

1 Multi-omics insights into the microbial oxidation of 2 seawater-soluble crude oil components

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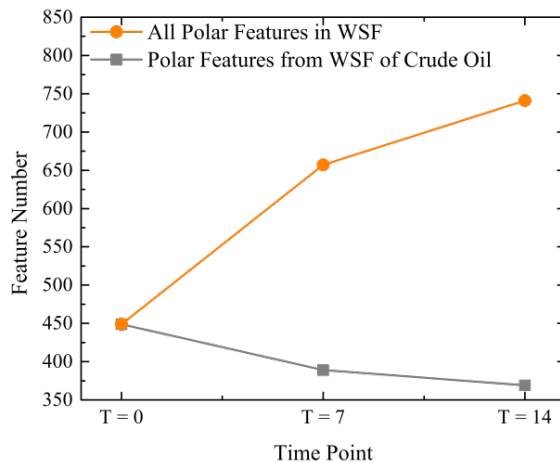
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TOC Art



Water-Soluble Fraction (WSF)



Incubation experiment

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20 ABSTRACT

21 Studies assessing the environmental impacts of oil spills focus primarily on the non-water-soluble
22 components, leaving the fate of the water-soluble fraction (WSF) largely unexplored. In this study,
23 we combined chemical and genomic analyses to examine the degradation of crude oil WSF in
24 seawater in the absence of light in a laboratory experiment. Over a 14-day incubation period,
25 microbes transformed WSF into various metabolic intermediates, without a significant change in
26 dissolved organic carbon concentrations. Microbial transformation processes increased the
27 chemical diversity and overall oxygen content of WSF compounds, consistent with an increase in
28 dioxygenase gene abundances. The majority of metabolites formed from the transformation of
29 WSF could not be structurally identified with existing databases, but elemental formulas suggest
30 that many of these compounds could be oxidation products of water-soluble non-polar compounds
31 such as PAHs. In particular, O₃ metabolites may represent a key transition point for WSF
32 degradation. Salicylic acid (O₃) is labile to generalist marine bacteria, and its dynamics and gene
33 products likely provide a route for complete WSF remineralization. The environmental persistence
34 and toxicity of WSF metabolic products are still unknown, but results from this study provide a
35 framework for further exploration of the fate of WSF in marine ecosystems.

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38 INTRODUCTION

39 Millions of barrels of crude oil are released to the ocean each year from unintentional spillages
40 and natural seepage.¹ A small, but significant, fraction of the oil dissolves in the water and behaves
41 differently than the bulk oil. The composition of dissolved oil is distinct from the total oil and is
42 enriched in small (<1000 Da), polar molecules.^{2,3} Despite decades of study on the fate of oil in the
43 environment, the water-soluble fraction (WSF) is vastly understudied because its components are
44 not resolved in traditional gas-chromatography-based analytical methods. Consequently, we know
45 much less about the factors affecting the fate and transport of crude oil WSF in marine ecosystems,
46 despite evidence suggesting that this fraction is enriched during weathering and is more toxic to
47 aquatic organisms than the parent oil.⁴⁻⁷

48 The best-studied oil spill occurred at the *Deepwater Horizon* (DWH) drill site in 2010, where
49 3.19 million barrels of oil spilled into the Gulf of Mexico over a period of 87 days,⁸ making it the
50 largest offshore oil spill in U.S. history.⁹⁻¹² Unlike many major oil spills, crude oil from the
51 Macondo well was injected into the water column from the deep sea. A widespread neutrally
52 buoyant subsurface plume, as thick as 200 m, was observed at 1100 m depth and persisted for
53 months after the blowout.⁹ Assessments during the early response estimated that water-soluble
54 crude oil components were preferentially enriched in this subsurface plume,¹³ with water-soluble
55 hydrocarbons such as low molecular weight n-alkanes and monoaromatic hydrocarbons
56 comprising ~69% by mass.^{9,12,14} The preferential enrichment of crude oil WSF in the deep ocean
57 underscores dissolution as an important process driving the distribution of spilled DWH oil.¹³
58 Similar enhancements are expected at any oil-water interface, such as oil slicks in surface water or
59 terrestrial spills.

60 In response to the DWH spill, numerous studies examined the fate, ecotoxicological impacts,
61 and degradation of the released oil.^{13, 15-21} For example, microbial composition and oil degradation
62 pathways deduced from genome sequences and transcripts were investigated in diverse impacted
63 environments, e.g. the oil plume, deep-sea sediments, and oiled beaches.^{14, 19, 20, 22-24} These
64 studies often have a focus on biologically mediated chemical transformations and degradation of
65 major compound classes in crude oils, such as n-alkanes, monoaromatic compounds (benzene,
66 toluene, ethylene, and xylene, or BTEX), and polycyclic aromatic hydrocarbons (PAHs).^{15, 25-27}
67 Absent from these studies, however, is information on the microbial response specific to the subset
68 of crude oil that is truly dissolved in water and more polar than hydrocarbons, i.e. compounds that
69 contain heteroatoms (N, S, and O). Early response assessments during the DWH spill suggested
70 that polar compounds accounted for ~10% of the spilled oil by mass, or ~0.41 million barrels of
71 largely uncharacterized polar crude oil.¹³ These compounds are not routinely monitored in oil
72 studies, because they are not readily accessible with traditional gas chromatography (GC)-based
73 analytical methods; thus, the chemical characteristics, fate, and transport of polar compounds
74 during DWH could not be included in early responses.¹³

75 Polar compounds account for < 15% by mass in bulk crude oil,²⁸ but may account for a
76 substantial fraction (70-82%) of the water-soluble fraction (WSF),^{4, 29} depending on the oil type
77 and weathering extent. Recent studies examining the dissolution of crude and weathered oils in
78 seawater have improved understanding of the chemical composition of WSF.^{2, 3, 30} Compounds
79 containing NSO preferentially partition into seawater.^{2, 3} These NSO-containing compounds are
80 more polar than hydrocarbons and appear to be resistant to degradation and toxic to organisms.⁴
81 ¹³ For example, uncharacterized compounds from WSF fractions generated from microbial
82 degradation of crude oil were found to be toxic to marine organisms (Crustacea),⁶ highlighting the

83 critical gap in understanding WSF chemical composition and its biogeochemical transformations
84 in the context of ecotoxicity. The microbial response to WSF, however, is relatively unexplored,
85 particularly in comparison to BTEX,^{31, 32} PAHs,^{33, 34} and alkanes^{24, 31}. During the DWH spill, next-
86 generation genomic techniques were employed to examine the sequential dominance of
87 hydrocarbon degraders in conjunction with traditional and emerging assessments of oil
88 degradation, but these tools were unable to link directly to WSF chemistry.^{14, 17, 20, 35}

89 Complex mixture analysis enabled by advanced mass spectrometry methods can provide
90 valuable information on the composition and fate of spilled oils, particularly the polar fraction
91 contained in WSF. Ultrahigh resolution mass spectrometry such as Fourier transform ion cyclotron
92 resonance mass spectrometry (FT-ICR MS) was widely used in response to the DWH spill, in
93 order to obtain insight into crude oil-derived compounds that could not be resolved with GC-based
94 methods.^{2, 25, 26, 30, 36} When applied to biological systems, these techniques identify and quantify
95 metabolites produced by organisms, thus providing metabolite profiles of microbial cultures or
96 communities under various conditions.^{22, 25} In this study, we combined metabolite profiling (i.e.
97 metabolomics) and metagenomics methods to examine the evolving chemical signature of WSF
98 and the dynamics of polar crude oil compounds within dark aerobic incubations with natural
99 seawater bacterial consortia. This approach combined with existing knowledge of hydrocarbon
100 degradation pathways enabled us to explore the microbial mechanisms responsible for WSF
101 degradation in aerobic seawater, analogous to oxic waters such as the surface ocean and the DWH
102 subsurface plume.³⁷

103

104 EXPERIMENTAL SECTION

105 **Incubation Experiment.** We created WSF by slow dissolution of Macondo oil surrogate
106 (source crude oil – MC252; Item IDs A0067V and A0067X) in filter-sterilized Vineyard Sound
107 seawater (VSW), following the low energy mixing methods described in Liu and Kujawinski²
108 (Supporting Information and Figure S1a). Briefly, we loaded the Macondo oil surrogate on the
109 surface of VSW at a 5:95 (v/v) ratio and allowed the oil dissolution to occur over 7 days in dark at
110 room temperature. Low energy was applied to ensure exchange across the water column, but no
111 oil droplets were entrained into the water. This method confined oil dissolution at the upper water
112 column of the vessel and the limited mixing provided a mean of dilution from upper to lower water
113 column, which mimicked the dilution of oil components across the concentration gradient in the
114 aquatic environment. After 7 days, we filtered the resulting water-accommodated fraction (WAF)
115 through a 0.7 μm glass fiber GF/F filter to collect the final, truly water-soluble oil component (i.e.
116 WSF). This method captures primarily low molecular weight PAHs and polar, water-soluble
117 compounds, because volatile hydrocarbons such as benzene, toluene, ethylbenzene, and xylene
118 (BTEX) will be lost over the 7-day mixing period as well as during the filtration process. The
119 dissolved organic carbon (DOC) concentration of WSF was comparable to that observed in the
120 field during DWH spill (Figures S3).³⁸

121 We established three parallel treatments, each in triplicate, to explore microbial community
122 changes in response to the addition of crude oil-derived components in the WSF (Figures S1b):
123 (1) the VSW control, which contained background seawater dissolved organic matter (DOM) and
124 natural bacterial consortia; (2) the succinic acid treatment, which contained seawater DOM,
125 succinic acid, and natural bacterial consortia; and (3) the WSF treatment, which contained seawater
126 DOM, crude oil WSF, and natural bacterial consortia. Succinic acid was used as a simple carbon
127 substrate control to distinguish opportunistic microbes responding to carbon addition,³⁹ relative to

128 microbes responding uniquely to WSF components. In treatments (2) and (3), we added the organic
129 substrates at similar DOC concentrations (succinic acid = $347 \pm 7 \mu\text{M-C}$; WSF $311 \pm 7 \mu\text{M-C}$).

130 We added filter-sterilized ammonium chloride (NH_4Cl ; 4 nM) and sodium phosphate (NaH_2PO_4 ;
131 0.3 nM) to all incubation chambers to mitigate nutrient limitation for cell growth. We inoculated
132 each treatment with natural bacterial consortia at a volume ratio of 10%, at the beginning of the
133 experiment (Supporting Information). One liter of headspace remained in each incubation
134 chamber, and we maintained aerobic conditions by swirling the incubation chambers gently three
135 times daily. We kept all incubations in the dark at room temperature ($\sim 24^\circ\text{C}$) throughout the 14-
136 day experiment. The experiment was conducted in the dark to minimize photo-oxidation;^{15, 40}
137 consequently, the observed chemical signatures over the experiment should be due primarily to
138 heterotrophic transformations. Details on the experiment setup are provided in the Supporting
139 Information.

140 **Sample Collection, Preparation, and Analyses.** We collected samples on three days (0, 7, 14).
141 On the sampling day, we swirled each bottle gently to ensure homogeneity and then filtered each
142 sample through a 0.2- μm Omnipore (Millipore Sigma) membrane filter under low vacuum.
143 Filtrates were used for external metabolite profiling, PAH analysis, and dissolved organic carbon
144 (DOC) analysis. We used microbial biomass retained on the filter for 16S rRNA gene and
145 metagenomics analyses. We enumerated bacteria in 10 mL of unfiltered water that was fixed with
146 borate-buffered formalin (2% final concentration) and frozen at -20°C . Our WSF protocol includes
147 GF/F filtration to remove oil droplets² and thus we do not expect significant amounts of
148 hydrophobic or sparingly hydrophilic compounds to be retained on the 0.2- μm membrane filters
149 used for biomass collection. We stored the filters at -80°C until extraction.

150 We acidified the filtrates (2L) to pH ~3 immediately after filtration, extracted the samples with
151 PPL solid-phase extraction (SPE) cartridges (Bond Elut, Agilent), and then eluted with 100%
152 methanol as described in Dittmar et al.⁴¹ and modified by Longnecker.⁴² The eluents were stored
153 at -20°C until mass spectrometry analysis. Immediately before analysis, we dried down the eluents
154 to near dryness, and reconstituted them in 250 µL of 95:5 water:acetonitrile. We added deuterated
155 biotin (5 µL; final concentration 0.05 µg/mL) to each sample as an internal standard. PPL resins
156 preferentially capture the aromatic compounds in WSF, but do not retain very small, highly polar
157 molecules such as succinic acid.⁴³ Therefore, to ensure similar extracted carbon concentrations
158 across all treatments and within the pooled samples, we diluted the WSF treatment eluents 50×
159 due to high concentrations of extracted carbon relative to the non-WSF treatments. We combined
160 50 µL of each sample to create a pooled sample as a reference for data quality control and
161 processing.

162 We divided all samples into two equal volumes, one for targeted analysis and one for untargeted
163 analysis, following methods described by Kido Soule et al.⁴⁴ The untargeted approach, using liquid
164 chromatography coupled to FT-ICR MS (LC-FT-ICR MS) equipped with an electrospray
165 ionization (ESI) source, allows examination of metabolite profiles without prior knowledge of
166 sample composition, and thus enables simultaneous identification of known compounds and
167 discovery of previously unknown metabolites. Metabolite profiles from different incubation
168 conditions at the three time points provided valuable information on how the microbes transformed
169 WSF components compared to other organic substrates over the 14-day experiment. Here, we
170 define a metabolite profile as all the features in a given sample, where a feature is defined as a
171 unique combination of mass to charge ratio (m/z) and retention time (RT). Each feature
172 corresponds to a specific metabolite or to a group of co-eluting isomers.

173 We subjected the top four features in each mass scan to tandem mass spectrometry (MS/MS or
174 MS2) for compound identification. We applied rigorous data quality control procedures to ensure
175 data robustness, e.g. removing features whose variability is driven by instrumental parameters
176 and/or are consistently present in all samples at similar intensities (see Supporting Information).
177 After these measures, approximately 40-48% of features in the dataset had associated MS2 spectra.
178 We used a step-wise approach to classify metabolites of interests based on the Metabolomics
179 Standards Initiative's (MSI) established four-level standard.⁴⁵ Specifically, a level-4 classification
180 includes unknown features that passed QA/QC but do not match any literature and database values;
181 a level-3 putative characterization requires a match between observed exact mass values and
182 elemental formulas; and a level-2 putative annotation requires a multi-component match between
183 exact mass and other physicochemical properties (e.g. RT and/or fragmentation pattern) with
184 literature or external libraries. The highest confidence level-1 identification requires at least two
185 of four independent confirmations of RT, exact mass, MS2 spectrum, or isotope patterns from a
186 chemical standard under identical analytical conditions.⁴⁵ We used the MetFrag *in silico* tool⁴⁶ to
187 search the fragmentation patterns and exact mass values to generate level-2 putative annotations.
188 We compared the search results with metabolites listed in Kyoto Encyclopedia of Genes and
189 Genomes (KEGG) for key hydrocarbon degradation pathways. For pathways with multiple level-
190 2 putative annotations, we searched our feature list for level-3 putative characterizations within
191 these pathways. We assigned elemental formulas for the level-3 features using an automated
192 compound identification algorithm (CIA) as described in Kujawinski and Behn⁴⁷ with parameters
193 from Liu and Kujawinski.² We did not consider pathways with only level-3 putative
194 characterizations, due to the inability to distinguish among structural isomers with the same mass.
195 Finally, we confirmed level-1 identities of key metabolites with commercial standards, including

196 six intermediates related to degradation of naphthalene and methylnaphthalenes, namely 3- and 4-
197 hydroxybenzaldehyde, salicylic acid, 3- and 4-methylsalicylic acid, and gentistic acid. These
198 metabolites were then quantified by LC triple-quadrupole-MS (see Supporting Information).

199 It should be noted that many observed features could not be identified with literature or database
200 searches (i.e. level-4). Additionally, many level-2 and level-3 features do not have commercial
201 standards available. Due to the lack of appropriate standards for most of the detected features,
202 feature intensities obtained with the untargeted method could not be converted to absolute
203 concentrations. Instead, we used relative abundances to compare concentration changes among
204 samples. We calculated relative abundance as: $\Sigma(\text{Intensity of feature groups}) / \Sigma(\text{Intensity of all}$
205 $\text{detected features}) \times 100$, where feature groups can be defined as specific compound classes or
206 those that contained elements of interest such as oxygen.

207 Compounds ionizable by ESI contain at least one polar function group; thus, features detected
208 by LC-FT-ICR MS are, by analytical definition, more polar than hydrocarbons containing no polar
209 functional groups. Therefore, we equate polar compounds with those observed within the LC-FT-
210 ICR MS analytical window in the subsequent discussions.

211 **Sample Collection for PAH Analysis.** We extracted 100 mL of WSF samples with
212 dichloromethane (DCM) for PAH analysis. We dried the extracts with anhydrous Na₂SO₄ and
213 removed excess DCM by rotary evaporation. We reconstituted the concentrated samples with
214 toluene for global chemical characterization through direct infusion FT-ICR MS (data not shown).
215 We took aliquots of ~250 μL from the remaining samples in toluene and performed solvent
216 exchange by drying down the toluene under a gentle stream of high purity nitrogen. The dried
217 samples were shipped to Haverford College for PAH analysis. The samples were reconstituted
218 with DCM before analysis by gas chromatography mass spectrometry (GC-MS). We expect that

219 our PAH concentrations are underestimates due to the sample drying steps. For example,
220 naphthalene was not detected, which is inconsistent with this oil's known composition.⁴⁸ From
221 previous work, we expect the less volatile PAHs should account for ~10% of the WSF.⁴ Individual
222 PAH analyzed in this study included: naphthalene, C1-C4 alkylated naphthalenes, biphenyl,
223 fluorene, C1-C3 alkylated fluorenes, dibenzothiophene, C1-C3 alkylated dibenzothiophenes,
224 anthracene, C1-alkylated anthracene, phenanthrene, C1-C3 alkylated phenanthrenes, chrysene,
225 C1-C3alkylated chrysenes, and triphenylene and benzo(a)anthracene. Detailed method for PAH
226 analysis is described in the Supporting Information.

227 **16S rRNA Gene and Metagenomics Analysis.** We extracted microbial DNA from all filters
228 using the MO BIO PowerWater (PW) kit (MOBIO Laboratories, Inc., Carlsbad, CA), according
229 to the manufacturer's instructions. Purified genomic DNA was submitted to the University of
230 Wisconsin-Madison Biotechnology Center. Further details of sample preparations for 16S rRNA
231 gene and metagenomics sequencing, library construction and bioinformatics analysis are provided
232 in Supporting Information.

233

234 RESULTS AND DISCUSSION

235 **Presence of WSF Selected for Distinct Microbial Communities.** Both chemical and microbial
236 community compositions within the same treatment type changed between the initial time point
237 and 7 and/or 14 days (Figures S2a and b), indicating selection of microbial community based on
238 the organic substrates and microbial alteration of the organic compounds in the starting incubation
239 media. Among the three treatments, the chemical and microbial community compositions were
240 different between non-WSF (i.e. VSW and succinic acid) and WSF incubations (Figures S2a and
241 b). The use of succinic acid as a control carbon source allowed us to determine that this observed

242 shift in microbial community is due specifically to the addition of WSF and not due to
243 opportunistic microbes. Indeed, we observed known hydrocarbon degraders uniquely in the WSF
244 treatment, including *Cycloclasticus*, *Oceaniserpentilla*, and *Rhodospirillales*, consistent with field
245 observations of microbial diversity during the DWH spill (Table S5 and S6).^{14, 24, 49}

246 **WSF is Transformed in Incubation Experiments.** We used changes in DOC concentrations
247 across time points to evaluate complete remineralization of organic carbon to CO₂ in each
248 treatment. DOC values were not statistically significantly different across the 14-day incubation
249 in the WSF treatments, based on a one-way ANOVA test at 95% confidence level (Figure S3). In
250 contrast, DOC concentrations decreased more than 100 μM-C by T = 14 in the succinic acid
251 treatment, suggesting catabolism of succinate for energy (Figure S3). DOC concentrations in the
252 VSW treatment were also not statistically significantly different across the three time points, which
253 was likely due to the overall lower biomass and microbial activity as a result of lower DOC
254 concentrations (Figures S4).

255 We used LC-FT-ICR MS to examine detailed chemical changes as the microbes altered WSF.
256 We define polar WSF-derived chemical features (P-WSF_{Total}), resolved by LC-FT-ICR MS, to be
257 those found in WSF treatment but not in any non-WSF treatments. We culled the list of features
258 to those that were found in all replicates at a time point. We then divided P-WSF_{Total} into P-WSF₀,
259 or the polar WSF compounds found at T = 0, and P-WSF_M, or the polar metabolites produced
260 during microbial degradation of WSF compounds (Figure S5).

261 To understand the chemical dynamics within WSF over the course of our experiment, we further
262 subdivided P-WSF₀ into four groups: P-WSF_{0-C}, or features that were likely consumed completely
263 (present only at T = 0); P-WSF_{0-U}, or features that were **unaltered** (similar relative abundance
264 values over 14 days); P-WSF_{0-I}, or features whose relative abundances **increased** over 14 days; and

265 P-WSF_{0-D}, or features whose relative abundances decreased over 14 days. The last three groups
266 (P-WSF_{0-U}, P-WSF_{0-I}, and P-WSF_{0-D}) include features present in WSF treatment samples at all
267 time points. We based our feature classifications on pair-wise one-tailed Student's t-test of feature
268 intensities. A visual overview of our classification scheme is shown in Figure S5.

269 Overall, the features in P-WSF_{Total} increased from 449 at T = 0 to 741 at T = 14 (Figure 1; Table
270 S1), indicating formation of new compounds as a result of microbial transformation of WSF crude
271 oil. Only 80 of 449 (<18 %) P-WSF₀ features were missing by the end of the experiment, due
272 either to complete degradation or to reduction below the detection limit (Figure 1; Table S1). In
273 contrast, P-WSF_M accounted for 41% and 50% of the total features observed in T = 7 and 14,
274 respectively (Tables S1 and S2). The similarity in DOC concentrations in WSF treatment samples
275 across time points, together with the increase in the number of P-WSF_{Total} and the small fraction
276 of P-WSF_{0-C}, implies that the majority of compounds initially found in WSF were transformed
277 into metabolic intermediates by microbial degradation rather than completely remineralized to
278 CO₂, within the time frame of the experiment.

279 Changes in the relative abundances of features in P-WSF_{0-I} and P-WSF_{0-D} were significant
280 between T = 0 and T = 7 but not significant between T = 7 and T = 14, based on paired one-tailed
281 Student's t-tests (Figure 2 and Table S3). In contrast, increases in relative abundances for P-WSF_M
282 features were significant between T = 0 and T = 7, and between T = 7 and T = 14 (Figure 2 and
283 Table S3). These findings suggest that processes that lead to a decrease or increase in abundances
284 of P-WSF₀ features proceed at slower rates, or not at all, after T = 7; while production of P-WSF_M
285 continues after T = 7 and potentially beyond T = 14. The source of P-WSF_M may be P-WSF₀ and/or
286 low-molecular weight, water-soluble non-polar compounds, such as PAHs. We can discount the
287 first possibility because the change in relative abundance in P-WSF_{0-D} is minimal between T = 7

288 and $T = 14$. In contrast, the second possibility could only be explained by microbial oxidation of
289 compounds that were originally outside the LC-FT-ICR MS analytical window, such as the non-
290 polar compounds in WSF.

291 **Degradation of PAHs is a Likely Source of Polar Metabolites in WSF.** One of the possible
292 sources of P-WSF_M is the oxidation of non-polar compounds. To investigate whether non-polar
293 compounds in WSF are the sources of polar compounds, we focused our data interpretation on the
294 degradation of water-soluble PAHs, i.e. typically those with less than 3-rings, which should
295 account for ~10% of the WSF.⁴ If all or a major fraction of PAHs were completely remineralized
296 into CO₂, such a process should result in statistically significant changes in DOC concentrations.
297 However, DOC concentrations did not change significantly while total PAH concentration
298 decreased and the number of P-WSF_{Total} increased (Figures 1 and S6). These observations support
299 the notion that the source of the increasing P-WSF_M was likely the non-polar compounds that were
300 not detected by LC-FT-ICR MS at $T = 0$.

301 We identified a total of 56 metabolites that occur within known aromatic compound degradation
302 pathways, such as xylene, toluene, naphthalenes, and PAHs with three or fewer rings (Table S4).
303 Twenty-four of the 56 compounds from P-WSF_{Total} were assigned a level-2 putative annotation
304 based on exact mass and fragmentation pattern matches (Table S4). Of the 24 Level-2 metabolites,
305 7 were associated with the degradation of naphthalene and methyl-naphthalenes (Table S4), even
306 though naphthalene was not detected in PAH analysis. Multiple naphthalene degradation products
307 were observed across all time points with either negligible abundances at $T = 0$ and elevated
308 abundances at $T=7$ and/or $T=14$ or with the highest abundances at $T = 7$ (e.g. Figures S7 and S8).
309 We observed multiple metabolites from the degradation of aromatic compounds with 1-3 rings,
310 but no metabolites associated with the degradation of high molecular weight PAHs. This is

311 consistent with the absence of high molecular weight PAHs in WSF based on their low aqueous
312 solubilities.²

313 **O3 and O4 Metabolites are Important Intermediates in WSF Transformations.** The number
314 of putatively annotated compounds related to WSF degradation accounted for only a small
315 percentage (<12%) of P-WSF_{Total} in each sample. To better understand the characteristics of the
316 broad range of compounds observed in WSF, existing chemical reaction information available in
317 the KEGG database and metagenomics data from this study was used to guide the interpretation
318 of the remaining P-WSF_{Total} features.

319 We focused on P-WSF_{Total} features with assigned formulas (Levels 1, 2, and 3) that contained
320 oxygen atoms, since oxidation is a well-known mechanism for oil degradation. Aerobic
321 biodegradation of PAHs is catalyzed by either monooxygenases or dioxygenases, enzymes that
322 add one or two oxygen atoms, respectively, in the initial steps.^{34, 50-53} In bacteria, dioxygenases are
323 the primary enzymes that initiate the PAH degradation.^{34, 51, 54} For PAHs with three or fewer rings,
324 dioxygenase-catalyzed oxygen addition occurs up to O4, at which point one ring opens. The open-
325 ring O4 intermediates break down into compounds with higher oxygen to carbon ratios, such as
326 O3 and O4 attached to only one aromatic ring (Figure 3). For example, degradation of naphthalene
327 initiated via the naphthalene 1,2-dioxygenase pathway produces metabolic intermediates with
328 oxygen numbers as follows: O0 → O2 (two isomers) → O4 → O4 (one ring opens) → O2 (lower
329 ring number) → O3 → O4 (towards central metabolism) (Figure 3).

330 In this experiment, three O3 compounds, salicylic and 3- and 4-methylsalicylic acids, were the
331 most abundant Level-1 metabolic intermediates (Table S4 and Figure S7). These compounds,
332 however, only account for a small fraction of the DOC (<0.1%), suggesting that: (1) a large

333 proportion of the WSF compounds were unknown and (2) these compounds likely represent a pool
334 of WSF organic carbon that has low concentrations but high flux.

335 To further examine oxygen-containing compounds beyond the level-1 identification, we
336 compared the oxygen distributions of compounds with $C_xH_yO_z$ formulas. Comparisons of relative
337 abundance between each oxygen compound class across time points were based on pair-wise one-
338 tailed Student's t-test. Within the oxygen number distributions of P-WSF_{Total} features, the relative
339 abundances of O4 compounds were significantly higher at T = 7 and 14 than T = 0 (Figure 4a).
340 Changes in relative abundance of P-WSF₀₋₁ features show that most of the production occurred in
341 the O3 and O4 classes (Figure 4b). The relative abundance of O3 P-WSF_M also increased
342 significantly at T = 14, compared to T = 7 (Figure 4d). The oxygen number distribution
343 characteristics from a broader range of compounds in P-WSF_{Total} features are similar to known
344 PAH degradation pathways. Therefore, the high relative abundance of O4 compounds in WSF is
345 likely due to multiple reactions from the degradation of polar and non-polar compounds.

346 The observed oxygen number pattern and complementary genomics data suggest that the
347 degradation of naphthalene and other PAHs in the experiment was likely attributed to dioxygenase-
348 catalyzed reactions. We observed enrichments of multiple genes encoding for dioxygenases in the
349 WSF samples (Figure 5). Although evidence of monooxygenase-catalyzed PAH metabolism is
350 present in some bacteria,^{31, 55} such reactions are predominantly observed in eukaryotic cells such
351 as fungi, yeast, and mammalian cells.⁵⁶⁻⁵⁹ Unlike dioxygenase-encoding genes, we did not observe
352 genes that encode for monooxygenases. The increase in O3 class relative abundance was,
353 therefore, not related to monooxygenases, and is instead more likely to be breakdown products
354 from O4 compounds after the first ring opens. We found that both P-WSF₀₋₁ and P-WSF_M features
355 include many O3 compounds (Figure 4b and d). The high abundance of O3 compounds may reflect

356 the production and accumulation of intermediates similar to salicylic acids (i.e. salicylic acid, its
357 methylated forms, and other modifications), which are key metabolites from naphthalene and
358 methyl-naphthalene degradation. Interestingly, the abundance of a subset of O3 compounds also
359 decreased substantially over the course of the experiment within P-WSF_{0-D} (Figure 4c), suggesting
360 some of these compounds were rapidly degraded. These findings suggest that the O3 compound
361 class is a dynamic group of compounds with production, accumulation, and degradation occurring
362 simultaneously.

363 **O3 Metabolites Such as Salicylic Acids Represent a Key Transition Point for WSF**
364 **Degradation.** Salicylic acid is a metabolic intermediate in multiple PAH degradation pathways
365 such as naphthalene, anthracene, fluorene, and phenanthrene; thus, it is reasonable to assume that
366 the degradation of naphthalene, anthracene, fluorene, and phenanthrene in WSF all contributed to
367 the observed concentration of salicylic acid in this study. The methylated forms of salicylic acid
368 (3- and 4-methylsalicylic acids) are key metabolic intermediates in the degradation of 1- and 2-
369 methylnaphthalenes, respectively.

370 The salicylaldehyde dehydrogenase gene (*nahF*, (KEGG, K00152)), which encodes the enzyme
371 that catalyzes the formation of salicylic acid in the naphthalene degradation pathway,^{60, 61} was
372 enriched in the WSF treatments (Figure 5). Further degradation of salicylic acid can proceed via
373 numerous mechanisms, depending on microbial community composition and their associated
374 genes.⁶² For example, in the naphthalene degradation pathway, salicylic acid is transformed into
375 one of two different products through competing reaction mechanisms, i.e. gentistic acid and
376 catechol. We detected trace amounts of gentistic acid in WSF samples (Figure S7), but not
377 catechol. Furthermore, the gene encoding the enzyme for converting salicylic acid to catechol,
378 namely salicylate hydroxylase (*nahG*, (KEGG, K00480)), was not enriched in the WSF samples.

379 Interestingly, the *nahG* gene was elevated in non-WSF samples relative to the WSF treatments
380 (Figure 5), particularly in the VSW treatment. The enrichment of *nahG* gene in the VSW treatment
381 suggest some of the bacteria in seawater retain the genetic capacity to metabolize salicylic acid
382 through the catechol pathway, even though the concentration of salicylic acid in seawater was low.
383 This finding suggests that while the production of salicylic acid via PAH degradation pathways
384 may require specific hydrocarbon-degrading microbial groups, i.e. those that thrive in the presence
385 of WSF, removal of salicylic acid may be mediated by generalist non-oil degrading bacteria. This
386 finding underscores the importance of community structure and succession in complete oil
387 degradation. It is known that during the DWH oil spill, distinct groups of oil-degrading microbes
388 bloomed at different stages of the oil spill.¹⁸ These microbes potentially produced an array of
389 metabolites that could be utilized by the general microbial community as carbon sources. We
390 propose that while specialized microbes initiate the degradation of WSF, the activity of generalists
391 may be important for the full remineralization of WSF compounds to CO₂. Interestingly, salicylic
392 acid has been shown to enhance the degradation of high molecular weight PAHs,^{34, 63-66} raising
393 the intriguing possibility that salicylic acid stimulates and facilitates degradation of PAHs.

394

395 CONCLUSIONS

396 WSF represents an important component of spilled oil that can play a role in ecotoxicology, and
397 can impact ecosystems,^{4, 6, 67} e.g. in subsurface plume of DWH and in the water column beneath
398 a surface oil slick (Figure 6). The molecular-level understanding of the fate of the polar compounds
399 in crude oil WSF, however, is still scarce. This study offers the first multi-omics insights into
400 microbial degradation of WSF, achieved by integrating a broad suite of chemical features with
401 hydrocarbon-degrading genes.

402 Although this is a controlled laboratory experiment, our results set important reference frames
403 for future oil spill studies and provide new compound targets for field monitoring. The wide range
404 of oxygen-containing WSF compounds share similar oxygen number distributions as those from
405 the degradation of PAHs through dioxygenase pathways. Intermediate metabolites with three
406 oxygen atoms may represent an important transition point in oil degradation in nature. We
407 hypothesize that O3 metabolites such as salicylic acids are funneling compounds for microbial
408 degradation of hydrocarbons. Initial degradation steps may require specialized microbes, but the
409 subsequent metabolism of intermediate products could be achieved by a more diverse group of
410 microbes present in seawater microbial consortia. Consistent with our laboratory findings, we
411 detected salicylic acids and gentistic acid in archived field samples collected in the DWH plume
412 (Table S7), suggesting these compounds may be used as markers for PAH degradation in the field.
413 Additional work with fresh samples or surface oil slicks is needed to confirm these results.
414 Nevertheless, our results highlight the complexity of uncharacterized polar compounds in WSF
415 and their transformation products. The ecotoxicity of this complex pool remains poorly
416 constrained, underscoring the need for improved understanding of the fate and ecotoxicity of WSF
417 on long and short timescales. With more comprehensive knowledge of WSF and its degradation
418 products, markers for assessing the fate and ecotoxicity of spilled oil in the environment can be
419 developed.

420

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428 The manuscript was written through contributions of all authors. All authors have given approval
429 to the final version of the manuscript. The authors declare no competing financial interest.

430 Supporting Information. Additional experimental details, figures and tables as noted in the text.

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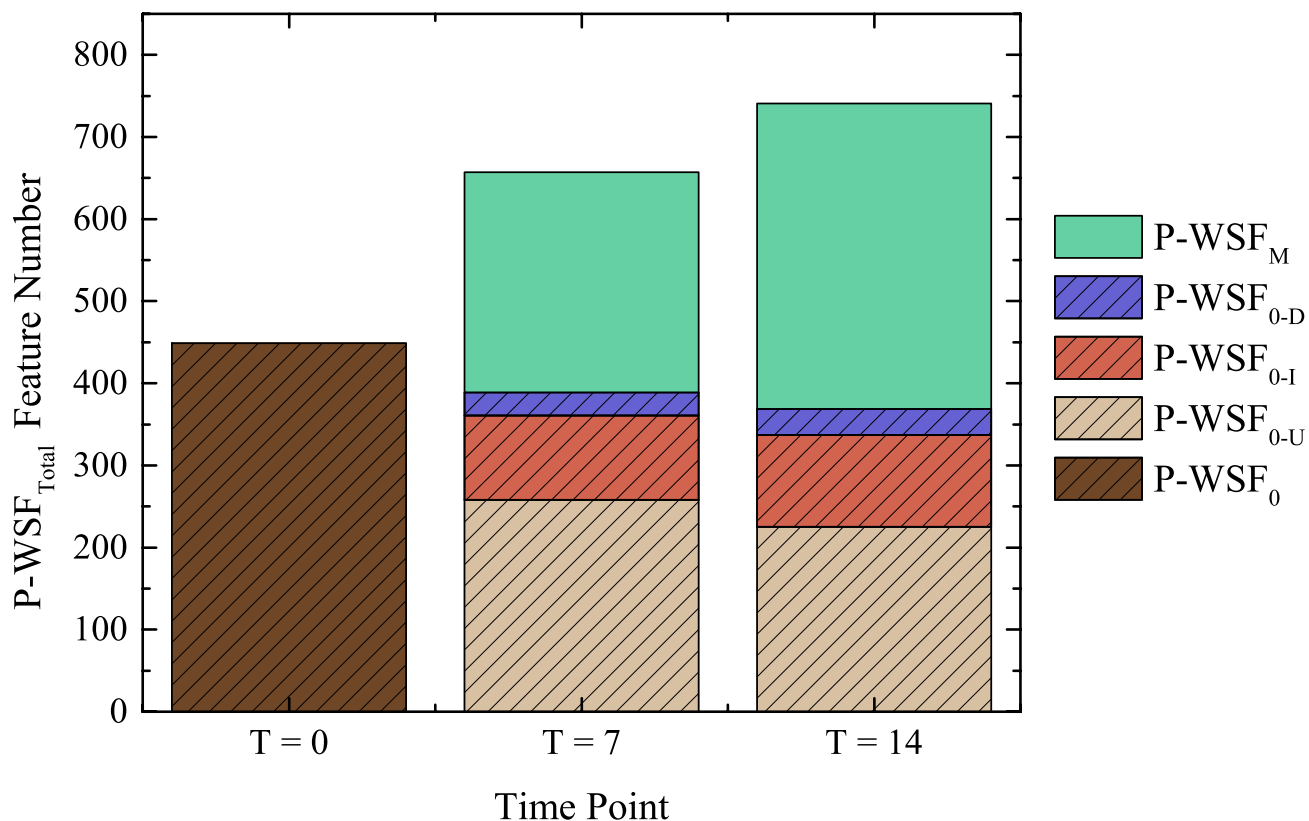
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439 doi:10.7266/N71C1TTK (nutrients and TOC data), doi:10.7266/N7N29TW0 (targeted
440 metabolomics data), doi:10.7266/N7WM1BBP (non-targeted metabolomics data), and
441 doi:10.7266/N7W95762 (metagenomic data).

442 ABBREVIATIONS.

443 FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; VSW, Vineyard
444 Sound seawater; WSF, water-soluble fraction; OTU, operational taxonomic unit; DOC, dissolved
445 organic carbon. WAF, water-accommodated fraction, DOM, dissolved organic matter.

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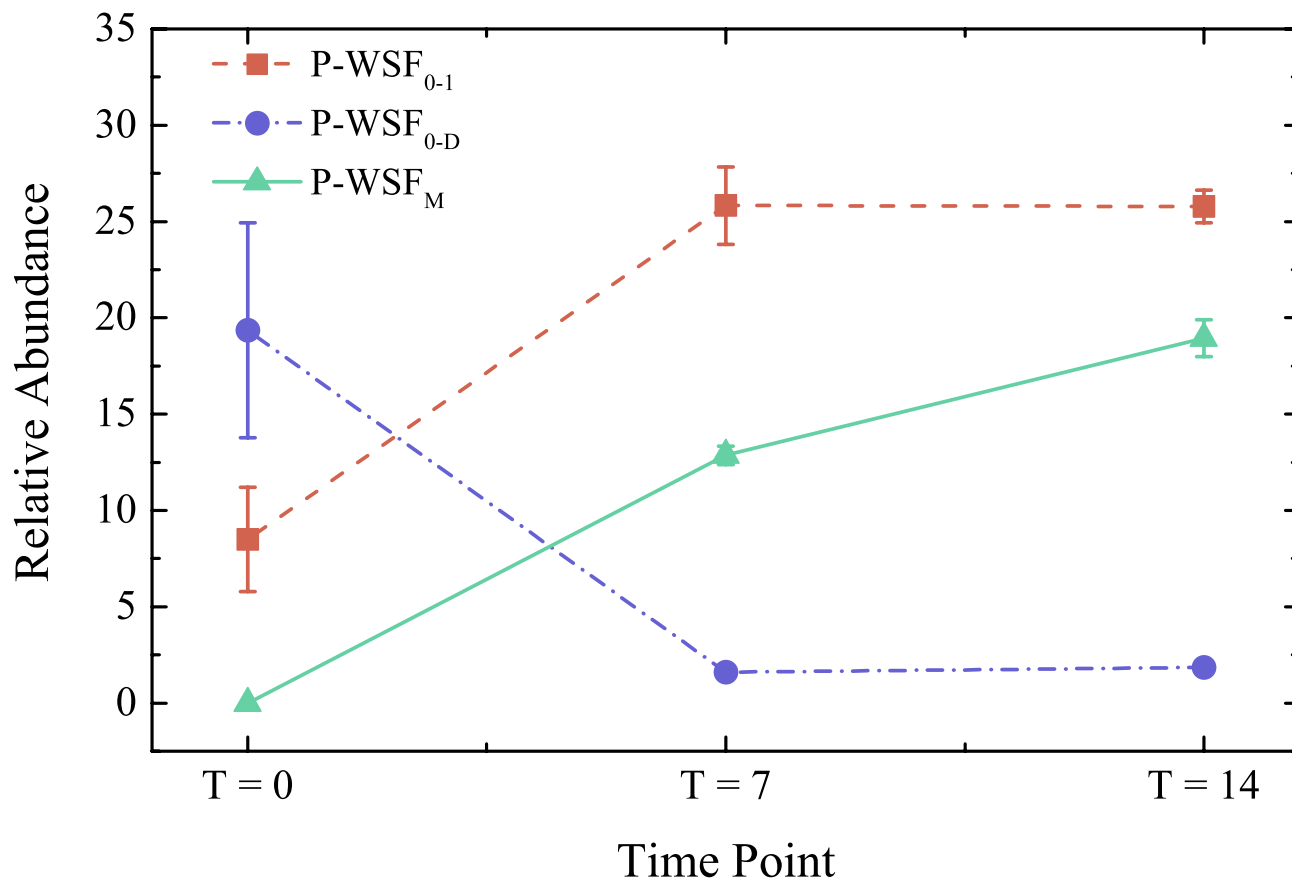
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450 **Figure 1.** Number of observed features across time points T = 0, T = 7, and T = 14 for P-WSF_{Total}.
451 All features shown here were present in all treatment replicates of a time point. Compounds derived
452 from WSF of crude oil at T = 0 (P-WSF₀) are indicated with stripes. Dark brown striped bar
453 represents P-WSF₀ features in the starting material of the incubation experiment. Light brown
454 striped bars highlighted features that persisted from T = 0 to T = 14 and whose relative abundance
455 did not change (P-WSF_{0-U}). Red striped bars highlighted features whose relative abundance
456 increased over the course of the experiment (P-WSF_{0-I}). Blue striped bars highlighted features
457 whose relative abundance decreased the course of the experiment (P-WSF_{0-D}). Green bars
458 highlighted new compounds present at T = 7 and T = 14, but not at T = 0 (P-WSF_M). The difference
459 between the initial striped bar and the sum of the three striped bars at T=7 and T=14 constitutes
460 the features that were lost (P-WSF_{0-C}).

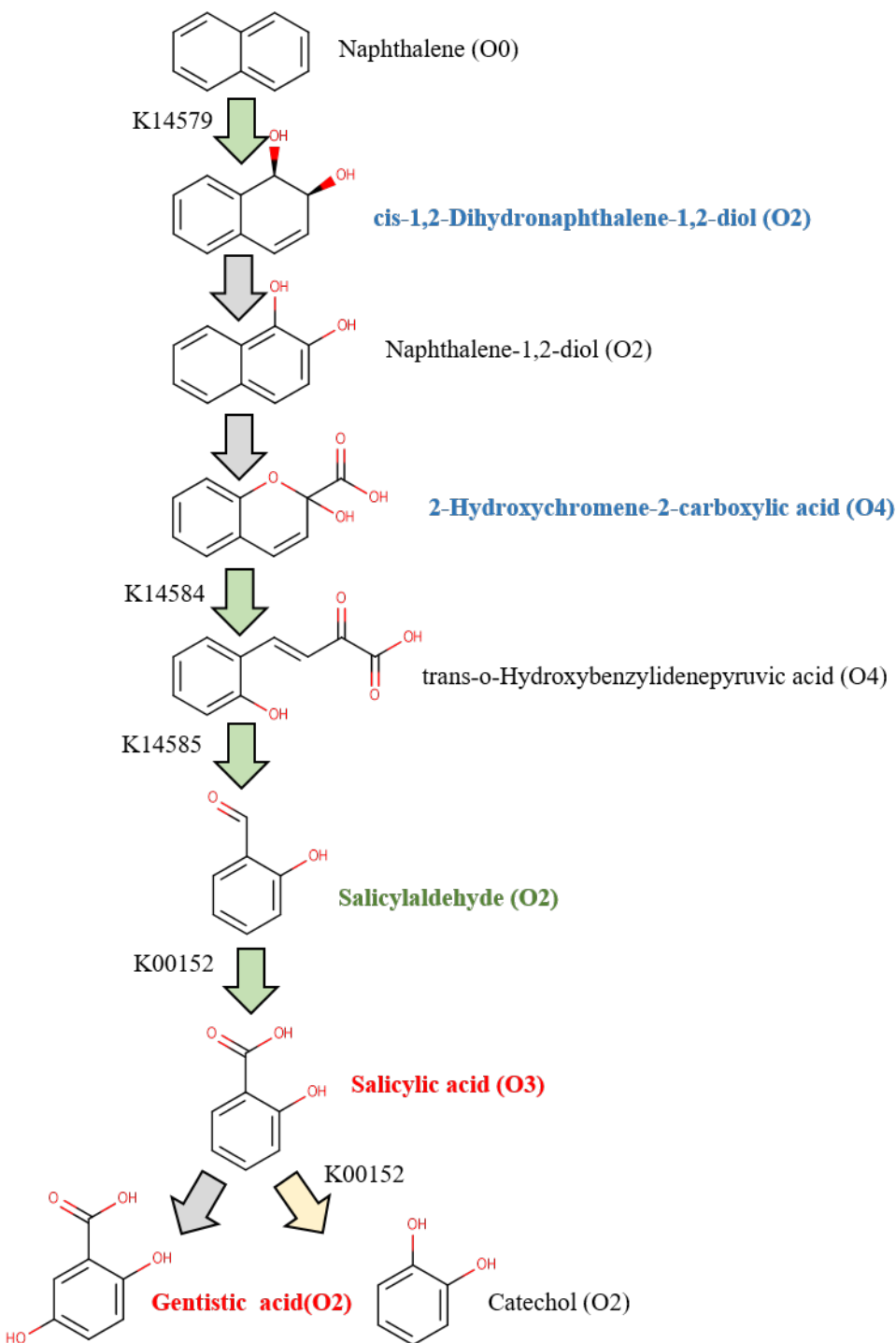
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463 **Figure 2.** Relative abundance of three compound groups observed in WSF treatment (P-WSF₀):
464 P-WSF₀₋₁ (red squares), P-WSF_{0-D} (blue circles), P-WSF_M (green triangles). Error bars represent
465 one standard deviation (n = 3) of relative intensity in each compound class at each time point.

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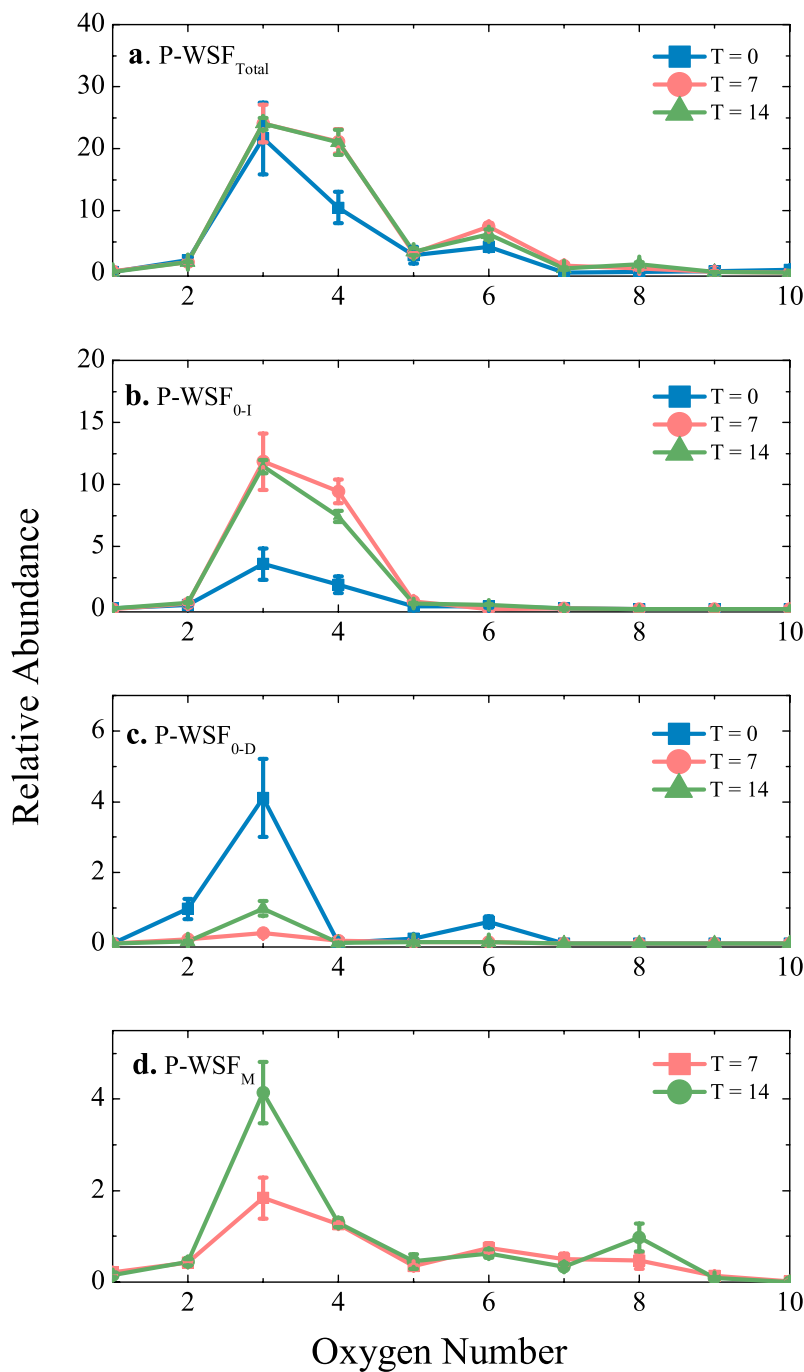
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468 **Figure 3.** Metabolic pathway of naphthalene degradation initiated through a dioxygenase, based
 469 on KEGG. Green arrows indicate the presence of the coding gene encoded in our samples. Grey
 470 arrows indicate absence of the coding gene in this study. A yellow arrow indicates the coding gene
 471 was observed but not enriched in WSF treatment samples. Chemical names for each structure was
 472 labeled with oxygen number indicated in parenthesis. Chemical names in red indicate a level-1
 473 identification. Chemical names in green indicate a level-2 putative annotation. Chemical in

474 blue indicate a level-3 putative characterization. Chemical names in black indicated that we did
475 not observe these compounds. BLAST identities of dioxygenase genes detected against an
476 experimentally validated database⁶⁸ are listed in Table S12.

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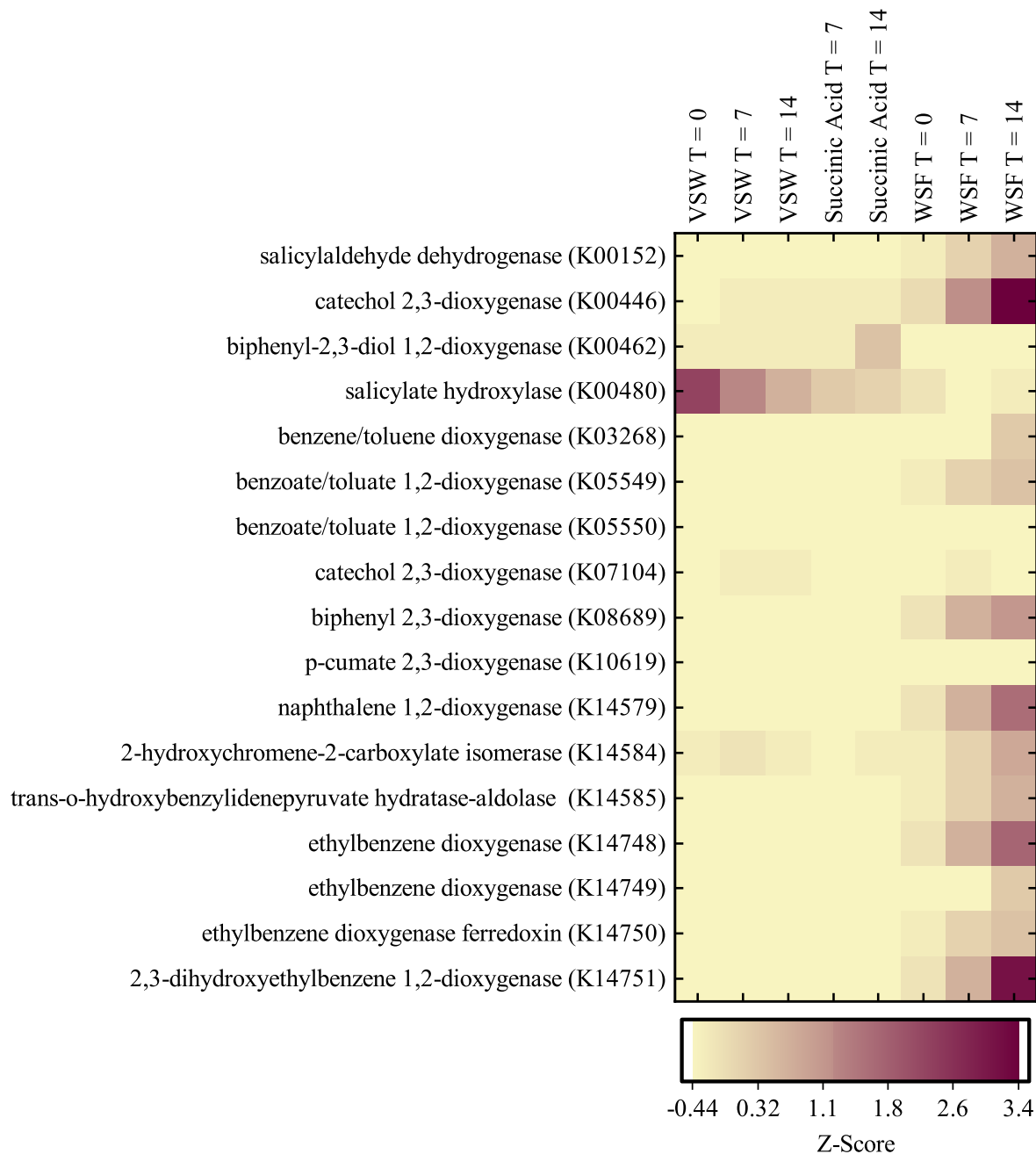
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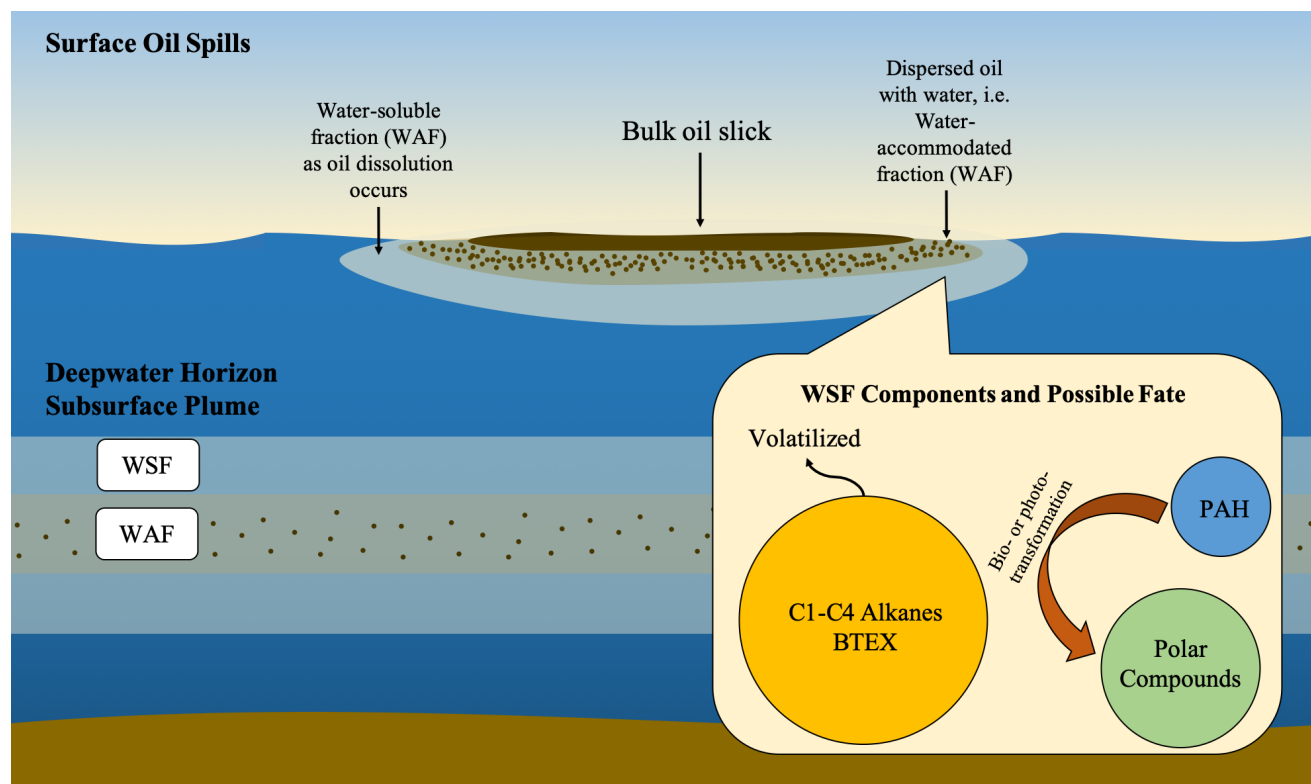
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481 **Figure 4.** Oxygen number distributions of C_xH_yO_z compounds observed in WSF. (a) P-WSF_{Total},
482 (b) P-WSF₀₋₁, (c) P-WSF_{0-D}, and (d) P-WSF_M in the three time-points (T = 0 in blue, T = 7 in red,
483 and T = 14 in green). Error bars indicate one standard deviation around the mean of triplicate
484 analyses.



485
 486 **Figure 5.** Heatmap of genes that encode for aromatic compound metabolisms identified using
 487 metagenomic analysis (described in Supporting Information). Only genes encoding for the
 488 degradation of oil-derived compounds are included. The darker color indicates higher Z-Score
 489 values for specific genes compared to the light yellow. Abundance and percentile of the gene are
 490 listed in Table S11. BLAST identities of dioxygenase genes detected against an experimentally
 491 validated database⁶⁸ are listed in Table S12.



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Figure 6. Proposed occurrence and fate of the water-soluble fraction (WSF) in the environment. Through dissolution, WSF is expected in the water around the surface oil slicks during an aquatic oil spill. The chemical fingerprint of WSF is distinct from the water-accommodated fraction (WAF), which contained emulsified oil droplets. During the DWH oil spill, we postulate both WAF and WSF were present in the subsurface plume. In the water column, WSF is expected to contain a larger fraction of BTEX, low molecular weight PAHs, and polar compounds. Based on our results, non-polar compounds such as BTEX and PAHs can contribute to polar compounds in WSF through bio-transformation in the water column. At the surface, BTEX may readily volatilize while PAHs are transformed to polar compounds through photo- and bio-mediated pathways.

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