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# Effect of Dissolved Aromatic Hydrocarbons on the Growth of Marine Bacteria in Batch Culture

JOHN A. CALDER* AND JUNE H. LADER

*Department of Oceanography, Florida State University, Tallahassee, Florida 32306*

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Dissolved aromatic hydrocarbons were found to decrease growth rate and maximum cell density of marine bacteria in batch cultures. The magnitude of the decrement was observed to be a function of concentration of the hydrocarbon and inherent toxicity. The inherent toxicity was observed to increase inversely with solubility such that naphthalene at 100 $\mu$M concentration demonstrated a toxic effect similar to benzopyrene at 0.02 $\mu$M. A partial oxidation product of naphthalene was found to be more effective in decreasing growth parameters than naphthalene at equivalent concentrations and to cause complete cessation of growth at the higher concentrations permitted by its polar structure.

Hydrocarbons are ubiquitous in the marine environment. They are derived from many sources, the largest fraction probably coming from organisms, especially the marine phyto- and zooplankton (3). In addition, petroleum hydrocarbons and heterocyclic compounds are introduced into the sea in yearly amounts estimated to be a least one million tons (4) and as high as ten million tons (2). The main portion of this petroleum pollution is thought to originate not from major disasters as might be expected, but from small daily influxes (6).

Many genera of organisms have been exposed to petroleum or its constituents to determine biological effects. Generally, bacteria have been considered as oil degraders rather than as organisms which could be adversely affected by petroleum (D. T. Gibson, Proc. Microb. Degrad. Oil Pollut., Atlanta, p. 21;11). However, Walker et al. (9) have recently demonstrated toxicity of a crude and refined oil to natural bacterial populations from sediments uncontaminated with oil. They report the refined to be more toxic than the crude oil, in agreement with the general observation that refined oils are more toxic.

Because bacteria play an important role in the energy budget and productivity of marine ecosystems, it is important to determine if nonhydrocarbon-degrading bacteria are adversely affected by dissolved petroleum hydrocarbons. The dissolution process is an important dispersal mechanism, especially for small scale inputs. Dissolution occurs preferentially with the lower- to medium-molecular-weight aromatic and heterocyclic hydrocarbons, since these are the most soluble in water (5).

## METHODS AND MATERIALS

Bacteria were grown on media containing varying amounts of hydrocarbon (Table 1) to determine the approximate concentration needed to produce an effect on their growth. The growth of the bacteria at each hydrocarbon concentration was compared to the growth in a control containing hydrocarbon-free medium.

The bacteria used in this analysis were *Serratia marinorubra* ATCC no. *19279* and *Vibrio parahaemolyticus* strain FC1011 obtained from R. Colwell, Department of Microbiology, University of Maryland.

The hydrocarbons (Table 1) used in this study were at least 97% pure and were obtained from Aldrich Chemical Co. Reagent grade organic solvents were double distilled in an all glass still before use.

Glassware was hexane rinsed, soaked in 5 N nitric acid for 24 h, rinsed with distilled water, and dried at 200°C for at least 12 h. Glassware (sealed with aluminum foil) was sterilized in a 200°C oven for at least 3 h.

**Culture media.** The bacteria were grown on a medium that consisted of 0.1% yeast extract, 0.05% glucose, 5% (vol/vol) artificial seawater ("Instant Ocean," Aquarium Systems Inc., Wickliffe, Ohio), 2.75% NaCl, 0.02% $MgSO_4$, 0.002% $CaCl_2$, 0.11% $(NH_4)_2SO_4$, 0.17% $K_2HPO_4$, and 0.042% $KH_2PO_4$. The medium was made up with double-distilled water with varying amounts of hydrocarbon-saturated water.

Difco 2216 marine agar was used for plate counting and for maintaining stock cultures.

**Hydrocarbon solutions.** A modification of the procedure of Eganhouse and Calder (7) was used to prepare saturated hydrocarbon solutions. An excess of the hydrocarbon to be analyzed (about 0.2 g) was placed in a ground glass-stoppered 1,000-ml flask with about 700 ml of double-distilled water and shaken on a gyratory shaker at 25.0 ± 0.5°C at 220

TABLE 1.

| Bacterium | Hydrocarbon | Δ% relative to control | | | | Δ% reduction in: | |
|---|---|---|---|---|---|---|---|
| | | Concn (μM) (A) | Increase in lag time (B) | Reduction in ΔA/24 h (C) | Reduction in maximum A (D) | ΔA/(24 h μmol) (E) = C/A | Maximum A/(μmol) (F) = D/A |
| *Serratia marinorubra* | Naphthalene | 46 | NS[a] | NS | NS | | |
| | | 86 | 25 | 33 | 14 | 0.38 | 0.16 |
| *Vibrio parahaemolyticus* | Naphthalene | 11 | NS | NS | NS | | |
| | | 45 | NS | 42 | 10 | 0.93 | 0.22 |
| | | 112 | 15 | 62 | 32 | 0.55 | 0.29 |
| *S. marinorubra* | Phenanthrene | 2.8 | NS | NS | NS | | |
| | | 4.5 | 9 | 31 | 10 | 6.9 | 2.2 |
| *V. parahaemolyticus* | Phenanthrene | 2.2 | 40 | NS | NS | | |
| | | 2.8 | 47 | NS | 8 | | 2.9 |
| *S. marinorubra* | Pyrene | 0.015 | NS | NS | NS | | |
| | | 0.060 | NS | +25 | 8 | +420 | 130 |
| | | 0.15 | NS | +20 | 7 | +130 | 47 |
| *V. parahaemolyticus* | Pyrene | 0.22 | 22 | +37 | NS | +170 | |
| | | 0.31 | 24 | +37 | NS | +120 | |
| *S. marinorubra* | Benzopyrene | 0.004 | NS | NS | 7 | | 1,750 |
| | | 0.020 | NS | NS | 13 | | 650 |
| *V. parahaemolyticus* | Benzopyrene | 0.016 | 24 | NS | NS | | |
| | | 0.024 | 32 | 18 | 9 | 750 | 375 |
| *S. marinorubra* | 2-Methyl-naphthalene | 1.8 | NS | NS | NS | | |
| | | 7.0 | 13 | 33 | NS | 4.7 | |
| | | 19 | 11 | 49 | 17 | 2.6 | 0.89 |
| *V. parahaemolyticus* | 2-Methyl-naphthalene | 1.8 | NS | NS | NS | | |
| | | 7.0 | 20 | NS | NS | | |
| | | 19 | 31 | 28 | 15 | 1.5 | 0.79 |
| *S. marinorubra* | 2,6-Dimethyl-naphthalene | 1.3 | 9 | 56 | NS | 43 | |
| | | 6.5 | 10 | 48 | 12 | 7.4 | 1.9 |
| *V. parahaemolyticus* | 2,6-Dimethyl-naphthalene | 1.3 | NS | 30 | 11 | 23 | 8.5 |
| | | 6.5 | NS | 28 | 11 | 4.3 | 1.7 |
| *S. marinorubra* | 2,3-Dihydroxy-naphthalene | 97 | 71 | 79 | 70 | 0.81 | 0.72 |
| | | 388 | ∞ | 100 | 100 | | |
| *V. parahaemolyticus* | 2,3-Dihydroxy-naphthalene | 92 | 72 | 67 | 42 | 0.73 | 0.46 |
| | | 277 | ∞ | 100 | 100 | | |

[a] NS, Not significant, differs from control by less than twice the average deviation about the mean.

rpm for 12 h. The flask was then removed from the shaker and allowed to stand at 25°C for at least 24 h to permit the hydrocarbon to equilibrate with the water. The hydrocarbon solution was filtered through a sterile Swinney filter containing a 0.45-μm membrane filter (Millipore Corp.) into a sterile flask. Various portions of the saturated hydrocarbon solution were used to prepare growth flasks of different hydrocarbon concentration. After aseptically removing the amount of hydrocarbon solution needed for the preparation of growth flasks, exactly 100 ml of the remaining solution were placed in a volumetric flask with an enlarged neck. The solutions were extracted with three 10-ml aliquots of hexane (chloroform was used when dihydroxynaphthalene was extracted). The first 10 ml of hexane contained a known quantity of an internal standard, which was a hydrocarbon similar in vapor pressure to the hydrocarbon being analyzed. After extraction, the 30 ml of hexane containing the hydrocarbon and internal standard were concentrated by evaporating the hexane under a purified nitrogen steam at room temperature. Usually evaporation to 10 ml was sufficient. One microliter of this extract was injected into a dual column gas chromatograph with flame ionization detectors, equipped with an automatic integrator. One microliter of an external standard containing known quantities of the particular hydrocarbon and the standard was also injected into the gas chromatograph. Peak areas of the unknown and the internal standard, as well as the hydrocarbon and standard in the external standard, were

automatically integrated.

The column used for all hydrocarbons was 4% OV-17 on Chromosorb G(AW, DMCS) (1 m by 2 mm inner diameter). The carrier gas was purified nitrogen flowing at 20 ml/min. The detectors and inlet ports were kept at 350°C and 300°C, respectively. The initial and final oven temperatures and the programming rate were optimized for the hydrocarbons being analyzed.

**Bacterial analysis.** Twenty-five milliters of medium was placed into sterile 125-ml screw-cap sidearm flasks. Duplicate flasks for each hydrocarbon concentration and for a control (containing no hydrocarbon) were prepared. The bacteria were pregrown on non-hydrocarbon-containing standard medium to an absorbance of 0.2 on a Bausch and Lomb Spectronic 20 spectrophotometer at 600 nm, which corresponds to about $7 \times 10^7$ cells/ml. The cells were diluted 100-fold, and 0.1 ml of this dilution was used to inoculate the test flasks. The flasks were incubated in the dark on a gyratory shaker at 25.0 ± 0.5°C at 200 rpm. Their absorbance was read on the spectrophotometer every 2 to 4 h to follow the growth curve. The number of bacteria (as determined by plate counts) was directly proportional to the absorbance over the range 0.05 to 0.6 units. The experiment was terminated when two successive readings showed no further increase in the absorbance of the bacterial culture.

The average absorbance of each pair of flasks was plotted as a function of time in hours to produce a growth curve for each hydrocarbon concentration as well as a standard curve obtained from the control. For each growth curve, the lag time (in hours), growth rate ($\Delta A$/24h) and maximum absorbance were determined. Data are expressed as Δ% of control where

$$\Delta\% = 10^2 \times \left(\frac{\text{control value} - \text{experimental value}}{\text{control value}}\right)$$

The average deviation about the mean for all replicate pairs of control flasks was ±3% for lag time, ±8% for growth rate, and ±3% for maximum absorbance.

## RESULTS

Representative results of the batch culture growth experiments in the absence and presence of aromatic hydrocarbons are presented as growth curves in Fig. 1 through 3. The data derived from all growth experiments are presented in Table 1. All of the hydrocarbons tested had an impact on one or more of the measured growth parameters. In most cases, this impact was negative, that is lag time increased, growth rate decreased, or maximum absorbance decreased. In the case of pyrene increases in growth rate were observed, whereas negative impacts were observed in one of the other growth parameters. Table 2 lists the molecular weight and solubility in distilled water for each hydrocarbon used in this study. The alkyl naphthalenes, pyrene and benzopyrene, caused significant effects at less than 20% of saturation. Phenanthrene required 68% of saturation to cause an effect, the highest percentage of saturation of the hydrocarbons tested.

The magnitude of the toxicity (Δ% of control) was normalized to unit (micromolar) concentration and tabulated in Table 1 (columns E and F). The normalized data will be referred to as the inherent toxicity of a given hydrocarbon. The average inherent toxicity for each hydrocarbon is tabulated in column B of Table 2. In Fig. 4, the log molar solubility and log average inherent toxicity are plotted versus molecular weight.

The impact of a partial oxidation product, dihydroxynaphthalene, was also investigated, and the results are included in Table 1.

## DISCUSSION

Aromatic hydrocarbons decrease the growth rate and maximum cell density of two representative marine bacteria when grown under laboratory conditions. The magnitude of the decrement is a function of both concentration of the hydrocarbon and its inherent toxic properties. The inherent toxicity increases inversely with solubility. Both log solubility and log inherent toxicity are plotted versus molecular weight in Fig. 4. As can be seen, the slopes of the lines are of opposite sign and approximately equal magnitude. The product of solubility and inherent toxicity for each hydrocarbon can be termed a toxicity index. Column E in Table 2 indicates numerically what Fig. 4 states visually, that a saturated solution of benzopyrene could have nearly the same impact as a saturated solution of naphthalene, although there are several orders of magnitude difference in their solubilities. The low aqueous solubilities of the medium- to high-molecular-weight aromatic hydrocarbons should not be allowed to obscure their potential toxicity. The overall impact on an ecosystem may reside primarily in the medium- to high-molecular-weight range since the low- to medium-molecular-weight range is subject to greater losses by volatilization and degradation. Recently, Atlas (1) observed that, after the removal of low- to medium-molecular-weight molecules by artificial weathering, weathered light oils were biodegraded by bacteria at a higher rate and to a greater extent than were weathered heavy oils. He states this may be due to resistance of the complex high-molecular-weight compounds to degradation.

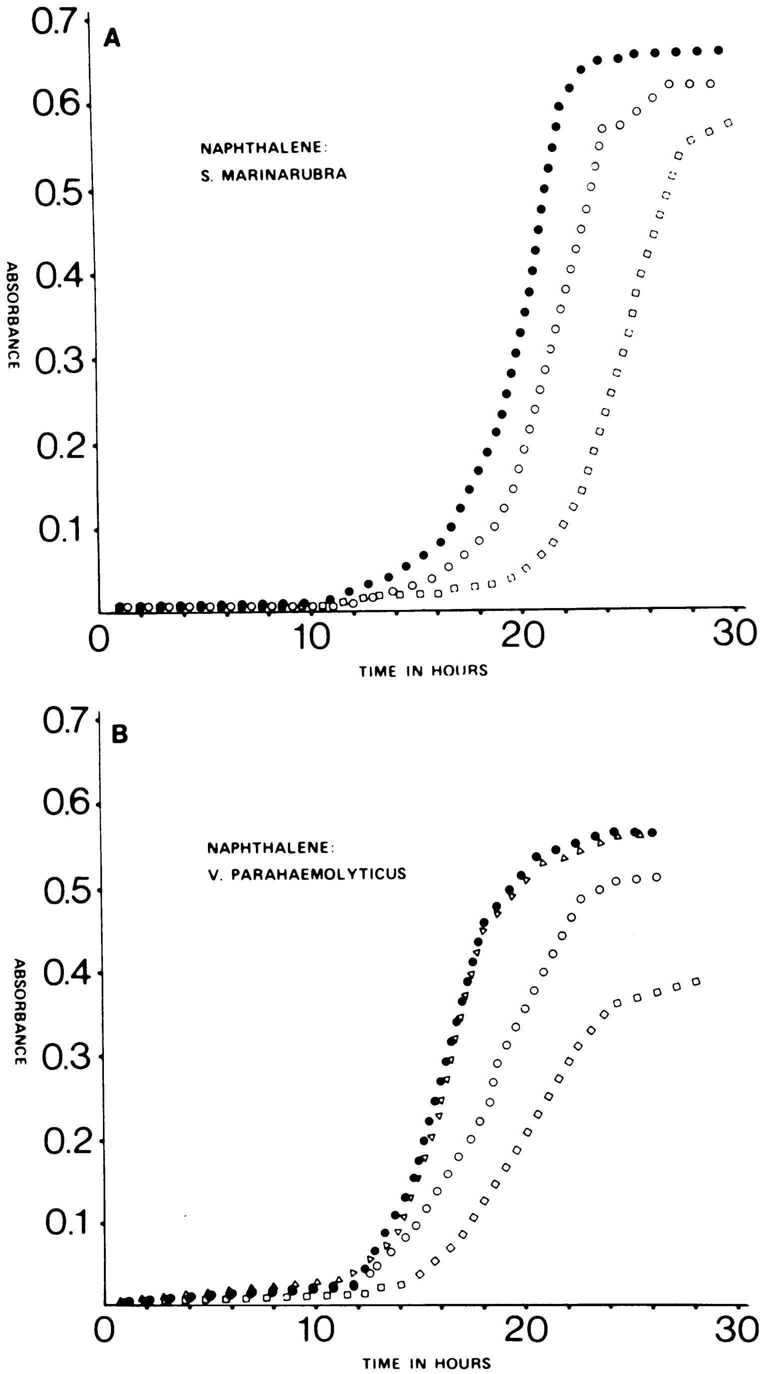


FIG. 1. *(A) Effect of naphthalene on growth of S. marinorubra: (●), control; (○), 45 μM; (□), 86 μM. (B) Effect of naphthalene on growth of V. parahaemolyticus: (●), control; (△), 11 μM; (○), 45 μM; (□), 112 μM.*

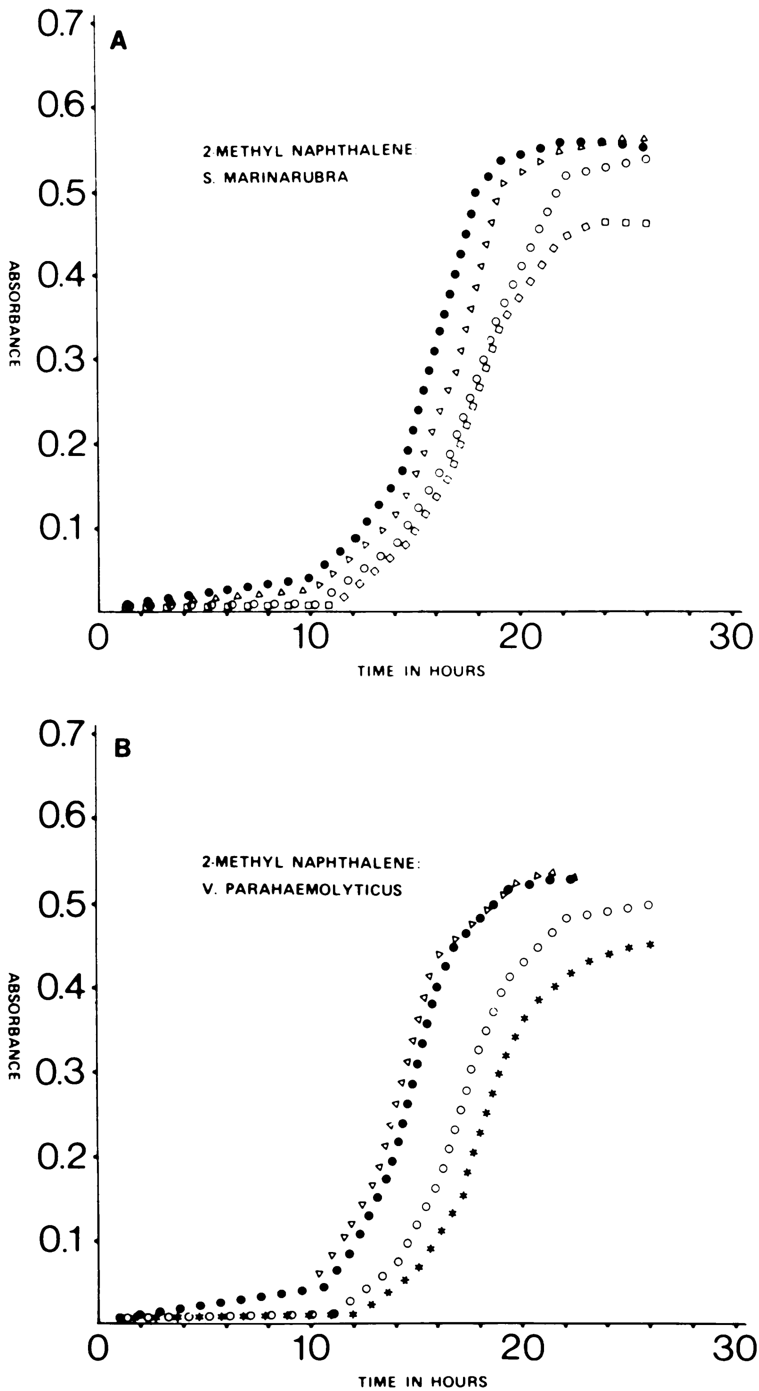


FIG. 2. *(A) Effect of 2-methylnaphthalene on growth of S. marinorubra: (●), control; (△), 1.8 μM; (○), 7.0 μM; (□), 19 μM. (B) Effect of 2-methylnaphthalene on growth of V. parahaemolyticus: (●), control; (△), 1.8 μM; (○), 7.0 μM; (★), 19 μM.*

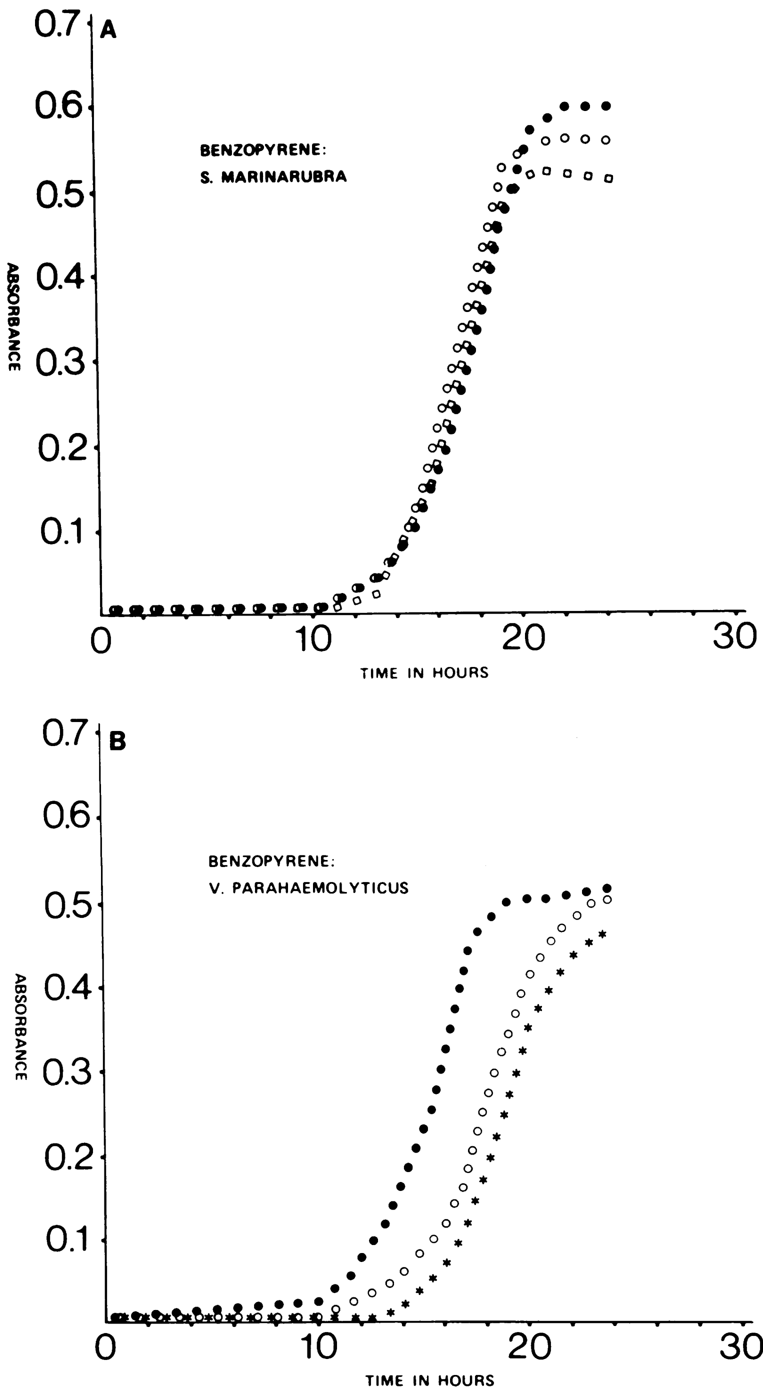


FIG. 3. *(A) Effect of benzopyrene on growth of S. marinorubra: (●), control; (○), 0.004 μM; (□), 0.020 μM. (B) Effect of benzopyrene on growth of V. parahaemolyticus: (●), control; (○), 0.016 μM, (★), 0.024 μM.*

TABLE 2. *Properties of hydrocarbons studied*

| Hydrocarbons | Mol wt | (A) Solubility[a] (M) | (B) Δ%/ μmole[b] | (C) Toxicity[c] index |
|---|---|---|---|---|
| Naphthalene | 128.16 | $2.43 \times 10^{-4}$ (7) | 0.4 | 97 |
| 2-Methylnaphthalene | 142.19 | $1.73 \times 10^{-4}$ (7) | 2.1 | 363 |
| 2,6-Dimethylnaphthalene | 156.22 | $0.83 \times 10^{-5}$ (7) | 12.8 | 106 |
| Phenanthrene | 178.22 | $0.66 \times 10^{-5}$ (7) | 4.0 | 26 |
| Pyrene | 202.24 | $0.73 \times 10^{-6}$ (10) | 170 | 124 |
| Benzopyrene | 252.3 | $\sim 0.30 \times 10^{-7}$ | 881 | >26 |
| 2,3-Dihydroxynaphthalene | 160.16 | $>16.5 \times 10^{-4}$ | .68 | >1,120 |

[a] In distilled water at 25°C, 1 atm.
[b] Average Δ% from columns E and F, Table 1.
[c] $C = A \times B \times 10^6$.

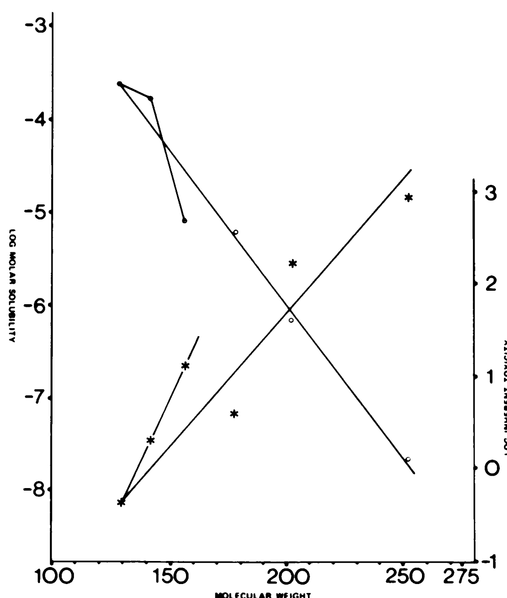


FIG. 4. *Molar solubility and inherent toxicity of aromatic hydrocarbons versus molecular weight. Symbols: (O), log molar solubility from Table 2, column A; (★), log inherent toxicity from Table 2, column B. In each case, the shorter line represents the series naphthalene, methylnaphthalene, dimethylnaphthalene, and the longer line represents the series naphthalene, phenanthrene, pyrene, benzopyrene.*

However, the observation is also consistent with a partial inhibition of bacterial growth by compounds in the heavy oil which possess a higher inherent toxicity.

When naphthalene is oxidized by bacteria, dihydroxynaphthalene is one of the early degradation products (8). It is interesting that both bacteria investigated here were affected to a greater extent by dihydroxynaphthalene than by naphthalene at equivalent concentrations. Moreover, because of its more polar structure the dihydroxy compound can achieve concentrations high enough to cause complete cessation of growth. If partial oxidation products were to build up in an environment, the impact of an oil spill may be significantly increased.

## ACKNOWLEDGMENT

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