. Pearson correlations of the total relative abundance (RA) and dominant bacterial ASVs of *Oleispira*, *Colwellia*, and *Cycloclasticus*, with microbial abundance (assessed by 16S rRNA gene abundance), bacterial production, hexadecane oxidation rate, and naphthalene oxidation rate in different treatments, displayed as heatmaps. Scale bars indicate correlation coefficients. Only significant correlations ($P < 0.05$) are shown. The red color in the heatmap indicates a significant positive correlation. The blue color in the heatmap indicates a significant negative correlation.

also observed in dispersant-only microcosms. Similar to *Colwellia*, *Cycloclasticus* ASVs varied significantly across treatments (Figure 2C). *Cycloclasticus* ASV_1 responded most to oil addition in the presence of rhamnolipid and Slickgone, whereas the relative abundance of *Cycloclasticus* ASV_2 increased most in the CEWAF treatments with Corexit.

Biosurfactants Stimulate the Microbial Activity. Immediate and vigorous responses of microbial activities were observed in all treatments (Figure 3). The rates of bacterial protein production were significantly stimulated, representing 3.6- to 7.9-fold increases by the addition of oil in the presence of rhamnolipid or dispersants on day 3. The rates varied among treatments, and they remained at relatively high levels by day 7 in rhamnolipid- and dispersant-amended oil treatments, whereas the rate decreased significantly in the WAF treatment. Furthermore, the rates of bacterial protein production significantly increased (day 3 and day 7, $P < 0.01$) in rhamnolipid-only or dispersant-only treatments, whose maximum rates were comparable to all other oil-amended treatments.

Biosurfactants Are More Efficient than the Two Chemical Dispersants in Stimulating the Oil Degradation Rate. Radiotracer assays provided direct quantification of alkane (*i.e.*, ^{14}C-hexadecane) and PAH (*i.e.*, ^{14}C-naphthalene) oxidation rates. Both the hexadecane and naphthalene oxidation rates were the highest in the rhamnolipid-dispersed oil treatment (Figures 3 and S8), underscoring that rhamnolipids stimulated oil hydrocarbon degradation the most. The hexadecane oxidation rates on day 3 and day 7 in the rhamnolipid-dispersed oil treatment were 37.4 and 16.1 times higher than those in the WAF treatment. Moreover, the hexadecane oxidation rate on day 3 in the rhamnolipid-dispersed oil treatment was remarkedly (106.6- and 6.9-fold) higher than those in treatments with CEWAF (*i.e.*, with Corexit and Slickgone). In the CEWAF treatment with

https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266

Slickgone, the rate was also higher than that of the WAF treatment. In contrast, the hexadecane oxidation rates were significantly reduced across the incubation in the CEWAF treatment with Corexit compared to the WAF treatment ($P < 0.05$). Stimulation effects of biosurfactants and dispersants on naphthalene oxidation rates were observed. Similarly, a maximum naphthalene oxidation rate occurred in the rhamnolipid-dispersed oil treatment on day 3. The CEWAF with Slickgone addition led to significantly higher naphthalene oxidation rates compared to the CEWAF with Corexit addition on days 7 and 28. In addition, results from GC-MS analysis further confirmed the patterns of hydrocarbon degradation across the treatments (Figure S9), showing that the degradation ratios of *n*-alkanes and PAHs in rhamnolipid-dispersed oil treatment were the highest observed across all of the treatments.

Factors Regulating the Abundance and Activities of Key Bacterial Taxa. We determined statistically significant relationships between the measured experimental data and the relative or absolute abundances of the key bacterial taxa (*i.e.*, *Colwellia*, *Oleispira*, and *Cycloclasticus*) and their dominant ASVs (Figure 4, Tables S1 and S2). The relative abundance of *Oleispira*, the key responder to the rhamnolipid-dispersed oil, CEWAF with Slickgone, and rhamnolipid-only additions, was significantly positively correlated with microbial abundance, hexadecane, and naphthalene oxidation rates during the incubation ($P < 0.05$). Additionally, the positive correlation of the absolute abundance of *Oleispira* with all of these microbial activity parameters further corroborated the important contribution of *Oleispira* to the hydrocarbon degradation activity in the rhamnolipid-dispersed oil treatment. Relative abundances of the majority of *Colwellia* ASVs and the absolute abundance of *Colwellia* in the WAF and CEWAF with Corexit treatments were positively correlated with bacterial production, as well as hexadecane and naphthalene oxidation rates ($P < 0.05$) (Table S2), suggesting hydrocarbon degradation by *Colwellia* in both the absence and presence of Corexit. Moreover, many *Cycloclasticus* ASVs positively correlated with microbial abundance and bacterial production and negatively correlated with hexadecane and naphthalene oxidation rates, as well as DOC concentration (Figure S10).

DISCUSSION

Biosurfactants (Rhamnolipid) Showed the Most Stimulation of Oil-Degrading Bacterial Activity. Based on direct radiotracer assay findings, rhamnolipid performed the best in stimulating hydrocarbon biodegradation compared to two widely used dispersants. Rhamnolipid has been previously shown to stimulate growth of hydrocarbon degraders since their addition enhanced the oil biodegradation efficiency by 9.22%.[18] Several reasons could explain this observation. First, rhamnolipid is less toxic than chemical dispersants.[18] The lower toxicity of biosurfactants is likely due to their simpler chemical structure and composition and the absence of solvents and other toxic chemicals that are present in chemical dispersant mixtures.[3] Certain biosurfactants exhibit selective toxicity toward specific organisms, but they may have a limited inhibitory impact in a natural remediation system involving a diverse indigenous microbial population.[37] For example, the suppression of *Oleispira* but not *Colwellia* by Corexit might be explained by a selective toxicity mechanism toward this taxon.[38]

Second, rhamnolipid is an excellent emulsifier of oil in seawater. Rhamnolipids are highly effective at forming small droplets (<0.15 μm) at low surfactant-to-oil (<1:10) ratios,[39] whereas an average oil droplet size generated by the addition of a chemical dispersant (*e.g.*, Corexit 9500)[40] is 5−150 μm. Rhamnolipid-coated oil droplets are very stable due to the modified structure and surface properties of the oil droplet, thereby providing an efficient barrier to droplet coalescence.[39]

Third, the concentration of rhamnolipid used in the seawater microcosms does play an important role in determining their efficacy. Microbial growth and oil biodegradation rate might be negatively affected by a suboptimal concentration of biosurfactants.[41] For example, previous studies in marine remediation indicated that a biosurfactant with low critical micelle concentration (CMC) is effective, whereas a concentration of the biosurfactant beyond 1−1.5 CMC of the biosurfactants becomes ineffective for oil biodegradation enhancement.[41,42] The rhamnolipid concentration in rhamnolipid-amended treatments here was ∼1/5 CMC of the rhamnolipids. This concentration could favor the oil microbial degradation activities in natural seawater. Furthermore, the optimal portfolio of efficient oil-degrading microbial groups might also contribute to the high oil biodegradation activities.

Additions of Corexit and Slickgone enhanced the microbial hydrocarbon degradation rates to a lesser extent compared to that of rhamnolipid. Varying impacts of the two dispersants on oil biodegradation were also observed, which have been reported previously.[24] This phenomenon is likely a result of a combination of chemical and biological factors. First, while the exact composition is proprietary, previous studies indicated that the Slickgone NS composition (1−10% anionic surfactant, > 50% odorless kerosene) is distinct from Corexit 9500 (18% DOSS, 4.4% Span 80, 18% Tween 80, and 4.6% Tween 85).[8,21] Second, the presence of chemical dispersants provides additional carbon sources for oil-degrading populations, which may also be capable of degrading dispersants.[9,43] Our results show that addition of Corexit enhanced the biodegradation of PAHs, but not *n*-alkanes. The Corexit-induced inhibition of alkane biodegradation was also found in previous studies,[9,44] which could be due to the negative impacts of some components of Corexit on oil degraders,[13] such as *Oleispira*, or the alteration of the microbial hydrocarbon metabolism.[45]

Fast Responses of *Oleispira* and *Colwellia* to Oil with or without Biosurfactants and Chemical Surfactants. The presence of biosurfactants and chemical dispersants altered the microbial community significantly and selected for different sets of oil-degrading bacterial groups. The varied time-dependent dynamics of microbial responses to the same initial DOC content could be caused by differing compositions of oil- and dispersed-oil-derived compounds. The proliferation of *Oleispira* and *Colwellia* highlights the importance of these two taxonomic groups to oil degradation in the presence of biosurfactants and dispersants, as previously reported.[22,43]

The key role of *Oleispira* in oil biodegradation in cold marine environments has been described previously.[46] The dominant *Oleispira* ASV_1 is affiliated with the psychrophilic *Oleispira antarcitica* strain RV-8 at a 100% 16S rRNA gene fragment sequence identity (Figure S11), which was isolated from a crude oil enrichment of Antarctic seawater and that exhibited optimal growth at 2−4 °C in cold and deep marine environments.[47] The *in situ* and incubation temperature of the

https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266

sampled seawater was 7−8 °C, which is slightly above the optimal condition for the psychrophilic *Oleispira*. The rapid growth of *Oleispira* in our study was potentially triggered by its psychrophilic enzymes that are similar to their more mesophilic homologues, allowing *Oleispira* to maintain activity at the experimental temperature.[48] *Oleispira* were also implicated in biosurfactant and dispersant degradation,[22,49] as shown by the increased relative and absolute abundances of *Oleispira* in rhamnolipid-only and Slickgone-only treatments and their positive correlation with hexadecane and naphthalene oxidation activity (Figure S10 and Table S2).

Similar to observations in seawater from the Norwegian fjord,[50] North Sea Byfjord, and Arctic seawater,[51] a bloom of *Colwellia* was observed in oil-amended seawater treatments. The growth of *Colwellia* was stimulated in the presence of Corexit, suggesting members within this genus are able to degrade Corexit compounds in addition to oil.[9] Furthermore, the significant correlation between the absolute abundances of *Colwellia* and naphthalene oxidation rates in rhamnolipid-only and Slickgone-only treatments indicates their potential in degrading rhamnolipid and Slickgone components (Table S2). The versatility of the genus *Colwellia* could be explained by their diverse genetic potentials to biodegrade a variety of hydrocarbons, such as gaseous, aromatics, *n*-alkanes, cycloalkanes, benzene, and oil biodegradation intermediates.[52,53] The varied compositions of *Colwellia* ASVs among the treatments support prior reports of the observed strain-specific carbon source preferences by the different *Colwellia* taxa[53,54] (Figure S11). This indicates that biosurfactants and dispersants drove the observed variation in *Colwellia* taxa.

Treatment-Specific Enrichment of Additional Potential Oil-Degrading Bacteria. The addition of biosurfactants and different dispersants to oil triggered the enrichment of specific microbial taxa. *Neptunomonas*, which are known for PAH degradation,[55] was more enriched in the WAF treatments compared to all other treatments. Another enriched genus in the WAF treatment was *Zhongshania*,[56] which was reported as an aliphatic degrader.[50] In contrast, rhamnolipid- or Slickgone-dispersed oil selectively promoted the growth of *C1-B045*, which is the most closely related to the PAH degrader *Porticoccus*.[57] Moreover, *Moritella* was especially enriched by the addition of Slickgone. *Moritella* was reported to play a role in the degradation of dispersant components.[58] This also agrees with our finding that *Moritella* was stimulated by the presence of Corexit. Furthermore, the addition of Corexit led to the enrichment of *Pseudoalteromonas*, which was reported to thrive in crude oil with Corexit.[12] These findings indicate that the selective forces of the biosurfactant and dispersant amendments drove a strong diversification of the microbial communities.

Successive Microbial Community Changes among Different Microcosms. The successive pattern of hydrocarbon-degrading bacteria is likely a result of a combination of the dynamically shifting availability of oil components and of the nutrients available for oil-degrading bacteria.[59] Both of the initially stimulated genera, *Oleispira* and *Colwellia*, are often associated with the early stages of oil spill situations.[22,60] The initial stimulation of *Oleispira* and *Colwellia* was probably due to their characteristic metabolic features, such as versatile metabolic enzyme systems and opportunistic growth strategies.[48,61]

One of the later bloomers, *Cycloclasticus*, is a cosmopolitan genus that is detected commonly in oil biodegradation studies[22,49] and is responsible for mineralization of aromatic compounds.[62] *Cycloclasticus* species could struggle to compete with fast-growing alkane degraders for nutrients, and typically, they do not dominate the community until after the early bloomers decline.[59] It is interesting to note that the addition of biosurfactants and dispersants led to different *Cycloclasticus* ASV compositions. The most stimulated *Cycloclasticus* ASV_1 in response to the additions of biosurfactant and the dispersant Slickgone was closely related to *Cycloclasticus pugetii* (Figure S11), which can use the aromatic hydrocarbons naphthalene, phenanthrene, anthracene, and toluene as the sole carbon sources.[62] In contrast, the main responder in the CEWAF with Corexit treatment, *Cycloclasticus* ASV_2, was closely related to a *Cycloclasticus endosymbiont*, which lacks genes needed for PAH degradation and degrades propane and short-chain alkanes as carbon and energy sources.[63] Furthermore, the predominance of *Cycloclasticus* ASV_2 in the *in situ* seawater combined with its increased abundance in all oil-amended treatments indicates that the seawater may be primed for the degradation of biologically or chemically dispersed oil. The stimulated growth of *Cycloclasticus* in dispersant-only treatments may be due to the presence of light aromatics or hydrotreated light petroleum distillates in the solvents of the dispersants.[21,63]

By day 28, the replacement of the dominant bacterial genera by the common and widespread marine uncultured SAR11 bacterial clade suggested that the microbial communities were changing from specialistic oil-degrading bacteria to more general heterotrophic bacteria later in the incubation.[64] This finding is also supported by the decrease of microbial hexadecane and naphthalene degradation rates on day 28. Furthermore, the varied microbial communities among different treatments in the late stage of the experiment may be structured primarily by stochastic processes resulting from the interaction between oil degraders and bacterial secondary consumers.[65]

Stimulating Oil Biodegradation in Nutrient-Rich, Temperate Seawater. We hypothesize that similar effects of rhamnolipid-based stimulation of oil biodegradation might be observed in real-world oil spill scenarios near the Helgoland Island, which is characterized by high nutrient concentrations.[66] However, other oceanic locations facing oil spill hazards with different physicochemical conditions might result in different effects of biosurfactants compared to chemical dispersants. For example, nutrient bioavailability might have a substantial impact on microbial performance during oil biodegradation.[67] A previous study showed that dispersant-induced inhibition of microbial growth and oil biodegradation was more significant under substrate-limited conditions (*e.g.*, carbon starvation).[11] This might also explain why the effects of chemical dispersants regarding potential inhibition of oil biodegradation were not as pronounced compared to microcosms with rather nutrient-poor seawater from the deep Gulf of Mexico.[9]

CONCLUSIONS

Overall, our results highlight the superiority of biosurfactants compared to the chemical dispersants Corexit and Slickgone in oil spill scenarios simulated with eutrophic and temperate seawater. Oil addition in the presence of rhamnolipid stimulated different microbial oil degraders (*e.g.*, *Oleispira*) in terms of both growth and oil biodegradation

https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266

activities. Therefore, high-yield and cost-effective rhamnolipid production to sustain large-scale production for oil spills is an important research topic. Although recent studies have developed combinations of biosurfactants and/or chemical dispersants to gradually replace chemical dispersants,[68] more research is still needed to develop and establish more efficient, practical, and environmentally friendly dispersant formulations. In addition, our findings highlight the need for additional research on the impacts of different biosurfactants and chemical dispersants and their impacts on microbial populations and oil biodegradation efficiencies in a variety of marine environmental settings.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.3c00048.

Method of hydrocarbon extraction and phylogenetic tree construction; location of the sampling site; changes of dissolved organic carbon, free-living cell abundances, microbial α diversity indexes, absolute abundance of dominant oil degraders, and total residual ratio of oil hydrocarbons during incubation; pictures showing the macroscopic particles and microbial cell aggregates in microcosms; principal coordinate analysis of bacterial communities among different treatments; DPM counts of $^{14}CO_2$ in radiotracer assays; and Pearson's correlation between the abundance of potential oil degraders and different environmental factors (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Ute Kieb and Moritz Holtappels from the Alfred-Wegener-Institut (AWI) for collecting the seawater used in the experiments, Ellen Röhm for conducing nutrient and DOC analyses, Franziska Schädler for assistance during the molecular analysis, and the Isotope Laboratory in Tuebingen University for providing training and access to the radiotracer laboratory. The authors further acknowledge Mittelplate, Wintershall Dea AG, for providing the crude oil, as well as the National Oceanic and Atmospheric Administration and DASIC International Ltd for providing Corexit 9500 and Slickgone NS, respectively. This research was supported by a fellowship from the China Scholarship Council (granted to L.L), an Emmy Noether fellowship from the Deutsche Forschungsgemeinschaft (German Research Foundation; DFG grant 326028733 provided to S.K.), the Institutional Strategy of the University of Tübingen (DFG; Zukunftskonzept [ZUK] 63), and Germany's Excellence Strategy (DFG; cluster of Excellence EXC2124; project ID 390838134).

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https://doi.org/10.1021/acsestwater.3c00048
ACS EST Water 2023, 3, 2255−2266