Short communication

Biodegradation-mediated alterations in acute toxicity of water-accommodated fraction and single crude oil components in cold seawater

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ABSTRACT

Hydrocarbon biodegradation may be slower in cold Arctic than in temperate seawater, and this will affect the toxicity time window of the hydrocarbons. In this study, the acute toxicities of water-soluble phases of 1,3-dimethylnaphthalene, phenanthrene, fluoranthene, and low energy water-accommodated fractions (LE-WAFs) of an evaporated (200°C+) crude oil, were screened by a Microtox bioassay during biodegradation in cold seawater (4-5°C). The water-solubility of fluoranthene was too low to provoke a toxic response at any time, whereas the toxicity of 1,3-dimethylnaphthalene and phenanthrene decreased over time in relation to biotransformation of these compounds. In LE-WAFs, the Microtox EC$_{50}$ was associated with biodegradation of the predominant hydrocarbons (naphthalenes, 2- to 3-ring PAH), as well as with phenol degradation products. The acute toxicities of single hydrocarbons and LE-WAFs persisted for a longer period in the cold seawater than previously shown at higher seawater temperatures. These results suggest implications for fate and effects assessment of hydrocarbons after oil spills in cold environments, like the Arctic. However, further biodegradation studies using Arctic seawater and relevant species for toxicity testing are needed for confirmation.
1. Introduction

Biodegradation of hydrocarbons (HCs) in seawater (SW) after oil spills is associated with oxidative processes. Aerobic $n$-alkane degradation is associated with monooxygenases, in which the alkane is converted to alcohols and further to acetyl-coA (Harayama et al., 1999), while aromatic HCs are degraded primarily by dioxygenases (e.g. Van Hamme et al., 2003). Resulting metabolites are more water-soluble and thus attributed with lower octanol-water partitioning coefficient ($K_{ow}$). Acute effect concentrations (e.g $LC_{50}$) of HCs, predicted as the relations between Log$K_{ow}$ and Log$LC_{50}$ (French-McCay, 2002), result in reduced acute toxicity after oxidative processes like biodegradation. Relations between biodegradation and acute toxicity have been investigated in several studies, mainly in soil or groundwater (Wang et al., 1990; Belkin et al., 1994; Tiehm et al., 1997; Renoux et al., 1999; Juhasz et al., 2000; Ruberto et al., 2006), or with bacterial cultures (Pagnout et al., 2006; Fernando Bautista et al., 2009). However, only few have studied these relationships in oil-polluted SW (Brakstad and Faksness, 2000). In cold SW environments like the Arctic, dissolution of oil, as well as biodegradation processes, are expected to be slower than in temperate environments (Faksness et al., 2008; Bagi et al., 2013). This may be compensated by the presence of cold-adapted (psychrophilic/psychrotrophic) bacterial communities (Yakimov et al., 2003; Yakimov et al., 2004).

The objective of this study was to determine the relation between biodegradation and acute toxicity in SW, using a rapid screening bioassay. Three aromatic HCs and the water-accommodated fraction (WAF) of a crude oil were used at low temperatures, relevant for Arctic conditions.
2. Materials and Methods

2.1 Biodegradation experiments

SW was collected from a depth of 80 m (below thermocline) in a non-polluted Norwegian fjord (Trondheimsfjord; 63°26'N, 10°23'E). The SW was supplied by a pipeline system from the source to our laboratories (salinity of 34%, temperature of 6-8°C, and dissolved oxygen (DO) of 8.5 mg/L when reaching our laboratory), passing a sand filter for removal of coarse particles. Nutrient analyses of the SW (Eurofins Environment Testing, Bergen, Norway) showed 23 µg/L total phosphorus, 20 µg/L PO$_4$-P, 940 µg/L total nitrogen, 160 µg/L NO$_2$+NO$_3$-N, 500 µg/L NH$_4$-N, 2.0 mg/L total organic carbon (TOC/NPOC), and <0.05 mg/L Fe. The SW was acclimated to 5°C (7 days before start of the experiments), aerated by bubbling with sterile-filtered air, and amended with mineral nutrients as described in OECD Guideline 306 (OECD, 1992).

Single HCs included 1,3-dimethylnaphthalene (1,3-DMN; CAS no. 575-41-7; 96% purity), phenanthrene (Phe; CAS no. 85-01-8; 98% purity) and fluoranthene (Fluor; CAS no. 206-44-0; 98% purity), all purchased from Sigma-Aldrich. HC properties are described in Table S1 (Supplementary Information), including physical data and predictions of biodegradability and acute ecotoxicity (Episuite, vs. 4.1, US EPA, 2011). Single HCs were dissolved in dichloromethane (DCM; 5.5 mg/ml), and were carefully spotted on 2.25 cm$^2$ Fluortex™ (Sefar AG, Heiden, Switzerland) hydrophobic adsorbents surfaces (100 µL), and adsorbent air-dried (30 min) to evaporate solvent. Adsorbents were then submerged in acclimated, aerated and amended SW in completely filled (no air bubbles) 275 ml flasks (nominal HC concentrations of 2 mg/L. Sterilized SW controls were poisoned with HgCl$_2$ (100 mg/L). Negative controls of DCM without HCs were also included.
Low-energy water-accommodated fractions (LE-WAFs) of a crude naphthenic oil (Troll 2007-0287), evaporated at 200°C to simulate 0.5-1 day on the sea (Daling et al., 1990), were prepared at an oil:SW ratio of 1:100 in acclimated, aerated and nutrient-amended SW, or in the same SW with HgCl$_2$ (sterilized controls), at 4-5°C for 96 hours as previously described (Singer et al., 2000). LE-WAFs were distributed on 275 ml flasks as described above.

Flasks with single HCs and LE-WAFs were incubated at 4-5°C in the dark for up to 63 days, with triplicate sampling after 0, 10, 14, 21, 28, 42 and 63 days of incubation.

2.2 Analyses and calculations

Primary biodegradation was determined using GC-MS analyses of DCM extracts of single HCs, or the LE-WAFs (SIM mode). In samples with single HCs, adsorbents were placed in 30 ml DCM with Na$_2$SO$_4$ and stored at 4°C until extracted, while SW phases were solvent-solvent extracted with DCM (see below). Semi-volatile organic compounds (SVOC) in LE-WAFs included 60 targeted compounds or compound groups of C0- to C4-alkylated naphthalenes, 2- to 6-ring PAH, C0- to C5-alkylated phenols, and C0-C4-alkylated decalines (Brakstad et al., 2014; Brakstad et al., 2015a). The SVOC analytes were quantified in a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent 6890 plus GC coupled with an Agilent 5973 MSD detector, operated in Selected Ion Monitoring [SIM] modus; Agilent Technologies). Deuterated SIS-PAH standards (naphthalene, phenanthrene, chrysene, perylene; 50-250 µg/ml) and RIS-PAH standards (acenaphthene, fluorene; 100 µg/ml) were used for the SVOC compound quantification. The response values for individual target analytes were determined, with a signal-to-noise ratio of 10 as the lower detection limit, and a lower limit of detection (LOD) of 0.01 µg/L was defined for individual oil compounds. Total extractable material (TEM) in DCM extracts was quantified by GC-FID analyses (Agilent
6890N with 30 mDB1 column; Agilent Technologies), using o-terphenyl as surrogate internal standard (SIS), and 5α-androstane as recovery internal standard (RIS), and a LOD of 0.1 µg/L (Brakstad et al., 2015).

Dissolved oxygen (DO) was measured in the flasks with an oxygen meter (YSI Inc., Yellow Springs, OH, USA), biochemical oxygen demand (BOD) determined, and theoretical oxygen demand (ThOD) calculated as a measure of ultimate biodegradation (OECD, 1992).

A closed vial Microtox™ bioassay was used to determine EC₅₀ concentrations in soluble fractions of single HCs or LE-WAFs (Hokstad et al., 1999), using the marine luminescent bacterium Aliivibrio fischeri.

Biotransformation (primary biodegradation) was determined by calculating the percentage concentrations of the compounds (1,3-dimethylnaphthalene and phenanthrene, and compound groups in the LE-WAF) in natural SW, compared to the concentrations of the same compounds in the sterilized SW at each sampling date. Mineralization (ultimate biodegradation) of single HCs and LE-WAFs was determined by comparison of BOD and ThOD as follows: \( y = 100 - \left( \frac{100}{\text{ThOD}} \times \frac{\text{BOD}_n}{C_0} \right) \), where BODₙ is BOD at day \( n \), and \( C_0 \) is the measured concentration of HCs or LE-WAF (TEM) at the start of the experiment (day 0). The calculated ThOD values of the single compounds are shown in Table S1, while a ThOD of 3.0 was used as a ThOD of the LE-WAF, being quantitatively predominated by aromatic HCs with ThOD values close to 3.0. Biotransformation kinetics were determined as first-order rate coefficients and half-lives (GraphPad Prism version 6.01; GraphPad Software, La Jolla CA, U.S.A).

3. Results and Discussions

3.1 Biodegradation and toxicity of 1,3-dimethylnaphthalene and phenanthrene
Initial Microtox studies showed no detectable EC$_{10}$ (Table 1) of the soluble fluoranthene fraction (EC$_{50}$ outside range), combined with concentrations in the SW phase below LOD of 0.01 µg/L (Table 1), and further analyses of this compound were therefore not performed in the study.

The concentrations of 1,3-dimethylnaphthalene and phenanthrene were separately measured on the adsorbents and in the water phase. Most of the compounds rapidly dissolved to the water phase, although measurements in sterilized SW also showed moderate dissolution of 1,3-dimethylnaphthalene from the adsorbents later in the experimental period. Results in natural SW showed faster depletion of both compounds in the water than on the adsorbents, and comparison to sterilized SW demonstrated that depletion was caused by biotransformation (Fig. S1, Supplementary Information). Biotransformation rates and half-lives were determined for the adsorbed fractions of 1,3-dimethylnaphthalene and phenanthrene, and for the total concentrations of the compounds (sum of the adsorbed and solubilized HCs). Since the relative depletion of 1,3-dimethylnaphthalene and phenanthrene was faster in the water than the adsorbed phase, faster degradation of the total concentrations was determined (Fig. 1). Biotransformation rates for the sum of adsorbed and solubilized compounds resulted in half-lives of 9 days for 1,3-dimethylnaphthalene, and 15 days for phenanthrene, while corresponding data for adsorbed HCs were 16 and 35 days, respectively (Fig. 1; Table S2). Mineralization of 1,3- dimethylnaphthalene and phenanthrene was determined from their ThODs (see Table S1). Mineralization half-lives of the HCs were 32 (1,3- dimethylnaphthalene) and 63 (phenanthrene) days (Table S2), resulting in mineralization:biotransformation ratios for 1,3- dimethylnaphthalene and phenanthrene of 3.5 and 4.2, respectively.

Higher Microtox EC$_{10}$ (%) of 1,3- dimethylnaphthalene and phenanthrene (lower toxicity) at day 0 than days 10 and 14 were related to the lower dissolved HC concentrations
at day 0, since dissolution continued during the biodegradation period. Comparison of $EC_{10}$ values as function of dissolved concentrations showed comparable results for 1,3-dimethylnaphthalene and phenanthrene (Table 1). After 21 days of incubation, no toxicity ($EC_{10}>100\%$) of 1,3-dimethylnaphthalene was measured, while no phenanthrene toxicity was measured after 28 days, both results in accordance with reduced HC concentrations as the result of biodegradation (Table 1; Fig. 1). The Microtox data are mainly in agreement with previous results, showing $EC_{50}$ values of 0.79 mg/L for 1,3-DMN and 0.14 mg/L for Phe (Renoux et al., 1999; Parvez et al., 2008). The toxicity reductions were in agreement with reductions in chemical concentration (biotransformation).

### 3.2 Biodegradation and toxicity of LE-WAFs

The LE-WAF contained 0.3 mg/L SVOC ($\sum$ decalines, naphthalenes, 2- to 6-ring PAH and C0- to C5-alkylated phenols). Biotransformation and mineralization half-lives of 11 and 33 days, respectively (Fig. 1; Table S2), resulted in a mineralization:biotransformation ratio of 3.0. Biotransformation of LE-WAF groups showed rapid naphthalene transformation, while PAH was first biotransformed after a lag-period of at least 10 days. Phenols/alkylphenols showed a rapid increase in WAFs after 10-14 days, with 4.5 times higher concentrations than in the sterilized controls, followed by a rapid decline after days 21 and 28 (Fig. S2). These increased phenol concentrations are assumed to be the result of degradation products from other aromatic compounds like naphthalenes and phenanthenes (Haritash and Kaushik, 2009). However, also degradation of monoaromatic hydrocarbons (BTEX; benzene, toluene, ethylbenzene, xylenes) may have contributed to the phenol concentrations (Brakstad and Faksness, 2000), although not measured here. In a separate experiment, using the same setup for preparation and degradation of LE-WAFs, BTEX
concentrations at T0 was 487 µg/L decreasing rapidly to 138 (day 10), 44.1 (day 14), 0.14 (day 21) and 0.05 (day 28) (Hansen et al., 2018).

The initial Microtox EC$_{50}$ of 25 % for the LE-WAF was maintained for 14 days, declined after 21 days, and was out of range (EC$_{50}$$\geq$100% WAF) after 28 days (Table 1). We were able to estimate EC$_{10}$-up until day 42, however, for the last two time points (days 28 and 42), the confidence intervals were very wide. The toxicity was associated with the combined effect of naphthalenes and phenol/alkylphenols and was reduced with the decline of phenol/alkylphenol concentrations after 21 days (Fig. S2). As mentioned above, also BTEX may have contributed to the phenol accumulation and toxicity (Brakstad and Faksness, 2000). Also in the LE-WAFs, the acute toxicity was associated with depletion measured by the chemical analyses (biotransformation) rather than with the oxygen consumption (mineralization).

The results from these studies show slower biotransformation and longer persistence of acute toxicity expressed by Microtox EC$_{50}$s than a previous biodegradation study of LE-WAFs (DOR 1:10000) in natural SW at 13°C, which resulted in naphthalene and PAH biotransformation half-lives of 1-2 days and a removal of Microtox toxicity after 7 days of incubation (Brakstad and Faksness, 2000).

The Microtox assay measures the toxicity of truly soluble compounds, and if compounds are attached to particles in the SW, or to the glass walls of flasks used for the biodegradation experiment, these may be unavailable for the bacteria in the Microtox assay. However, the TOC concentrations in the SW was low (2 mg/L), while sterilized controls of single compounds and LE-WAFs showed negligible depletion of soluble compounds, demonstrating negligible glass wall attachment (Fig. S1).

4. Conclusions
This biodegradation study of 1,3-dimethylnaphthalene, phenanthrene and and LE-WAF of a crude oil in cold SW, showed that the acute toxicities of single compounds, determined by a Microtox screening bioassay, were mainly associated with the biotransformation of the compounds, rather than the slower mineralization process, which involves the complete mineralization of the original compounds and its degradation products. In the LE-WAF, phenol/alkylphenols as degradation products, may have contribute to the toxicity, together with the measured aromatic HCs and BTEX. The cold SW used in these studies resulted in slower biodegradation and longer periods of acute toxicity compared to previous studies with higher SW temperatures. SW temperature may therefore be expected to affect the time window of toxicity after an oil spill, for instance in the Arctic. However, these results should be confirmed through studies with Arctic SW and relevant species for toxicity testing.

Acknowledgements

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References


Tables and Figures

Table 1. Microtox EC\textsubscript{10} concentrations (% of undiluted solutions), concentrations of the single HCs 1,3-dimethyl-naphthalene (1,3-DMN), phenanthrene (Phe), fluoranthene (Fluor) and a LE-WAF. The concentrations of dissolved single HCs in SW (µg/L) are shown, and toxicity related to concentrations (EC\textsubscript{10} in µg/L) during the biodegradation period. For LE-WAF, EC\textsubscript{10} and EC\textsubscript{50}-values are given as % of undiluted solutions. SW controls (adsorbents with DCM) showed EC\textsubscript{10} > 100 %. Results are shown with 95% confidence intervals (C.I.).

<table>
<thead>
<tr>
<th>Inc. (days)</th>
<th>1,3-DMN</th>
<th>Phe</th>
<th>Fluor</th>
<th>LE-WAFs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC\textsubscript{10} (95% C.I.) (µg/L)</td>
<td>EC\textsubscript{10} (95% C.I.) (µg/L)</td>
<td>EC\textsubscript{10} (95% C.I.) (µg/L)</td>
<td>EC\textsubscript{10} (95% C.I.) (µg/L)</td>
</tr>
<tr>
<td>0</td>
<td>28,50 (21,81-37,24)</td>
<td>115 ± 40</td>
<td>32,78 (11,27-15,55)</td>
<td>296 ± 107</td>
</tr>
<tr>
<td>10</td>
<td>10,56 (6,821-16,34)</td>
<td>201 ± 82</td>
<td>21,23 (2,912-5,090)</td>
<td>381 ± 70</td>
</tr>
<tr>
<td>14</td>
<td>13,00 (10,26-16,47)</td>
<td>185 ± 6</td>
<td>24,05 (3,375-22,14)</td>
<td>332 ± 66</td>
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<tr>
<td>21</td>
<td>&gt;100</td>
<td>45 ± 31 ND</td>
<td>&gt;100</td>
<td>89,07 (43,28-183,3)</td>
</tr>
<tr>
<td>28</td>
<td>&gt;100</td>
<td>8.0 ± 12 ND</td>
<td>&gt;100</td>
<td>60 ± 76 ND</td>
</tr>
<tr>
<td>42</td>
<td>&gt;100</td>
<td>&lt;0.01 ND</td>
<td>&gt;100</td>
<td>&lt;0.01 ND</td>
</tr>
<tr>
<td>63</td>
<td>&gt;100</td>
<td>&lt;0.01 ND</td>
<td>&gt;100</td>
<td>&lt;0.01 ND</td>
</tr>
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\[A^\dagger\] nd, not detected
Fig. 1. Biotransformation and mineralization of 1,3-dimethylnaphthalene (A), phenanthrene (B) and LE-WAFs (C), shown as first-order rates. Calculations of % biotransformation and mineralization are described in Materials and Methods. The reference used for calculation is sterilized controls (biotransformation) or ThOD (mineralization). In samples with 1,3-dimethylnaphthalene and phenanthrene, biotransformation rates are shown for the sum of the HCs on the adsorbents and in the SW (Ads + SW) or attached to the adsorbents (Ads).
Highlights

- Acute toxicity of oil compounds were reduced during biodegradation in cold seawater
- Two PAH compounds showed toxicity reductions in relation to their biotransformation
- The toxicity reduction of a crude oil LE-WAF followed depletion of the predominant PAH
- Acute toxicities persisted for longer periods than in previous tests with warmer seawater