1 **BIOLOGICAL SCIENCES - Environmental Sciences**

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3 **Chemical dispersants can suppress the activity of natural oil-degrading microorganisms**

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Abstract

During the Deepwater Horizon oil well blowout in the Gulf of Mexico, the application of 7 million liters of chemical dispersants aimed to stimulate microbial crude oil degradation by increasing the bioavailability of oil compounds. However, the effects of dispersants on oil biodegradation rates are debated. In laboratory experiments, we simulated environmental conditions comparable in the hydrocarbon-rich, 1100m deep, plume that formed during the Deepwater Horizon discharge. The presence of dispersant significantly altered the microbial community composition through selection for potential dispersant-degrading *Colwellia*, which also bloomed *in situ* in Gulf deep-waters during the discharge. In contrast, oil addition lacking dispersant stimulated growth of natural hydrocarbon-degrading *Marinobacter*. Dispersants did not enhance heterotrophic microbial activity or hydrocarbon oxidation rates. Extrapolating this comprehensive data set to real world scenarios questions whether dispersants stimulate microbial oil degradation in deep ocean waters and instead highlights that dispersants can exert a negative effect on microbial hydrocarbon degradation rates.

45   ***Significance Statement***

46   *Oil spills resulting from anthropogenic activity, such as the explosion and sinking of the*

47   *Deepwater Horizon drilling rig, are a significant source of hydrocarbon inputs into the marine*

48   *environment. As a primary response to oil spills, chemicals are applied to disperse contiguous*

49   *oil slicks into smaller droplets that may be more bioavailable to microorganisms. We provide*

50   *compelling evidence that chemical dispersants applied to deep-sea waters in the Gulf of Mexico*

51   *do not stimulate oil biodegradation. Direct measurements of alkane and aromatic hydrocarbon*

52   *oxidation rates revealed instead that dispersants suppressed microbial activity. Dispersants*

53   *impacted the microbial community composition and enriched bacterial populations with the*

54   *ability to utilize dispersant-derived compounds as growth substrates, while oil-alone enriched*

55   *for natural hydrocarbon degraders.*

56   Crude oil enters marine environments through geophysical processes at natural

57   hydrocarbon seeps (1) at a global rate of ~700 million liters per year (2). In areas of natural

58   hydrocarbon seepage, such as the Gulf of Mexico (hereafter Gulf), exposure of indigenous

59   microbial communities to natural oil fluxes can select for microbial populations that utilize oil-

60   derived hydrocarbons as carbon and energy sources (3, 4). The uncontrolled deep-water oil well

61   blowout that followed the explosion and sinking of the *Deepwater Horizon* (DWH) drilling rig in

62   2010 released more than 750 million liters of oil into the Gulf; roughly 7 million liters of

63   chemical dispersants were applied at the sea surface and seabed (5) to disperse hydrocarbons and

64   stimulate oil biodegradation. A deep-water (1000-1300 m) plume, enriched in aliphatic and

65   aromatic hydrocarbons (6-11) and the anionic surfactant dioctyl sodium sulfosuccinate (DOSS)

66   (12, 13) a major component of the dispersants (14), formed early in the discharge (7). The

67   chemistry of the hydrocarbon plume significantly altered the microbial community (11, 15-17),

68   driving rapid enrichment of low-abundance bacterial taxa such as *Oceanospirillum*,

69   *Cycloclasticus*, and *Colwellia* (18). In contrast, the major hydrocarbon degraders from Gulf

70   waters that are adapted to slow-diffusive natural hydrocarbon seepage were present in low-

71   abundance or absent in DWH deep-water plume samples, suggesting an inability to cope with

72   plume conditions (18).

73   Chemical dispersants break up surface oil slicks, reduce oil delivery to shoreline

74   ecosystems (19), and increase oil dissolution in the water column, presumably making it more

75   bioavailable (20) and potentially stimulating biodegradation (21). The efficacy of dispersants in

76   achieving these aims remains poorly documented (22) and, in some cases, dispersant application

77   led to substantial negative environmental effects (e.g. *Torrey Canyon* oil spill (23)). Dispersant

78   application often requires ecological trade-offs (24) and little is known about the impacts of

79 dispersants on the activity and abundance of natural hydrocarbon-degrading microorganisms

80 (25). This work addressed three key questions: 1) Do dispersants influence microbial community

81 composition? 2) Is the indigenous microbial community as effective at oil biodegradation as

82 microbial populations resulting from dispersants exposure? And, 3) Do dispersants and

83 chemically dispersed oil affect hydrocarbon biodegradation rates?

84 Laboratory experiments were employed to unravel the effects of oil-only (supplied as a

85 water-accommodated fraction; 'WAF'), Corexit 9500 ('dispersant-only'), oil-Corexit 9500

86 mixture (supplied as a chemically enhanced water-accommodated fraction; 'CEWAF') or a

87 CEWAF with nutrients ('CEWAF+nutrients') (26) on Gulf deep-water microbial populations (*SI*

88 *Appendix* Fig. S1 and S2). Experimental conditions (*SI Appendix* Table S1) mimicked those

89 prevailing in the DWH deep-water hydrocarbon plume (6-13, 18). The results show that

90 dispersant application selected for specific microbial taxa and oligotypes with 16S rRNA gene

91 sequences similar to those recovered *in situ* during the DWH discharge. Surprisingly, when

92 CEWAF (±nutrients) was added to deep seawater, microbial activity was not stimulated nor were

93 microbial oil-degradation rates enhanced.

94

95 **Results and Discussion**

96 **Dispersant significantly altered microbial community composition**

97 We hypothesized that dispersants would alter microbial community composition and that the

98 selection of one population over another would drive differences in hydrocarbon-degradation

99 rates, altering the oil-degradation efficiency. We therefore explored patterns in microbial

100 abundance (Fig. 1a) using microscopy and community composition using Illumina paired-end

101 sequencing of bacterial 16S rRNA gene amplicons (Fig. 1b). We resolved closely related

102 bacterial taxa that would otherwise group into a single operational taxonomic unit (OTU) using

103 oligotyping analysis (27) (Fig. 2). We furthermore highlighted the ecological preference of

104 specific microbial taxa using statistical correspondence analysis (CA) (*SI Appendix* Fig. S3-7).

105 All dispersant-amended treatments showed ingrowth of *Colwellia* (*SI Appendix* Fig. S3), a

106 group containing both hydrocarbon and dispersant degraders (28). After one week of incubation,

107 the relative abundance of *Colwellia* compared to other Bacteria increased from 1% to 26-43% in

108 dispersant-only and CEWAF (±nutrients) treatments (Fig. 1b). In contrast, *Colwellia* was a

109 minority (1-4%) in WAF treatments. Selective enrichment of *Colwellia* in dispersant-only

110 treatments could indicate that dispersant components served as growth substrates. Detailed

111 analysis revealed that the relative abundance of *Colwellia* oligotypes 01, 02, and 05 increased in

112 dispersant treatments (Fig. 2a, *SI Appendix* Fig. S4). Phylogenetic analysis of the 16S rRNA

113 gene amplicons confirmed that these oligotypes were closely related to species detected in DWH

114 plume samples *in situ* (9, 16, 18) (*SI Appendix* Fig. S8), verifying the environmental relevance of

115 these organisms.

116 Though *Colwellia* oligotypes 03 and 10 increased in WAF treatments, the dominant

117 microbial responder to WAF addition was *Marinobacter*, whose relative abundance increased

118 from 2% to 42% of all Bacteria after 4 weeks (Fig. 1b). In contrast, in dispersant-only and

119 CEWAF (±nutrients) treatments, *Marinobacter* comprised only 1-5% of all sequences. The CA

120 analysis emphasized the dominance of *Marinobacter* in WAF samples (*SI Appendix* Fig. S5) and

121 the same *Marinobacter* oligotypes occurred across all treatments, illustrating that dispersants did

122 not select for specific *Marinobacter* oligotypes, as was the case for *Colwellia* (Fig. 2b). The

123 *Marinobacter* (*SI Appendix* Fig. S9) degrade a wide variety of hydrocarbons, including pristane,

124 hexadecane, octane, toluene, benzynes, phenanthrene, etc. (29-31) and are likely dominant

125   hydrocarbon degraders under natural conditions. However, their abundance clearly declined in

126   the presence of dispersants. Whether *Colwellia* outcompetes *Marinobacter* or whether

127   *Marinobacter* is inhibited by some component of Corexit 9500 or the CEWAF remains to be

128   resolved (26).

129   Like *Marinobacter*, the abundance of *Cycloclasticus* increased primarily in the absence of

130   dispersants. In WAF treatments, the relative abundance of *Cycloclasticus* increased from 12% to

131   23% after 1 week and an oligotype (type 03) closely related to *Cycloclasticus pugetii* (Fig. 2c

132   and *SI Appendix* Fig. S10), which degrades naphthalene, phenanthrene, anthracene, and toluene

133   as sole carbon sources (32), increased substantially. *Cycloclasticus* also increased slightly in

134   relative abundance in the CEWAF+nutrients treatment (Fig. 1b), but less so than in the WAF

135   treatment.

136   *Oceaniserpentilla* (a.k.a. DWH *Oceanospirillum* (33)) abundance decreased consistently

137   across treatments and their abundance did not correlate with the presence or absence of WAF,

138   dispersant or CEWAF (±nutrients) (Fig. 1b, 2d, and *SI Appendix* Fig. S7). The *Oceaniserpentilla*

139   oligotypes closely resembled those observed *in situ* during the DWH incident (18) (*SI Appendix*

140   Fig. S11). The DWH *Oceanospirillum* oxidize *n*-alkanes and cycloalkanes (17); the latter were

141   lacking in the microcosms because they are absent in surrogate Macondo oil, possibly explaining

142   the low abundance of *Oceanospirillum* in these experiments.

143   **Stimulation of cell growth and exopolymer formation**

144   At the start of the experiment, all treatments exhibited similar cell abundance

145   (3×10^5 cells mL^{-1}; Fig. 1a). At the end of the experiment, microbial abundance in the WAF

146   treatment increased by a factor of 60, which was significantly higher (T_4: $p < 0.0001$) relative to

147   microbial abundance in CEWAF (±nutrients) treatments. Microbial abundance in dispersant-only

148   treatments increased by a factor of 29, far below levels in WAF treatments but clearly showing

149   stimulation of microbial growth by dispersant alone.

150   Marine snow, here defined as particles >0.5 mm in diameter, formed in WAF, dispersant-

151   only and CEWAF (±nutrients) microcosms, but differed in appearance, size and abundance

152   across treatments (*SI Appendix*, *Supplementary Results and Discussion*). Microbial exopolymeric

153   substances, including transparent exopolymer particles (TEP) serve as the matrix for marine

154   snow formation (34). Oil-degrading bacteria produce copious amounts of TEP as biosurfactants

155   (35). TEP production increased in the WAF microcosms relative to controls, underscoring the

156   metabolic activities of oil-degrading bacteria (*SI Appendix* Table S1). The abundance of TEP

157   could not be quantified in dispersant treatments (26) but massive formation of oil snow was

158   observed in the CEWAF+nutrients treatments (*SI Appendix*, *Supplementing Results and*

159   *Discussion*), inferring that TEP levels were likely elevated. The different types of macroscopic

160   particles that formed resembled marine oil snow observed *in situ* during the DWH oil spill (*SI*

161   *Appendix* Fig. S12 f, g). Fluorescence *in situ* hybridization in combination with catalyzed

162   reporter deposition (CARD-FISH) revealed that *Gammaproteobacteria* and *Alteromonadales,*

163   including *Colwellia* dominated micro-aggregate populations in CEWAF+nutrients treatments (*SI*

164   *Appendix* Fig. S12q-r and *SI Appendix*, *Supplementary Results and Discussion*). These findings

165   point towards *Colwellia's* involvement in marine oil snow formation when dispersants were

166   present.

167   **Microbial activity and oil and dispersant degradation**

168   Addition of dispersants did not enhance bacterial oil degradation or general microbial activity as

169   reflected by rates of hydrocarbon oxidation, bacterial protein production, and exoenzyme

170   activities. Radiotracer assays allowed direct quantification of alkane ($[1-^{14}C]$-hexadecane) and

171 polycyclic aromatic hydrocarbon (PAH; [1-^{14}C]-naphthalene) oxidation rates across treatments

172 (26) (Fig. 3 a, b). These two hydrocarbon classes are chemically distinct and PAHs are inherently

173 toxic and mutagenic (36). Naphthalene concentrations in the WAF treatments exceeded

174 hexadecane concentrations, as expected given the relative solubility of the two compounds (e.g.

175 naphthalene and hexadecane solubility at 25ºC are 31.6 and 9×10^{-4} mg L^{-1}, respectively).

176   Hexadecane oxidation rates were significantly (T_3 and T_4: $p = 0.004$) lower in dispersant-

177 only and CEWAF (±nutrients) treatments (Fig. 3a), implying that dispersants suppressed

178 hexadecane degradation. Similarly, naphthalene oxidation rates in the WAF treatments were

179 significantly (T_3 and T_4: $p < 0.0001$) higher than those in dispersant-only and CEWAF

180 (±nutrients) treatments, indicating that dispersants inhibited also microbial naphthalene

181 degradation (Fig. 3b). Biodegradation of other n-alkanes and PAHs could be similarly decreased

182 or inhibited by dispersants.

183   Rates of ^{3}H-leucine incorporation showed that bacterial protein synthesis was highest in

184 WAF treatments, particularly at later time points (Fig. 3c; *SI Appendix* Table S1), underscoring

185 that dispersant-only and CEWAF (±nutrients) did not stimulate bacterial production to the same

186 degree (T_3 and T_4: $p < 0.001$). We observed similar patterns for exoenzyme activities indicative

187 of potential bacterial degradation rates of carbohydrate- and protein-rich exopolysaccharides

188 (EPS). All enzyme assays exhibited up to one order of magnitude higher activities in the WAF

189 and dispersant-only treatments compared to the CEWAF (±nutrients) treatments (Fig. 3d-f, *SI*

190 *Appendix* Table S1).

191   Results from gas chromatography-mass spectrometry (GC-MS) and excitation/emission

192 matrix spectra (EEMS) confirmed variable rates of oil-derived hydrocarbon degradation across

193 treatments. Concentrations of n-alkanes and hexadecane decreased more significantly in WAF

194   treatments (*SI Appendix* Fig. S13). However, addition of dispersant led to changes in degradation

195   patterns for individual compounds. In the WAF treatment, microorganisms preferentially

196   degraded low molecular weight *n*-alkanes (<C20) relative to high molecular weight (≥C21)

197   compounds and the isoprenoids, pristane and phytane. In the dispersant treatments, this pattern

198   was not observed (*SI Appendix* Fig. S14). The temporal changes in *n*-alkane concentration (*SI*

199   *Appendix* Fig. S13) supported the rate data (*SI Appendix* Table S1), and underscored the fact that

200   oil degradation was highest in WAF treatments and that addition of CEWAF+nutrients did not

201   generate higher overall hydrocarbon degradation rates.

202   Liquid chromatography tandem mass spectrometry (LC–MS/MS) enabled quantitative

203   detection of distinct dispersant compounds: the anionic surfactant DOSS and the nonionic

204   surfactants Span 80, Tween 80, Tween 85, as well as, α/β-ethyhexylsulfosuccinate (EHSS), the

205   hydrolysis products of DOSS (13, 37). Biodegradation of DOSS to EHSS occurs under aerobic

206   conditions (37). In the dispersant-only treatment, a significant (p <0.05) decrease (8%) of DOSS

207   and an increase of EHSS (15%) was detected at T_3 (*SI Appendix* Fig. S15a, b). At all other time

208   points, no significant (p <0.05) change in DOSS or EHSS was observed in the dispersant-only

209   treatments (*SI Appendix* Fig. S15a, b). However, the nonionic surfactants were consumed within

210   1 week driving concentrations below detection (20 µg L^{-1}; *SI Appendix* Fig. S15c, d). Though the

211   carrier solvent dipropylene glycol butyl ether (DGBE) was not analyzed, it could have served as

212   an additional growth substrate for microorganisms (38) in the dispersant treatments.

213   In the CEWAF (±nutrients) treatments, DOSS decreased significantly (p <0.05) after

214   6 weeks (*SI Appendix* Fig. S15a). No significant change in EHSS concentrations was observed in

215   CEWAF (±nutrients) treatments (*SI Appendix* Fig. S15 b), indicating that DOSS was converted

216   to other products. This observation was supported by the formation of sulfur-containing

217 compounds detected by ultra-high resolution Fourier transform ion cyclotron resonance mass

218 spectrometry (FT-ICR-MS) (39) (Fig. 4f and 4g). In the CEWAF (±nutrients) treatments, the

219 nonionic surfactants were at or below detectable levels at time zero, inferring that they probably

220 associated with residual organic phase that was removed during CEWAF preparation. However,

221 similarly to dispersant-only setups, low concentrations of nonionic compounds and DGBE could

222 have served as additional microbial growth substrates in CEWAF (±nutrients) amended

223 treatments.

Molecular characterization of dissolved organic matter

225 Most compounds remaining in weathered oil-contaminated fluids fall outside the GC-amenable

226 analytical window (40), and conventional GC analysis (41) did not detect roughly 60% (on a

227 mass basis) of compounds in Macondo crude oil. The FT-ICR-MS analysis further supported the

228 conclusion that significantly more oil-derived dissolved organic molecules were degraded in the

229 WAF compared to CEWAF (±nutrients) treatments, underscoring a more extensive degree of oil

230 biodegradation in the absence of dispersant (Fig. 4).

231 Between 50 and 74% of the degraded compounds were highly unsaturated CHO molecular

232 formulae (Fig. 4a, b), which include the common aromatic hydrocarbons abundant in Macondo

233 crude oil (41). Oil-derived nitrogen-containing dissolved organic matter (DOM) compounds also

234 decreased during the incubations (between 26 and 43% of the decreasing formulae, Fig 4c, d),

235 agreeing with previous studies reporting that crude oil (42), including Macondo oil (41), contains

236 numerous biodegradable polar and water-soluble organic nitrogen compounds. The WAF

237 incubations exhibited the highest rates of degradation of oil-derived nitrogen-containing

238 compounds (ca. 8% of the initially present formulae vs. ~1% in the CEWAF treatment,

239 respectively) (39). In the WAF treatments, protein synthesis rates significantly exceeded those in

11

240   the dispersant-amended treatments (T_4: $p = 0.0002$), and a 31% decrease of seawater- and oil-

241   derived dissolved organic nitrogen (DON) concentrations in these treatments indicates that the

242   generation of microbial biomass was supported by significant rates of nitrogen uptake (*SI*

243   *Appendix* Table S1). The enhanced uptake of oil-derived organic nitrogen underscores that oil

244   can serve as an important nitrogen source when oil-degrading microbial communities are

245   nitrogen limited (43).

246   Organic sulfur compounds are abundant in Macondo oil (41). The FT-ICR-MS results

247   imply complex processing of sulfur-containing oil-derived and dispersant-derived DOM,

248   including degradation of oil-derived sulfur compounds and formation of new organic sulfur

249   compounds (Fig. 4e-g). The FT-ICR-MS detected DOSS (molecular formula $C_{20}H_{38}O_7S$; see

250   arrow in Fig. 4f, g) in all dispersant-amended treatments after six weeks of incubation. The

251   formation of new organic sulfur-compounds was particularly pronounced in the CEWAF

252   ($\pm$nutrients) samples (circled area in Fig. 4f, g), signaling that their formation was stimulated by

253   dispersant addition. Elevated relative abundances of *Colwellia* in post-DWH discharge seawater

254   along with enhanced expression of genes involved in the degradation of sulfur-containing

255   organic matter (e.g., alkanesulfonate monooxygenase) (44) infer a role for *Colwellia* in organic

256   sulfur cycling *in situ*. The genome of *C. psychrerythraea* 34H has a remarkable potential for

257   sulfur metabolism (45). Thus, we hypothesize that *Colwellia* were important in the observed

258   turnover of DOSS-derived sulfur compounds as a result of their capability to metabolize the

259   organic sulfur compounds in dispersants; they may have exhibited similar metabolic abilities *in*

260   *situ* during the DWH incident.

261   **Factors regulating microbial activity**

262   Substantial variations in the inorganic nitrogen-containing compounds were observed throughout

263   the experiment. Nitrite (NO_2^-) concentrations increased from below detection limit to 0.6 μM (*SI*

264   *Appendix* Table S1) while nitrate (NO_3^-) concentrations decreased significantly in the WAF

265   (from 23 µM to 2 µM; $p < 0.0001$) and dispersant-only (from 23 µM to 14 µM; $p = 0.002$)

266   microcosms (*SI Appendix* Table S1), implying active nitrate uptake and potentially incomplete

267   denitrification. While denitrification is generally considered to occur under anoxic or suboxic

268   conditions, *Marinobacter hydrocarbonoclasticus* is classified as an aerobic denitrifier and may

269   have denitrified in the presence of oxygen (46) in the WAF treatments. Likewise, *Colwellia*

270   *psychrerythraea* has the genetic potential to denitrify. Genes for hydrocarbon degradation under

271   nitrate-reducing conditions (*bbs*) as well as genes for denitrification (*narG, nirS, nirK and nosZ*)

272   were observed *in situ* in the DWH deep-water plume (43). The presence of mucus-rich, microbial

273   aggregates could further promote denitrification through formation of anoxic microzones (47).

274   Microbial communities, especially in WAF treatments, assimilated phosphate but were never

275   phosphate limited (*SI Appendix* Table S1).

276   To further unravel factors that regulate activity of key bacterial taxa, we determined

277   statistically significant relationships between experimental conditions (geochemistry, cell counts

278   and microbial activity) and oligotype abundances. Distinct trends were apparent for *Colwellia*,

279   *Marinobacter, Oceaniserpentilla,* and *Cycloclasticus* as were correlations for specific oligotypes

280   (*SI Appendix* Table S2). Of the 24 detected *Colwellia* oligotypes, many correlated positively with

281   concentrations of dissolved organic carbon (DOC) (88%), NH_4^+ (50%), cell counts (46%), and

282   bacterial production (79%) as well as peptidase, glucosidase and lipase (38-79%) activities. The

283   majority of *Colwellia* oligotypes correlated negatively with concentration of total *n*-alkanes,

284   hexadecane, naphthalene and phenanthrene (71-79%), supporting the hypothesis that oligotypes

285   of this taxon are predominantly responsible for dispersant breakdown. A considerable number of

286   the 24 *Marinobacter* oligotypes correlated positively with cell counts (79%), bacterial production

287   (79%) as well as peptidase and lipase (67-71%) activities. In contrast to *Colwellia*, *Marinobacter*

288   oligotypes correlated positively to total petroleum concentrations (83%) and hexadecane

289   oxidation (71%), highlighting a key role for these microorganisms in hexadecane degradation in

290   the absence of dispersants. *Oceaniserpentilla* and *Cycloclasticus* oligotypes (30 and 31 types,

291   respectively) correlated positively with nitrate and total *n*-alkanes, hexadecane, naphthalene, and

292   phenanthrene (71-80%) concentrations. In addition, *Cycloclasticus* abundance positively

293   correlated with naphthalene oxidation (61%), supporting their involvement in PAH degradation.

294   **Evaluating the utility of dispersants**

295   Dispersants are used globally as a response action after oil spills to disperse oil slicks, enhance

296   the relative oil surface area in water, and to stimulate microbial hydrocarbon degradation. During

297   the DWH, the deep-sea application of dispersants was unprecedented. The data shown here do

298   not support dispersant stimulation of oil biodegradation, questioning the utility of dispersant

299   application to pelagic ocean ecosystems. Different results could be expected in pelagic

300   environments that are not characterized by natural oil seepage. However, it seems unlikely that

301   dispersants would stimulate hydrocarbon degradation in a system that lacks a substantial

302   population of hydrocarbon degraders when they had no effect in samples from a system that was

303   primed for oil degradation (e.g., oil degraders account for 7-10% of the natural microbial

304   population at GC600 (18)). In fact, the presence of dispersant selected against the most effective

305   hydrocarbon degrading microorganisms (*Marinobacter*). This multi-disciplinary data set strongly

306   suggests that dispersants negatively influenced microbial hydrocarbon-degradation rates, with

307   maximal oil-degradation rates occurring in WAF treatments. Though we quantified degradation

308   rates of only two hydrocarbons, hexadecane and naphthalene, biodegradation of other *n*-alkanes

309   and PAHs may be similarly decreased or inhibited by dispersants. Quantification of the total

310   crude oil showed that the highest levels of oil biodegradation occurred in treatments without

311   dispersants. While microbial activities in CEWAF (±nutrients) microcosms were comparable for

312   1 week, rates were stimulated by nutrients in the later time points (e.g. hydrocarbon oxidation

313   rates after 4 and 6 weeks), suggesting progressive nutrient limitation. Clearly, there was no need

314   to chemically jump-start oil biodegradation through dispersant application in deep Gulf waters.

315   Therefore, caution is advised when considering dispersant applications as a primary response for

316   future oil spills in deep-water environments similar to the Gulf. A full understanding of

317   dispersant impacts on microbial populations requires immediate and careful evaluation of

318   dispersant impacts across a variety of oceanic and terrestrial habitats.

319

320   **Material and Methods**

321   **Microcosm setup and sampling**

322   Seawater (160 L) was sampled from 1178 m at an active natural hydrocarbon seep in the

323   northern Gulf on 7th of March 2013 (site GC600, latitude 27.3614, longitude -90.6018; Fig. S1).

324   After sampling, seawater was transferred to 20 L carboys and stored at 4°C onboard the ship for

325   3 days. The carboys were transported at 4°C to the laboratory at UGA where the experiment and

326   sampling was conducted in an 8°C cold room. Setup and sampling of microcosms are described

327   in detail in the *SI Appendix* and *Supplementary Material and Methods*. In brief, we incubated 72

328   2-L glass bottles (1.6 L sample per bottle) on a roller table (Fig. S2). Treatments (WAF,

329   dispersant-only, and CEWAF±nutrients) and controls (abiotic, biotic) were set up in triplicate for

330   each time point. Sampling (except for the CEWAF+nutrients treatment) was performed after 0

331 days (T_0), 1 week (T_1), 2.5 weeks (16 days; T_2), 4 weeks (T_3), and 6 weeks (T_4);

332 CEWAF+nutrients treatments were sampled at T_0, T_1 and T_4. Water accommodated fractions

333 (WAFs) were prepared by mixing pasteurized seawater with oil and/or dispersants for 48 h at

334 room temperature and subsequently sub-sampling WAFs, excluding contamination by oil or

335 dispersants phases; see also *SI Appendix*.

Molecular, microbiological and geochemical analyses

337 Nutrients (nitrate, nitrite, phosphate, and ammonium), DIC and oxygen as well as hydrocarbons

338 (48) and dispersants concentrations were monitored during the course of the experiment (see *SI*

339 *Appendix*). Microbial community evolution and cell numbers were investigated for each sample

340 using 16S rRNA amplicon Illumina sequencing (Bioproject accession PRJNA253405),

341 computational oligotyping analysis (27), and total cell counts (see also *SI Appendix*). Activity

342 measurements were performed using enzyme assays (peptidase, glucosidase, lipase) (49), ^{3}H-

343 leucine incorporation analysis (50), as well as a newly developed method for the analysis of ^{14}C-

344 hexadecane and ^{14}C-naphthalene oxidation (see *SI Appendix*). TEP analyses were carried out for

345 controls and oil-only treatments (51) and CARD-FISH analysis (52) were performed in particular

346 for microbial-aggregate formations in nutrient treatments (*SI Appendix*). Oil-derived

347 hydrocarbons were extracted from water samples using a mixture of hexane:dichloromethane

348 (1:1, v/v). After concentration, hydrocarbon compounds were identified and quantified by Gas

349 Chromatography/Mass Selective Detector (GC/MSD) using conditions described previously (53)

350 (see *SI Appendix*). Analysis of the surfactant components of the dispersant Corexit was

351 performed as described elsewhere (13), with minor modification (see *SI Appendix*). Fourier

352 transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) was carried out to analyze

353   DOM (54) (see *SI Appendix*). Statistical analyses were used to unravel factors that drive

354   microbial community evolution and microbial activities (see *SI Appendix*).

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369   **Author Contributions** S.K. and S.B.J designed the experiments and wrote the manuscript with

370   input from all authors. S.K. and M. Seidel setup and sampled the microcosms. S.K. and S.G.

371   accomplished DNA extraction, sequencing, oligotyping and phylogenetic analyses. S.K.

372   performed bacterial production and ^{14}C-hydrocarbon oxidation rate assays, total cell counts and

373   CARD-FISH analyses. K.Z. performed enzyme assays. U.P generated TEP data and S.K. and

374   U.P. described micro-aggregate formations M.P. and J.F. conducted Corexit surfactant analyses.

375   M.Seidel, P.M.M. and T.D. carried out FT-ICR-MS analyses. P.M.M., M.Seidel and K.M.L.

376 conducted hydrocarbon analyses (P.M. and M.S. *via* GC-MS and K.M.L. *via* EEMS). M. Sogin,

377 S.G., S.K. and M.P. carried out statistical analyses. All authors discussed the results and their

378 interpretation.

379

380 **References**

381 1.   Kvenvolden KA, Cooper CK (2003) Natural seepage of crude oil into the marine

382   environment. *Geo-Mar Lett* 23(3):140-146.

383 2.   National Research Council (2003) *Committee on oil in the sea III: inputs, fates, and*

384   *effects* (The National Academic Press, Washington, D.C.) p 280.

385 3.   Widdel F, Knittel K, Galushko A (2010) Anaerobic hydrocarbon-degrading

386   microorganisms: an overview. *Handbook of Hydrocarbon and Lipid Microbiology*, eds

387   Timmis KN, McGenity T, van der Meer JR, de Lorenzo V (Springer Berlin Heidelberg),

388   Vol 3, pp 1997-2021.

389 4.   Head IM, Jones DM, Roling WFM (2006) Marine microorganisms make a meal of oil.

390   *Nat Rev Micro* 4(3):173-182.

391 5.   National-Commission-on-the-BP-Deepwater-Horizon-Oil-Spill-and-Offshore-Drilling

392   (2011) The use of surface and subsea dispersants during the BP Deepwater Horizon oil

393   spill

394 6.   Diercks A-R, et al. (2010) Characterization of subsurface polycyclic aromatic

395   hydrocarbons at the Deepwater Horizon site. *Geophys Res Lett* 37(20):L20602.

396 7.   Camilli R, et al. (2010) Tracking hydrocarbon plume transport and biodegradation at

397   Deepwater Horizon. *Science* 330:201-204.

398   8.   Kessler JD, et al. (2011) A persistent oxygen anomaly reveals the fate of spilled methane

399   in the deep gulf of Mexico. *Science* 331:312-315.

400   9.   Valentine DL, et al. (2010) Propane respiration jump-starts microbial response to a deep

401   oil spill. *Science* 330:208-211.

402   10.   Joye SB, MacDonald IR, Leifer I, Asper V (2011) Magnitude and oxidation potential of

403   hydrocarbon gases released from the BP oil well blowout. *Nature Geosci* 4(3):160-164.

404   11.   Reddy CM, et al. (2012) Composition and fate of gas and oil released to the water

405   column during the Deepwater Horizon oil spill. *Proc Natl Acad Sci USA* 109(50):20229–

406   20234.

407   12.   Kujawinski EB, et al. (2011) Fate of dispersants associated with the Deepwater Horizon

408   oil spill. *Environ Sci Technol* 45(4):1298-1306.

409   13.   Place BJ, et al. (2014) Trace analysis of surfactants in Corexit oil dispersant formulations

410   and seawater. *Deep-Sea Res Pt II* (0).

411   14.   U.S.-Environmental-Protection-Agency (2014) Questions and Answers on Dispersants.

412   15.   Valentine DL, et al. (2012) Dynamic autoinoculation and the microbial ecology of a deep

413   water hydrocarbon irruption. *Proc Natl Acad Sci USA* 109(50):20286-20291.

414   16.   Redmond MC, Valentine DL (2012) Natural gas and temperature structured a microbial

415   community response to the Deepwater Horizon oil spill. *Proc Natl Acad Sci USA*

416   109(50):20292-20297.

417   17.   Mason OU, et al. (2012) Metagenome, metatranscriptome and single-cell sequencing

418   reveal microbial response to Deepwater Horizon oil spill. *ISME J* 6(9):1715-1727.

419   18.   Kleindienst S, et al. (in review) Diverse, rare microbial taxa responded to the Deepwater

420   Horizon deep-sea hydrocarbon plume.

421 19. Lunel T, et al. (1996) Shoreline clean up during the Sea Empress incident: the role of surf

422   washing (clay-oil flocculation), dispersants and bioremediation. *Proceedings of the*

423   *nineteenth arctic and marine oilspill program (AMOP) technical seminar*, (Environment

424   Canada), p 2v.

425 20. Mu J, Jin F, Ma X, Lin Z, Wang J (2014) Comparative effects of biological and chemical

426   dispersants on the bioavailability and toxicity of crude oil to early life stages of marine

427   medaka (Oryzias melastigma). *Environ Toxicol Chem* 33(11):2576-2583.

428 21. Prince R, Butler J (2014) A protocol for assessing the effectiveness of oil spill dispersants

429   in stimulating the biodegradation of oil. *Environmental Science and Pollution Research*

430   21(16):9506-9510.

431 22. National-Research-Council (*Committee on Understanding Oil Spill Dispersants: Efficacy*

432   *and Effects. Oil spill dispersants: efficacy and effects.* (National Academies Press,

433   Washington, DC).

434 23. Smith JE (1968) *'Torrey Canyon' pollution and marine life. A report by the Plymouth*

435   *Laboratory of the Marine Biological Association of the United Kingdom* (Cambridge

436   University Press, Cambridge (UK)).

437 24. Peterson CH, et al. (2012) A tale of two spills. *Biosci Biotech Bioch* 62:461-469.

438 25. Kleindienst S, Paul J, Joye SB (in press) Using dispersants following oil spills: impact on

439   the composition and activity of microbial communities. *Nat Rev Microbiol*.

440 26. Kleindienst S, et al. (Supporting Online Material for "Chemical dispersants suppress the

441   activity of natural oil-degrading microorganisms".

442 27. Eren AM, et al. (2013) Oligotyping: differentiating between closely related microbial

443   taxa using 16S rRNA gene data. *Methods Ecol Evol* 4(12):1111–1119.

444 28. Chakraborty R, Borglin SE, Dubinsky EA, Andersen GL, Hazen TC (2012) Microbial

445   response to the MC-252 oil and corexit 9500 in the Gulf of Mexico. *Front Microbiol*

446   3:357.

447 29. Singer E, et al. (2011) Genomic potential of *Marinobacter aquaeolei*, a biogeochemical

448   "opportunitroph". *Appl Environ Microbiol* 77(8):2763-2771.

449 30. Huu NB, Denner EB, Ha DT, Wanner G, Stan-Lotter H (1999) *Marinobacter aquaeolei*

450   sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. *Int J Syst*

451   *Bacteriol* 49 Pt 2:367-375.

452 31. Gauthier MJ, et al. (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a

453   new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst*

454   *Bacteriol* 42(4):568-576.

455 32. Dyksterhouse SE, Gray JP, Herwig RP, Lara JC, Staley JT (1995) *Cycloclasticus pugetii*

456   gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine

457   sediments. *Int J Syst Bacteriol* 45(1):116-123.

458 33. Hazen TC, et al. (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria.

459   *Science* 330(6001):204-208.

460 34. Passow U (in press) Formation of rapidly-sinking, oil-associated marine snow. *Deep-Sea*

461   *Res Pt II* (0):doi:10.1016/j.dsr1012.2014.1010.1001.

462 35. Gutierrez T, et al. (2013) Role of bacterial exopolysaccharides (EPS) in the fate of the oil

463   released during the Deepwater Horizon oil spill. *PLoS ONE* 8(6):e67717.

464 36. Pickering RW (1999) A toxicological review of polycyclic aromatic hydrocarbons.

465   *Journal of Toxicology - Cutaneous and Ocular Toxicology* 18:101–135.

466   37.   Campo P, Venosa AD, Suidan MT (2013) Biodegradability of Corexit 9500 and
467   dispersed South Louisiana crude oil at 5 and 25 degrees C. *Environ Sci Technol*
468   47(4):1960-1967.

469   38.   Mason OU, et al. (2014) Metagenomics reveals sediment microbial community response
470   to Deepwater Horizon oil spill. *ISME J* 8(7):1464-1475.

471   39.   Seidel M, Kleindienst S, Dittmar T, Joye SB, Medeiros PM (accepted) Influence of
472   dispersant on the biodegradation of crude oil in deep sea water from the Gulf of Mexico:
473   Insights from ultra-high resolution mass spectrometry.

474   40.   Aeppli C, et al. (2012) Oil Weathering after the Deepwater Horizon Disaster Led to the
475   Formation of Oxygenated Residues. *Environ Sci Technol* 46(16):8799-8807.

476   41.   McKenna AM, et al. (2013) Expansion of the Analytical Window for Oil Spill
477   Characterization by Ultrahigh Resolution Mass Spectrometry: Beyond Gas
478   Chromatography. *Environ Sci Technol* 47(13):7530-7539.

479   42.   Corilo YE, et al. (2013) Oil Spill Source Identification by Principal Component Analysis
480   of Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectra.
481   *Anal Chem* 85(19):9064-9069.

482   43.   Lu Z, et al. (2011) Microbial gene functions enriched in the Deepwater Horizon deep-sea
483   oil plume. *ISME J* 6:451-460.

484   44.   Rivers AR, et al. (2013) Transcriptional response of bathypelagic marine
485   bacterioplankton to the Deepwater Horizon oil spill. *ISME J* 7(12):2315-2329.

486   45.   Methé BA, et al. (2005) The psychrophilic lifestyle as revealed by the genome sequence
487   of Colwellia psychrerythraea 34H through genomic and proteomic analyses. *Proc Natl*
488   *Acad Sci USA* 102(31):10913-10918.

489   46.   Dell'Acqua S, Pauleta SR, Moura JJ, Moura I (2012) Biochemical characterization of the

490   purple form of *Marinobacter hydrocarbonoclasticus* nitrous oxide reductase. *Philos*

491   *Trans R Soc Lond B Biol Sci* 367(1593):1204-1212.

492   47.   Ploug H, Kuehl M, Buchholz-Cleven B, Jorgensen BB (1997) Anoxic aggregates - an

493   ephemeral phenomenon in the pelagic environment? *Aquat Microb Ecol* 13:285-294.

494   48.   Joye SB, Bowles MW, Samarkin VA, Hunter KS, Niemann H (2010) Biogeochemical

495   signatures and microbial activity of different cold-seep habitats along the Gulf of Mexico

496   deep slope. *Deep-Sea Res Pt II* 57(21–23):1990-2001.

497   49.   Hoppe HG (1983) Significance of exoenzymatic activities in the ecology of brackish

498   water: measurements by means of methylumbelliferyl-substrates. *Marine Ecology-*

499   *progress Series* 11:299-308.

500   50.   Smith DC, Azam F (1992) A simple, economical method for measuring bacterial protein

501   synthesis rates in seawater using ^{3}H-leucine. *Mar Microb Food Webs* 6(2):107-111.

502   51.   Passow U, Alldredge AL (1995) A dye-binding assay for the spectrophotometric

503   measurement of transparent exopolymer particles (TEP). *Limnol Oceanogr* 40(7):1326-

504   1335.

505   52.   Kleindienst S, Ramette A, Amann R, Knittel K (2012) Distribution and in situ abundance

506   of sulfate-reducing bacteria in diverse marine hydrocarbon seep sediments. *Environ*

507   *Microbiol* 14(10):2689-2710.

508   53.   Medeiros PM, Bícego MC (2004) Investigation of natural and anthropogenic

509   hydrocarbon inputs in sediments using geochemical markers. I. Santos, SP-Brazil. *Mar*

510   *Pollut Bull* 49(9–10):761-769.

511   54.   Seidel M, et al. (2014) Biogeochemistry of dissolved organic matter in an anoxic

512   intertidal creek bank. *Geochim Cosmochim Acta* 140(0):418-434.

513

514   **Fig. 1. Dispersants affect the evolution of oil-degrading microbial populations a**, Average

515   and standard deviation of cell numbers from sample triplicates (log scale) monitored for 6 weeks

516   in microcosms. **b**, Relative abundance of bacterial groups in *in situ* Gulf of Mexico deep-water

517   and in the microcosm (average of triplicate samples). Reads of the V4V5 regions of the 16S

518   rRNA gene were clustered into OTUs and taxonomy was assigned with GAST.

519

520   **Fig. 2. Different microbial oligotypes respond to dispersants or oil (WAF). a-d**, Oligotyping

521   enabled the interpretation of 16S rRNA gene sequence diversity at the level of specific

522   oligotypes. Relative abundance averaged across biological triplicates of **a,** *Colwellia,* **b,**

523   *Marinobacter,* **c,** *Cycloclasticus* and **d,** *Oceanisperpentilla* oligotypes in microcosms, simulating

524   DWH spill-like plumes (biotic control, dispersant-only, CEWAF, WAF, CEWAF+nutrients)

525   monitored for 6 weeks.

526

527   **Fig. 3. Microbial activity, hydrocarbon oxidation and enzymatic activities are not enhanced**

528   **by dispersed oil (CEWAF ± nutrients). a**, **b**, Oxidation rates of ^{14}C-hexadecane and ^{14}C-

529   naphthalene as model compounds for alkanes and PAHs degradation, respectively (Table S1). **c**,

530   Rates of bacterial production increased up to three orders of magnitude in the two weeks

531   between the first and second sampling point (see also Table S1). **d-f**, Potential activities of

532   peptidase, glucosidase and lipase measured using fluorogenic substrate analogs were up to one

533   order of magnitude higher in the WAF and dispersant-only compared to the CEWAF ± nutrients

534   treatments. All data are illustrated as average of biological triplicates and error bars show

535   standard deviation of the mean (note that a lack of error bars means indicates standard deviations

536   too small to be shown on the plot scale).

537

538   **Fig. 4. Dispersants impact microbial turnover of dissolved organic matter.** Analysis of

539   molecular-level patterns in van-Krevelen diagrams (hydrogen-to-carbon, H/C, and oxygen-to-

540   carbon, O/C ratios; each circle represents a molecular formula). **a, b,** Molecular formulae present

541   in all treatments ($n = 1205$) and that significantly changed ($p \leq 0.01$, determined on triplicates

542   using Student's t-test) relative signal intensities between the initial and last time points. The

543   color scales represent changes in relative intensities (open circles, no significant change), **c, d,**

544   Van-Krevelen diagrams showing nitrogen-containing formulae (color scale depicts N/C ratios;

545   open circles, formula contained no nitrogen). **e-g,** Van-Krevelen diagrams presenting changes in

546   the presence or absences of sulfur-containing compounds (red circles, produced compounds, i.e.,

547   absent at T_0 but present at T_4; blue circles, degraded compounds , i.e. absent at T_4 but present at

548   T_0, open circles, common compounds present at T_0 and T_4). DOSS (molecular formula

549    $C_{20}H_{38}O_7S$, marked by arrow) was present at T_0 and T_4. Several sulfur-containing compounds

550   were exclusively produced in the dispersant-amended treatments (molecular formulae marked by
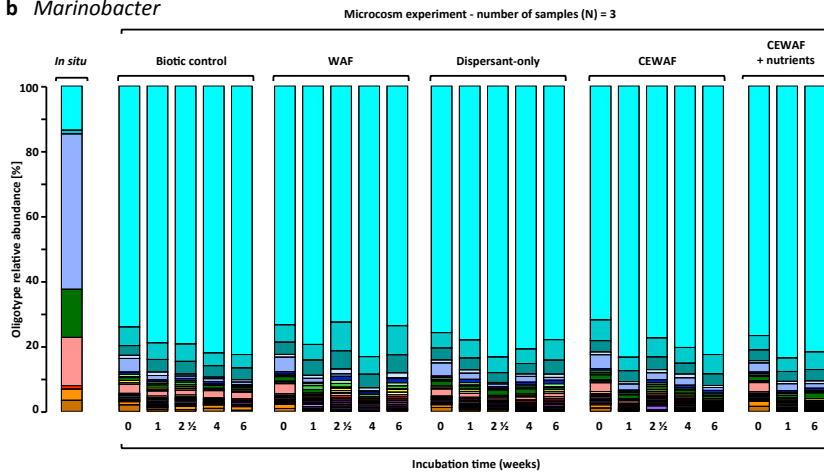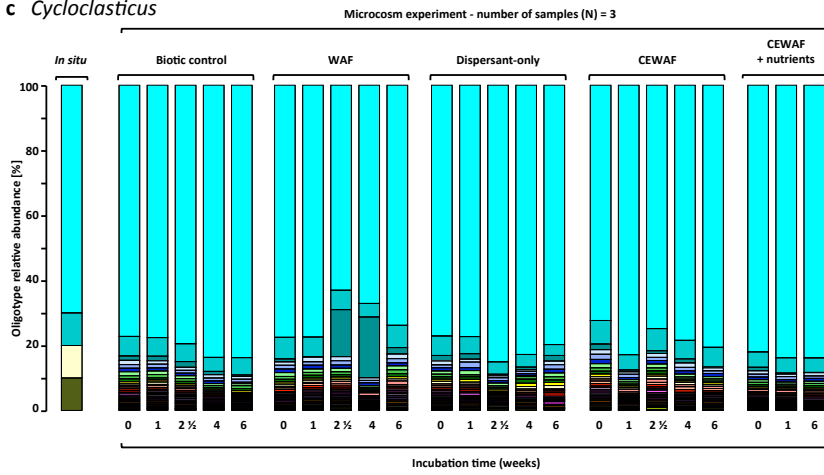
551   an ellipse).
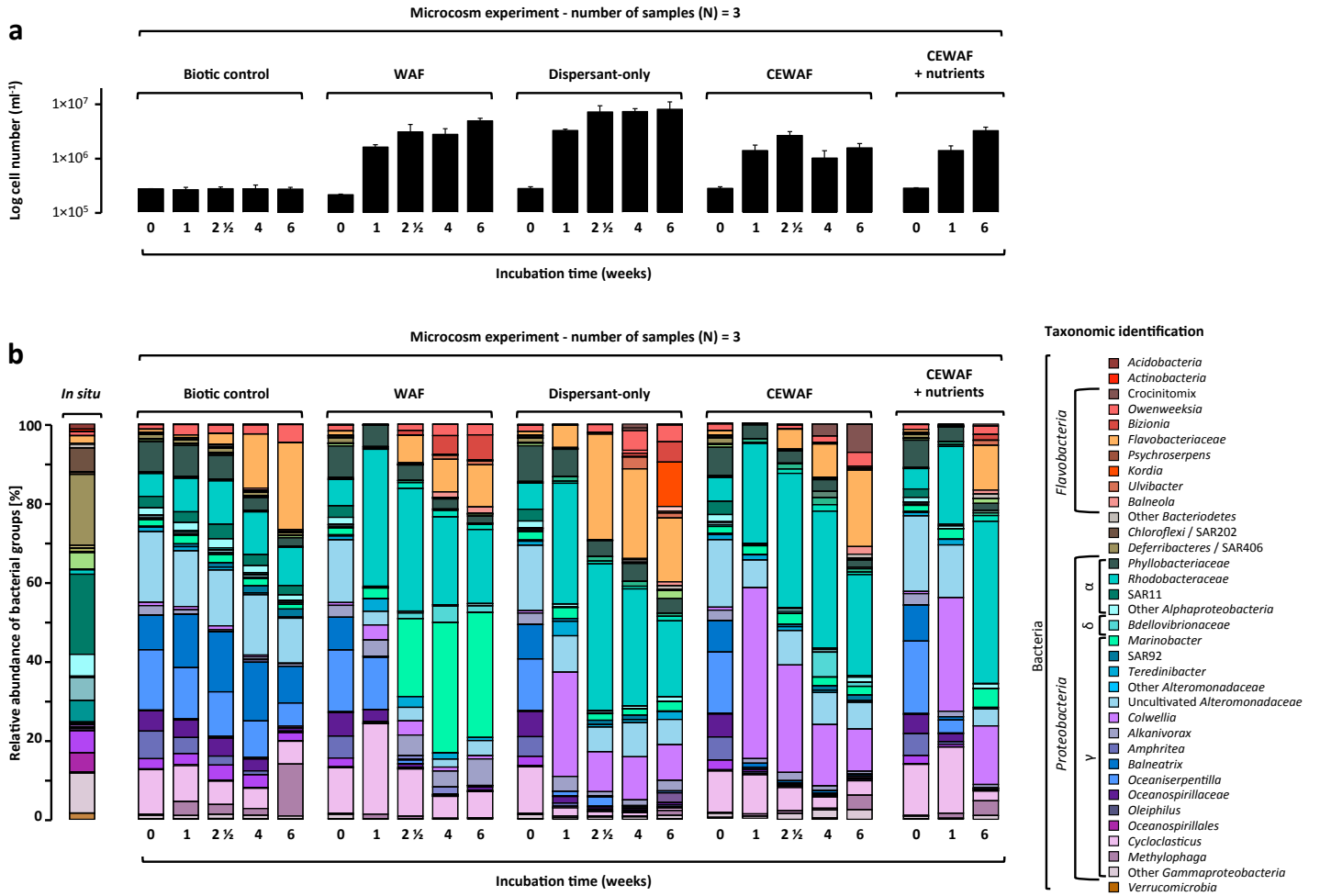
Kleindienst et al. Fig. 1

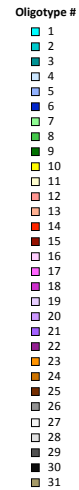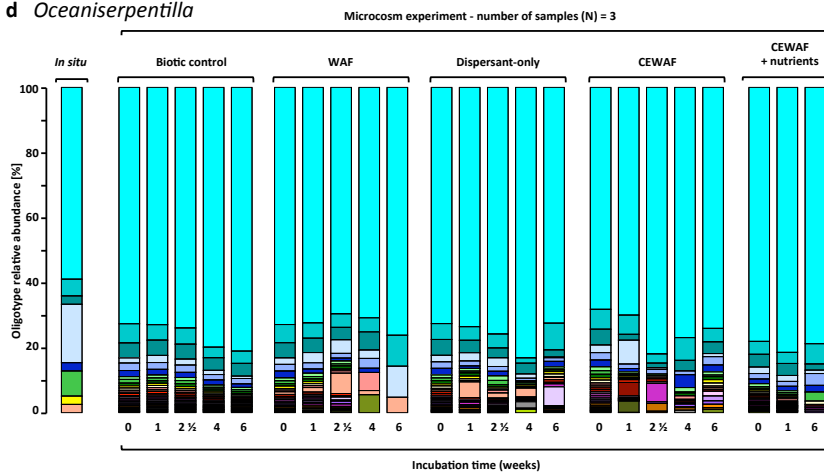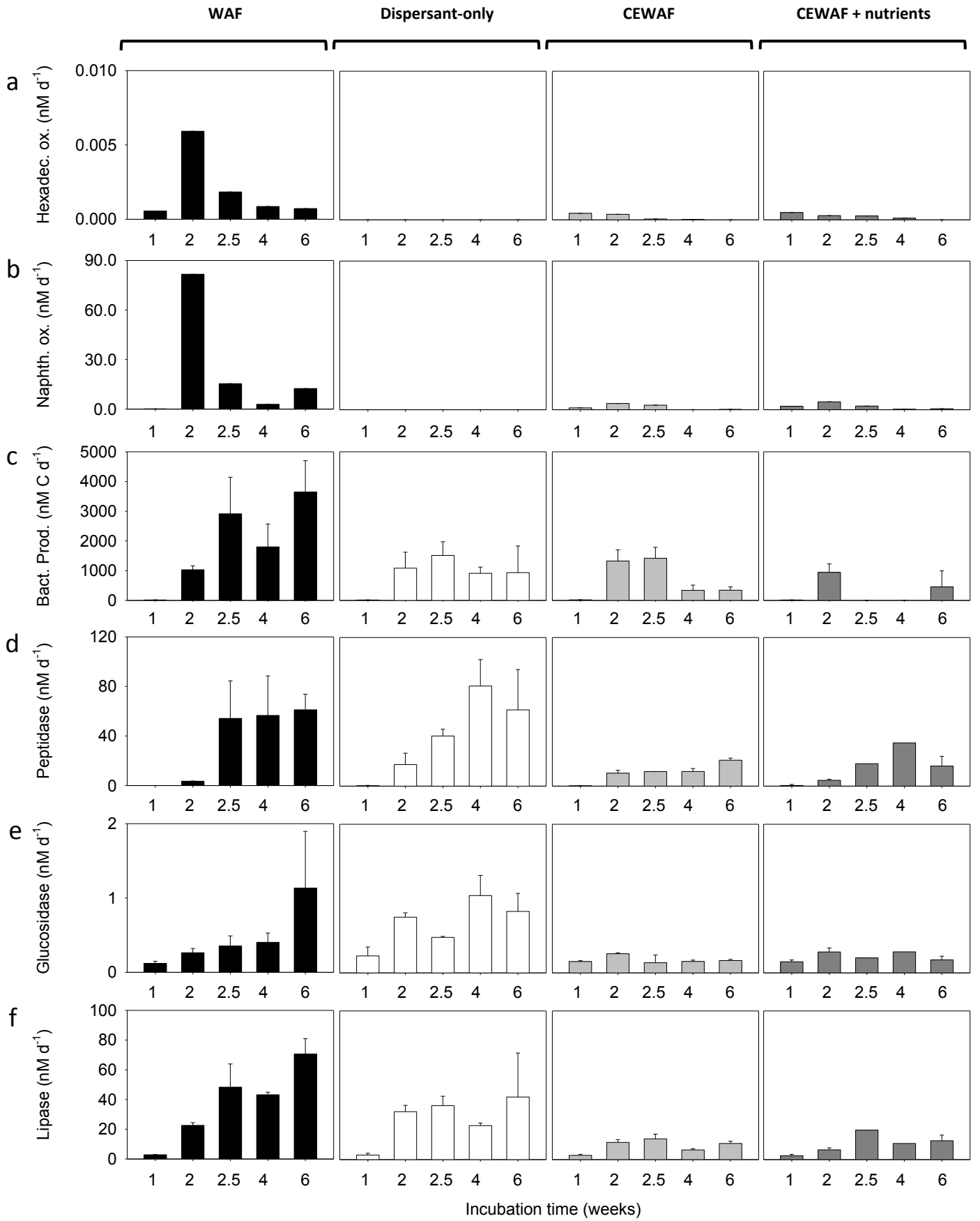**a** *Colwellia*
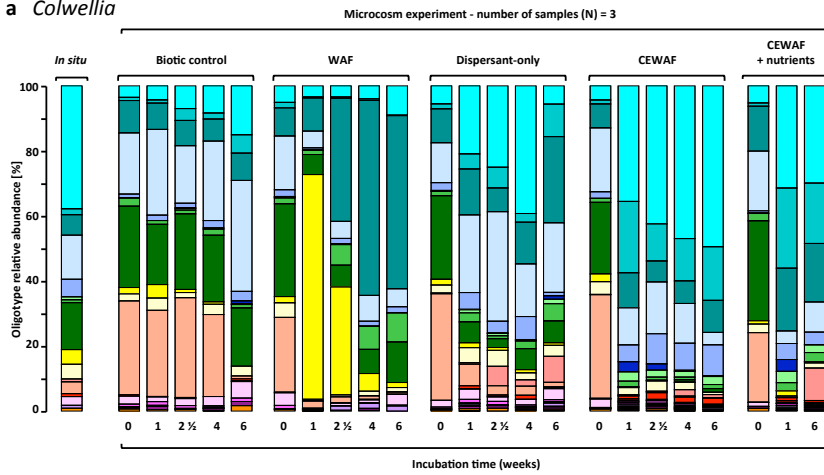
b *Marinobacter*
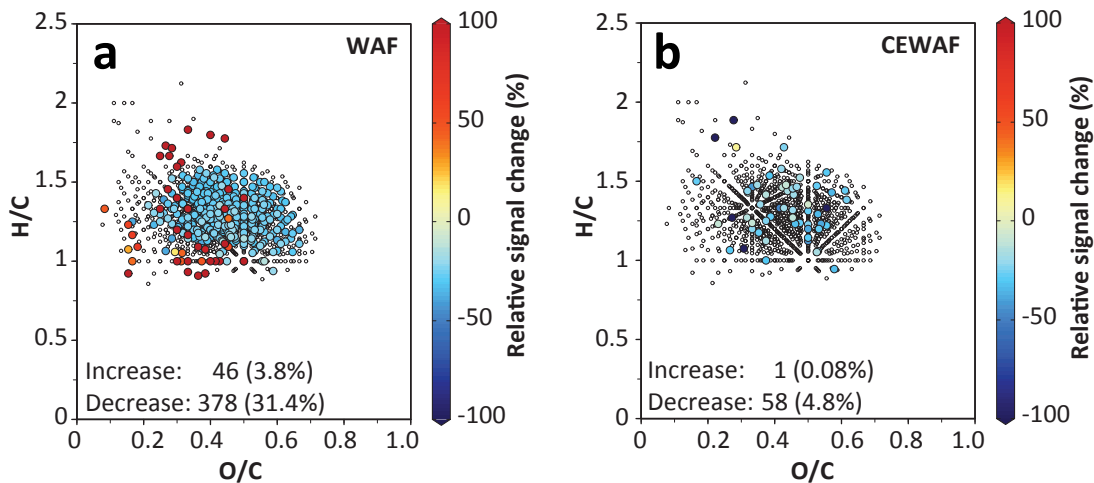
c *Cycloclasticus*

d *Oceaniserpentilla*

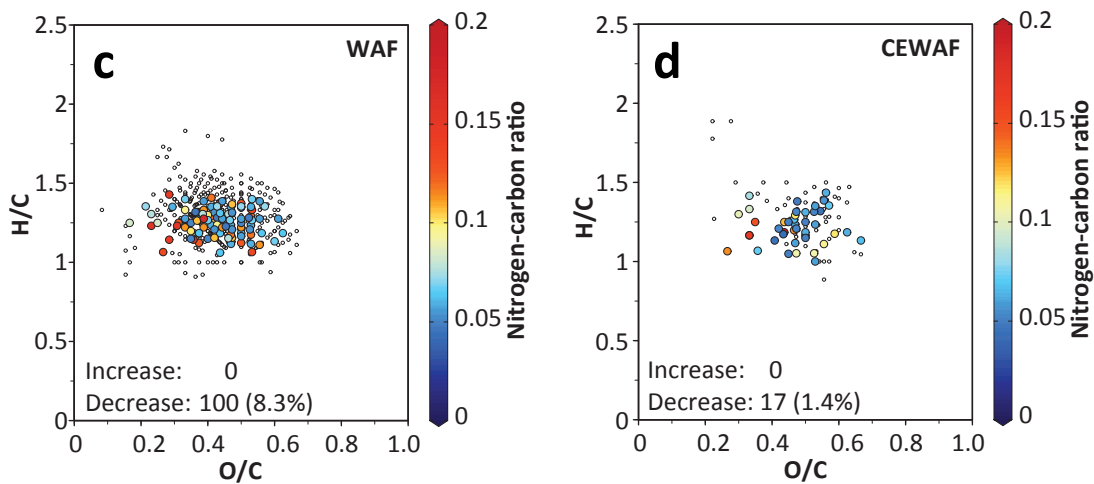Kleindienst
et al. Fig. 2

Kleindienst et al. Fig. 3

All compounds

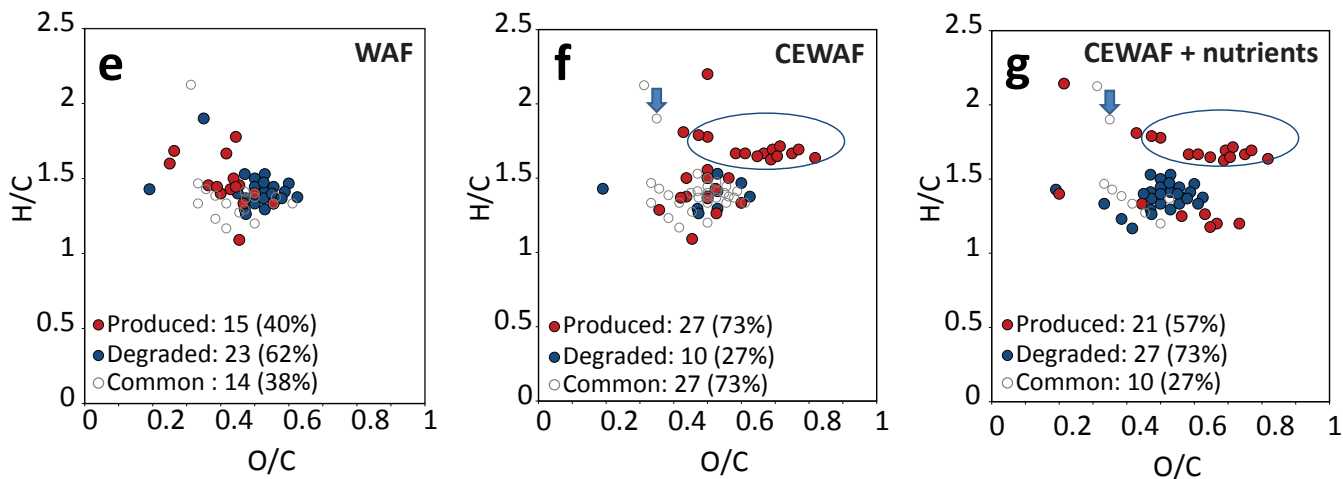**N-containing compounds**

S-containing compounds

Kleindienst et al. Fig. 4