

# Effect of toluene exposure time and concentration on induction of high affinity values for toluene oxidation by bacteria of estuarine seawater samples

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**ABSTRACT:** Upon amending fresh seawater samples with toluene, metabolism began immediately; autoradiography indicated that a minimum of about 7 % of the bacteria were responsible. Rate of toluene metabolism increased with time, maximal rate of increase occurring about 48 h following exposure. There was no concomitant increase in bacterial populations and, consistent with observations of marine isolates in predator-free systems, the increase in rate was interpreted as induction. Induction followed saturation kinetics as might be expected if the rate of inducer penetration also followed saturation kinetics;  $K_{ind}$  observed was the very small value of  $1.9 \mu\text{g l}^{-1}$ , but was consistent with Michaelis constants for toluene transport observed in other studies.

## INTRODUCTION

The metabolism of hydrocarbons by bacteria is known to be inducible (Claus & Walker 1964, Connors & Barnsley 1980). Murray & Williams (1974) found that 2.5 mM catechol, a common degradation product of aromatic substrates, was required to induce enzymes of the *meta* pathway; but that 'growth limiting' concentrations were sufficient to induce only *ortho* pathway enzymes. Spain et al. (1980) found that exposure of river bottom core samples to  $60 \mu\text{g l}^{-1}$  *p*-nitrophenol for 100 h was sufficient to stimulate its metabolism. However, the minimum intercellular hydrocarbon concentrations necessary for enhancement of the capacity of aquatic microflora to metabolize aromatic hydrocarbons remain unknown.

Our interest in induction kinetics for toluene metabolism stems from the wide range of toluene oxidation rates observed in 8 estuarine seawater samples (Button et al. 1981a, Button & Robertson unpubl.), the short turnover times for terpenes in seawater (Button 1984), and the structural similarity to toluene of the styryl moiety of stilbene, a common antiherbivore parent-compound in hardwoods (Bryant et al. 1983). It appears that concentrations where induction is half-maximal ( $K_{ind}$ ) lie near the Michaelis constants for

transport of the substrates. Both of these values appear to be relatively small for hydrocarbons as compared to other substrates which suggests an unusual mechanism for transport (Button et al. 1984). Moreover, an understanding of the concentration dependency of the induction process is useful in evaluating the responses to given concentrations of hydrocarbons in the environment, in evaluating the metabolism rates of hydrocarbons by natural microflora from oxidation rate data, in inferring ambient concentrations of hydrocarbons from the state of induction of the bacteria contained, and in describing the general nature and mechanism of hydrocarbon transport into bacteria.

In this paper we explore the concentration- and time-dependent kinetics of hydrocarbon metabolism induction in an estuarine microbial assemblage, and examine the populations involved.

## METHODS

*Site.* Seawater was collected in Resurrection Bay, a pristine Alaskan fjord which borders the Gulf of Alaska and receives tidal input that is rich in fresh-water runoff from extensive conifer-forested slopes further south. This fresh surface water is retained

shoreward and is carried north by the Alaska Coastal Current (Royer 1982). Mid-June was selected for these experiments because toluene oxidation rate measurements were undetectable during the previous fall. We thought that if the spring runoff contained part of the winter's accumulation of conifer-derived terpenes, populations of hydrocarbon oxidizers in the receiving estuaries might be sufficiently high for convenient measurements of the kinetics of induction.

**Sample collection.** Samples were collected on June 12 to 22, 1983, using a rowed skiff at a point 100 m offshore, 500 m upwind and upcurrent from the town of Seward. Seawater was dipped from the surface with a glass carafe into 20 l glass carboys. Most glassware had been heated to 550°C for 6 h for final cleaning.

**Substrate.** [U-ring-<sup>14</sup>C]-toluene, 60 mCi mmol<sup>-1</sup> (New England Nuclear) and [4-<sup>3</sup>H]-toluene, 75 mCi mmol<sup>-1</sup> (Amersham) were distilled onto a cold finger to separate toluene from less volatile contaminants and stored as previously described (Button et al. 1981b). The distilled, tritiated substrate was diluted 300-fold with unlabeled toluene (reagent grade, Baker) to reduce a contaminant (possibly <sup>3</sup>H<sub>2</sub>O) that survived distillation and remained at the 10% level. The toluene used for induction was therefore diluted with the usual <sup>1</sup>H isotope of toluene.

**Incubation.** A walk-in incubator was carefully cleaned and flushed with seawater to minimize sources of hydrocarbon vapor, and adjusted to the approximate temperature of the estuary, 10°C. Samples were placed in the incubator and amended with toluene within 1.5 h after collection. The diluted, tritiated toluene was added as the inducer to each of 5 carboys, with concentrations ranging from 0 to 142 µg l<sup>-1</sup>. The carboys were shaken daily, and 4 l subsamples were removed. Resulting subsamples were placed in a separate constant-temperature bath to prevent toluene exchange, and sparged with filtered air for 2.5 h at 1 l min<sup>-1</sup> to remove the <sup>3</sup>H-toluene (removal rate = 0.95 h<sup>-1</sup>). <sup>14</sup>C-toluene was then added to obtain a concentration of 0.9 to 2.0 µg l<sup>-1</sup>. To determine the extent of induction, incubation of subsamples began immediately: 2 l portions were collected after 5 and 10 h, acid was added to each, and the liberated <sup>14</sup>CO<sub>2</sub> was collected in an air stream, dried, purified with chilled hydrophobic resin and trapped in 20 ml of scintillation mixture containing (per liter) 270 ml methanol, 270 ml phenethylamine, 460 ml toluene and 5 g Omnifluor (New England Nuclear); the radioactivity was measured as previously described (Button et al. 1981b).

In 1 control experiment designed to observe the effect of sample manipulation, preincubation with tritiated toluene was omitted; <sup>14</sup>C-toluene was added to the seawater immediately following collection, and the

<sup>14</sup>CO<sub>2</sub> production rate measured over time. In another control experiment, seawater was filtered (0.2 µm 'Mini-Capsule', Gelman Instrument Co.) to observe any abiotic toluene oxidation. To evaluate the magnitude of the 'bottle effect' (Ferguson et al. 1984), a seawater sample was carefully collected and divided into four 2 l bottles. Manipulations were outdoors to avoid contamination with laboratory air, and special care was taken to avoid the introduction of organic carbon. Incubation was also outside in a seawater-fed pond and in the shade. Each day, one of the series was amended with <sup>14</sup>C-toluene at about 2 µg l<sup>-1</sup>, and the rate of <sup>14</sup>CO<sub>2</sub> production measured as described above.

**Populations.** Samples for determination of total bacterial populations were fixed with formaldehyde (to 1%) and counted using epifluorescence microscopy (Hobbie et al. 1977). One ml subsamples were diluted to 2 ml with filtered (0.2 µm) artificial seawater medium and stained with acridine orange.

The population of toluene oxidizers was estimated from autoradiographic determinations in conjunction with epifluorescence microscopy based on the procedures of Tabor & Neihof (1982) using a portion of the incubation mixture from the toluene induction sample that contained 142 µg l<sup>-1</sup> of the tritiated toluene. A washed suspension of *Escherichia coli*, for which toluene is not a growth substrate, was added to 1 sample giving a final population of 0.6 × 10<sup>9</sup> l<sup>-1</sup>. Incubation was at 10°C in the dark. Subsamples were taken at 24 and 116 h, fixed with formalin, and stored refrigerated for about 3 wk. These were then filtered and treated with photographic emulsion. Representative autoradiograms were developed and observed over a period of 7 wk until the silver grain count became constant as compared to the <sup>3</sup>H-toluene-free control. <sup>3</sup>H-beta-emission-exposed silver grains in the emulsion (Paerl 1984) were clearly apparent after photographic development.

**Formulations.** The toluene (A<sub>out</sub>) removal rate (v) from solution is given by the biomass (X) and the affinity (a<sub>A</sub>) of the biomass for toluene by the second order rate equation (Button 1983) by

$$v = dA_{out}/dt = a_A X A_{out} \quad (1)$$

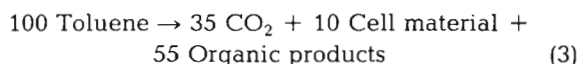
If the substrate (A<sub>out</sub>) becomes sufficient for saturation to occur, the affinity is reduced correspondingly as specified by the relation between v and A<sub>out</sub>. For example if the transport system obeys Michaelian kinetics, then the value of the affinity at the Michaelis constant for transport K<sub>t</sub> is reduced to

$$a_A^{Kt} = \frac{V_{max}}{K_t + A_{out}} = \frac{a_A^0}{2} \quad (2)$$

where V<sub>max</sub> is given in grams A per gram of cells (wet weight) per hour. At low concentrations where satura-

tion is insignificant and  $v$  is proportional to  $A_{out}$ , the affinity  $a_A^o$  at the concentration specified by its superscript lies near its maximal values and serves as a base on which to compare the substrate transport ability of various organisms. The alternative of formulating affinity as  $a_A^o = A_{max}/K_t$  is not used because transport kinetics often have a first order leg in concentration, making  $V_{max}$  indeterminate.

Toluene consumed and cell material produced were estimated from  $^{14}CO_2$  production according to Eq. (1) and laboratory mass balance data for toluene metabolism by the marine isolate *Pseudomonas* sp. strain T2 (to be presented separately):



Carbon dioxide produced from toluene is given in units of toluene on a carbon basis:  $ng \text{ CO}_2 \times 92 / (7 \times 44)$ .

Growth is given assuming 0.18 g carbon (g cell wet weight) $^{-1}$ , based on C/N analysis (Perkin-Elmer CHN analyzer) of *Pseudomonas* sp. strain T2 with size determined by Coulter volume (Model Z<sub>BI</sub>) as standardized with red blood cells supplied by Coulter Electronics. Biomass was calculated using an average mass of 0.25 pg organism $^{-1}$  according to microscopic measurements.

RESULTS

Toluene oxidation vs population

$^{14}CO_2$  formation from  $^{14}C$ -toluene from 2 experiments is shown in Fig. 1. The lower rate was obtained

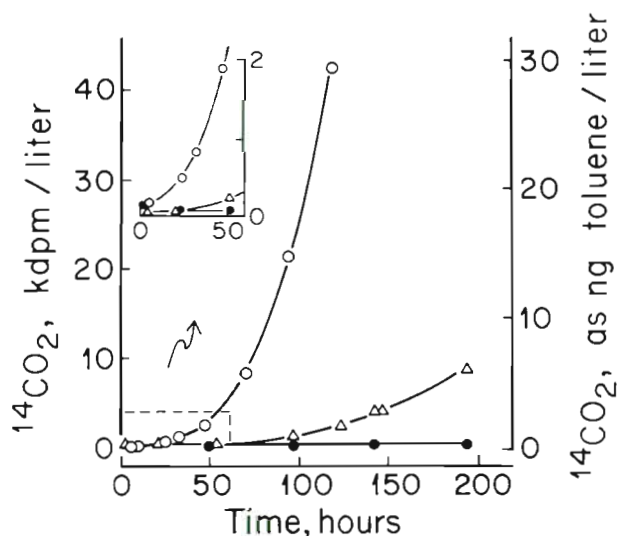


Fig. 1.  $^{14}C$ -toluene metabolism to  $^{14}CO_2$  in fresh surface seawater from Resurrection Bay near Seward, Alaska. ( $\Delta$ ) 12 June 1983, 2  $\mu g$  toluene  $l^{-1}$ ; ( $\circ$ ) 22 June 1983, 0.9  $\mu g$  toluene  $l^{-1}$ ; ( $\bullet$ ) 12 June 1983, 2  $\mu g$  toluene  $l^{-1}$  filtered (0.2  $\mu m$ ) control

from a preliminary experiment to determine if measurable activity existed. The higher rate was obtained a few days later from a 20 l portion of the induction experiment detailed below. Rates increased over the duration of the experiments shown. Initial rates, when normalized to 2  $\mu g$  toluene  $l^{-1}$  differed by a factor of 10 whereas the bacterial populations were about the same (Table 1). The composition of the population may have been different, however. During this spring season, a daytime onshore breeze usually retains onshore a surface layer that is rich in fresh water, seston and glacial silt. Upwelling (Heggie & Burrell 1981), wind and tides combine to give dramatically visible changes in turbidity. These changes are likely to affect the microbial composition of the surface water.

The population of toluene oxidizers, determined after incubation with the  $^3H$ -toluene at 142  $\mu g l^{-1}$  for 24 h, was 7 % of the total. Association of silver grains with bacteria for reasons other than toluene incorporation did not appear to be a problem because in these experiments (Table 2) and in similar experiments using a  $^3H$ -terpene mixture (not shown), silver grains were 20 times as likely to be associated with the indigenous organisms as with *Escherichia coli*, and silver grains were not associated with indigenous

Table 1. Toluene oxidation rates and bacterial populations

Sample date	Toluene conc. ( $\mu g l^{-1}$ )	Time (h)	Initial rate ( $pg l^{-1} h^{-1}$ ) <sup>a</sup>	Population $\times 10^9 l^{-1}$
12 Jun 1983 filtered	2.0	0	0.0	0.05 ( $\pm 0.05$ ) <sup>b</sup>
		148	-	0.71 ( $\pm 0.24$ )
		193	-	1.43 ( $\pm 0.44$ )
12 Jun 1983	2.0	0	3.0	0.62 ( $\pm 0.18$ )
		148	-	1.08 ( $\pm 0.29$ )
		193	-	1.13 ( $\pm 0.37$ )
22 Jun 1983	0.0	0	-	0.68 ( $\pm 0.18$ ) <sup>c</sup>
		0	-	0.53 ( $\pm 0.13$ ) <sup>d</sup>
		117	-	1.18 ( $\pm 0.34$ )
	0.7	117	-	0.95 ( $\pm 0.19$ )
		0.9 <sup>e</sup>	0	41.2
	117	117	-	1.07 ( $\pm 0.31$ )
		7.5	117	-
	14.2	117	-	1.21 ( $\pm 0.19$ )
		142.	117	-

<sup>a</sup> Rate of  $^{14}CO_2$  production measured from 3 subsamples during a 24 h incubation, expressed as toluene and normalized with respect to a toluene concentration of 2.0  $\mu g l^{-1}$  according to Eq. 1

<sup>b</sup> Number in parenthesis is the standard deviation

<sup>c, d</sup> Duplicate counts from the same sample

<sup>e</sup> Preincubation with  $^3H$ -toluene was omitted;  $^{14}C$ -toluene was added immediately after sample collection, and the population was therefore the same as that measured for 0.0  $\mu g$  toluene  $l^{-1}$  at 0 h

Table 2. Portion of the total bacterial population, according to epifluorescence microscopy, capable of metabolizing toluene according to autoradiography data

Incubation time (d)	Total particles counted	Silver grains	Organisms with silver grains	% Toluene oxidizers
— <sup>a</sup>	3	68	0	0
1	308	—	24	7.7
1	51 <sup>b</sup>	197 <sup>b</sup>	4 <sup>b</sup>	7.8
5	328	—	36	9.1
5	249 <sup>c</sup>	331 <sup>c</sup>	20 <sup>b</sup>	8.0
5	276 <sup>b, d</sup>	257 <sup>b</sup>	40	14
	( <i>E. coli</i> added)			
5	151 <sup>e</sup>	—	1	0.7
	( <i>E. coli</i> added)			

<sup>a</sup> From autoradiogram of filtered artificial seawater  
<sup>b</sup> Number 0.01 mm<sup>-2</sup>  
<sup>c</sup> Number 0.005 mm<sup>-2</sup>  
<sup>d</sup> Organisms morphologically different from *Escherichia coli* in a sample with added *E. coli* at a population of  $0.6 \times 10^9$  l<sup>-1</sup>  
<sup>e</sup> Organisms in the above sample that were similar to *E. coli*

organisms if the samples were treated with formaldehyde before incubation. The proportion of apparent toluene oxidizers increased when the incubation with <sup>3</sup>H-toluene was extended from 1 to 5 d, but only to 14%. It should be noted that where silver grains were associated with organisms there was only an average of 1 grain organism<sup>-1</sup>. Low <sup>3</sup>H-toluene specific activity and low toluene metabolism rates combined to reduce sensitivity in this experiment, resulting in a probable underestimation of toluene oxidizers – those organisms that contained some toluene-derived tritium but emitted insufficient electrons to sensitize a silver grain. It is therefore estimated that toluene oxidizers represent at least 7% of the total bacterial population visible by epifluorescence microscopy.

Table 1 indicates that the total bacterial population increased with incubation time, about doubling over the duration of the experiments. Even the population of the filtered control returned to its original level prior to filtration after 6 d of incubation and double that level after 8 d. This could have represented contamination, or more likely passage of small bacteria through the 0.2 μm filter because of the large inoculum required to replace the original population in 6 d. Toluene oxidation in this filtered control, however, remained at baseline levels throughout the experiment (Fig. 1) indicating removal of the responsible organisms. Data also indicate that this special, non-toluene-oxidizing bacterial population can grow in a presumably bacterivore-free system in the absence of significant added substrate (2 μg toluene l<sup>-1</sup> as compared to about

700 μg bacteria l<sup>-1</sup>) with a doubling time of about 2 d (Table 1).

### Induction

<sup>14</sup>CO<sub>2</sub> accumulation from <sup>14</sup>C-toluene in the various <sup>3</sup>H-toluene-induced samples is related to time in Fig. 2. Although special precautions were taken to avoid contamination of the samples with hydrocarbon vapors, there was a significant increase in the oxidation activity with time in the toluene-free control. The samples incubated in the outdoor pond, free from laboratory contamination, also showed a systematic increase in oxidation rate with time (Table 3). We therefore attribute a small amount of the increase in

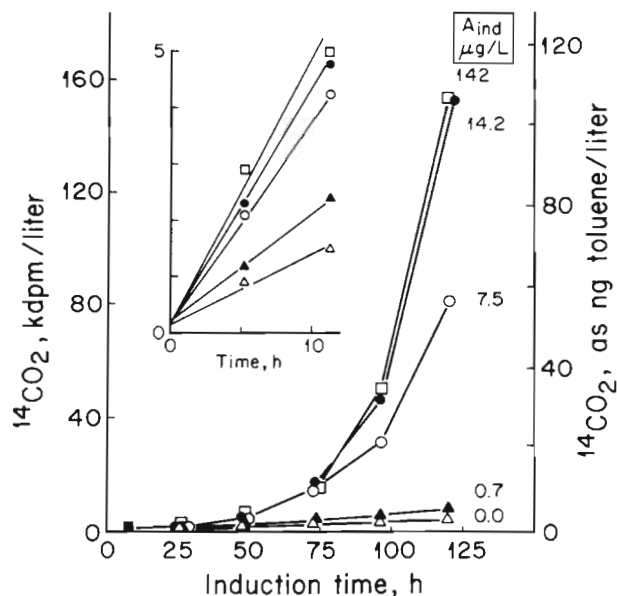


Fig. 2. Toluene metabolism to <sup>14</sup>CO<sub>2</sub> 10 h after addition of <sup>14</sup>C-substrate following 10 to 120 h exposure to (Δ) 0.0, (▲) 0.7, (○) 7.5, (●) 14.2, and (□) 142 μg <sup>3</sup>H-toluene l<sup>-1</sup>. (■) control, 0.9 μg <sup>14</sup>C-toluene l<sup>-1</sup> added to freshly collected seawater. Inset shows typical time course for <sup>14</sup>CO<sub>2</sub> production from 2 μg toluene l<sup>-1</sup> following 48 h exposure to inducing toluene

Table 3. Effect of confining seawater samples in bottles for various periods before measuring activity of toluene-oxidizing bacteria contained

Time (h)	Toluene (μg l <sup>-1</sup> )	Rate (pg l <sup>-1</sup> h <sup>-1</sup> ) <sup>a</sup>
0	2.0	36
24	0.8	70
48	2.0	113
72	2.0	639

<sup>a</sup> Rate of <sup>14</sup>CO<sub>2</sub> release over a 10 h period following the confinement period and calculated as toluene

activity to a 'bottle effect' of unknown cause rather than air-borne contamination.

Rates of toluene oxidation to carbon dioxide as a function of exposure time and inducing substrate concentration are shown by the surface of a 3-dimensional histogram (Fig. 3). Both concentration and rate are on logarithmic scales in order to demonstrate the low level effects. Detailed data are given in Table 4. The maximal rate of change in oxidation rate occurred between 24 and 48 h following exposure to the inducer. The concentration-dependent kinetics of toluene induction, as evaluated from the rate of toluene oxidation at 48 h, are shown in Fig. 4. A small rate of  $v_0 = 0.08 \text{ ng l}^{-1} \text{ h}^{-1}$  obtained from the inducing toluene-free control was subtracted from the measured rates ( $v$ ) obtained before plotting, in order to demonstrate the increase in rate due to induction. The affinity  $a_A^2$  ( $\approx a_A^0/2$ ) of the hydrocarbon oxidizers for toluene was calculated from the  $\text{CO}_2$  production rate at 48 h and the total observed population of  $0.6 \times 10^9 \text{ l}^{-1}$  (Table 1) by assuming that (i) half of those present were obscured by detritus in the epifluorescence counting samples, (ii) only 7 % were toluene oxidizers (autoradiography data), (iii) the mass of the organisms was 0.25 pg each.

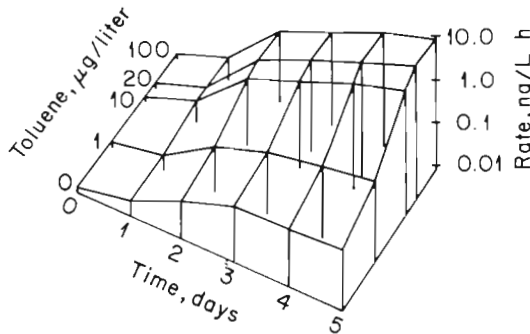


Fig. 3. Rate of toluene metabolism vs inducing toluene concentration and exposure time to substrate

Table 4. Rates of  $^{14}\text{CO}_2$  production ( $\text{ng l}^{-1} \text{ h}^{-1}$ ) with respect to both inducing toluene concentration and exposure time. Rates are normalized to a toluene concentration  $2 \mu\text{g l}^{-1}$  used in the incubation period following induction

Inducing toluene conc. ( $\mu\text{g l}^{-1}$ )	Toluene exposure duration (h)					
	5	24	48	72	96	120
0.0	—	0.02	0.08	0.15	0.20	0.25
0.7	—	0.03	0.14	0.23	0.36	0.56
7.5	—	0.04	0.26	0.91	2.05	6.20
14.2	—	0.03	0.28	1.12	2.99	10.37
142.0	—	0.04	0.30	1.04	3.29	9.50
0.9 <sup>a</sup>	0.04	—	—	—	—	—

<sup>a</sup> Inducing substrate was  $^{14}\text{C}$ -toluene

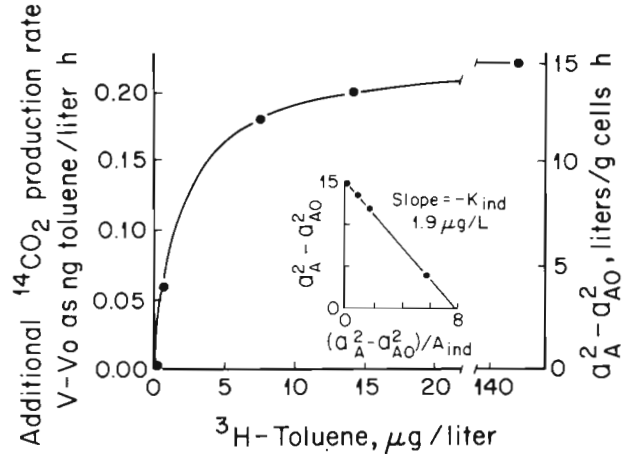


Fig. 4. Increase in rate of  $^{14}\text{CO}_2$  production vs inducing  $^3\text{H}$ -toluene concentration following 48 h exposure to substrate. Initial rates without added toluene,  $v_0$ , were subtracted from observed rates,  $v$  (left ordinate). Corresponding net changes in affinities  $a_{A0}^2$  are shown (right ordinate). Subtracted values are  $a_{A0}^2 = 1.4 \text{ l (g cell)}^{-1} \text{ h}^{-1}$  and  $v_0 = 0.08 \text{ ng l}^{-1} \text{ h}^{-1}$

Data were fitted to a hyperbola based on the hypothesis that the rate of induction was proportional to an internal metabolite concentration that in turn depended on accumulation through a transport system that was saturatable. This gave a concentration ( $K_{ind}$ ) of  $1.9 \mu\text{g l}^{-1}$  as sufficient to cause the affinity to increase to half its maximal value. This very small concentration range was confirmed indirectly in experiments to be reported separately where we examined the toluene-concentration-dependent kinetics of toluene oxidation of natural seawater assemblages. Experiments showed increased rates of the type shown in Fig. 2 after 18 h exposure to sub- $\mu\text{g l}^{-1}$  concentrations of toluene.

## DISCUSSION

### Toluene oxidizing population

In unperturbed marine systems, heterotrophic bacteria as determined by direct count epifluorescence microscopy are comparatively homogeneous in population, ranging only from 0.1 to  $10 \times 10^9 \text{ l}^{-1}$  (Bowden 1977, Hobbie et al. 1977, Meyer-Reil 1978) as controlled by microflagellate predation (Fenchel 1982, Davis & Sieburth 1984). Populations in surface samples taken from Resurrection Bay within a 2 wk period were about the same,  $0.6 \times 10^9 \text{ l}^{-1}$  (Table 1), but initial toluene oxidation rates differed by a factor of 10. This observation is consistent with that of Meyer-Reil (1978) that bacterial direct counts alone do not always correlate directly with substrate uptake rate. Initial oxidation rates of a  $^{14}\text{C}$ -terpene mixture measured in Resurrection Bay over the same time period varied directly with

total bacterial biomass measured by epifluorescence microscopy (Button 1984), suggesting that the metabolically active biomass toward these substrates was relatively constant. A likely explanation for the variation in rate of toluene metabolism lies therefore in changes in the extent of induction toward toluene.

Sensitivity of the autoradiographic method for measuring the population of toluene oxidizers was probably low since the specific activity of  $^3\text{H}$ -toluene used was low. This resulted in a maximum of only 1 attached silver grain for most organisms scored as positive as shown. Even so, data indicated that at least 7% ( $21 \mu\text{g l}^{-1}$ ) of the total population oxidized toluene and the total could have been higher if a range of affinities for toluene were generated and only the organisms with the highest values observed.

### Growth

Toluene metabolism contributed little toward the growth of the bacteria during incubation. Although the population, as determined by epifluorescence microscopy, doubled (an increase of  $300 \mu\text{g l}^{-1}$ ) in 5 d, during that same period only  $30 \text{ ng } ^{14}\text{CO}_2$  (as toluene)  $\text{l}^{-1}$  was produced from the  $2 \mu\text{g l}^{-1}$   $^{14}\text{C}$ -toluene supplied. Even if only 7% of the population, or  $21 \mu\text{g l}^{-1}$ , were actually responsible for the metabolism of toluene (autoradiography data), the amount of toluene consumed represents an insignificant contribution (0.1%) to that biomass and a significant new population of toluene oxidizers probably did not develop.

After passing seawater through Gelman  $0.2 \mu\text{m}$  'Mini Capsule' filters a population of epifluorescence-visible bacteria did form in the filtrate; it had a doubling time of about 1 d and reached a population of about  $1 \times 10^9 \text{ l}^{-1}$ ; however,  $^{14}\text{CO}_2$  production from toluene by the renewed population was not detectable. Extrapolating backward in time, this suggests that about 1% of the original population, comprised of 'ultramicrobacteria' incapable of metabolizing toluene, passed through the filter, grew to a normal  $5 \times 10^{-13} \text{ g organism}^{-1}$  size and multiplied with a doubling time of about 1 d at the expense of dissolved indigenous substrates.

Observations with unamended samples (Table 3) are consistent with the existence of a 'bottle effect' (Ferguson et al. 1984) – the increase in microbial biomass and activity at the expense of indigenous carbon made available following water-sample containment. Like induction, the maximal rate of change due to bottle effect occurred between 24 and 48 h. The change in biomass was the same in all the incubations (Table 1) regardless of the concentration (0 to  $142 \mu\text{g l}^{-1}$ ) of  $^3\text{H}$ -toluene supplied for induction. While relative activity

of hydrocarbon-exposed populations is the subject of this report, absolute rates and turnover times calculated from experiments lasting longer than a day must be questioned.

Since toluene metabolism activity determined from daily initial rates increased from 20- to 300-fold over the 5 d duration (depending on  $^3\text{H}$ -toluene concentration) as compared to only a 12-fold increase observed in the control without  $^3\text{H}$ -toluene, the great change in activity must have reflected an increased affinity of the toluene-oxidizing population for the substrate. This induction occurred at a maximal rate after 24 h and was characterized by a  $K_{\text{ind}}$  of  $2 \times 10^{-8} \text{ M}$ , a value which is the first measured for any substrate and which is remarkably low. The general kinetics of induction for toluene reported here have been confirmed using the marine isolate *Pseudomonas* sp. strain T2, although  $K_{\text{ind}}$  is larger (unpubl.). Use of  $^3\text{H}$ -toluene diluted extensively with the non-radioactive form helped give confidence that reported inducing concentrations were accurate. However, this precaution resulted in a lowering of the sensitivity of the autoradiographic measurements.

### Induction kinetics

The relation between induction rate and inducing substrate concentration is well described by the constant  $K_{\text{ind}}$  ( $= 1.9 \mu\text{g l}^{-1}$ ) and a maximal value for the affinity  $a_{\text{A max}}^2$  ( $= 2.6 \text{ l}[\text{g cells}]^{-1} \text{ h}^{-1}$ , computed from initial rate data). Although data are limited, it appears that  $K_{\text{ind}} \approx K_t$ , the half-saturation constant for transport. The substrate concentration dependency ( $K_t = 1.9 \mu\text{g l}^{-1}$ ) of toluene metabolism in stored seawater has been reported (Button et al. 1981b). Toluene metabolism and induction in *Pseudomonas* sp. strain T2 are similarly related ( $K_t \approx K_{\text{ind}} \approx 40 \mu\text{g l}^{-1}$ , this laboratory unpubl.).

Induction can be described by a model in which toluene is accumulated by the membranes of microorganisms according to its partition coefficient ( $\xi$ ), oxidized at a half-maximal rate at an intracellular Michaelis concentration  $K_M$  ( $\approx K_t \xi$ ) to more polar intermediate products which may in turn be lost to the exterior medium and subsequently retransported by conventional transport mechanisms for polar substrates. If the induction rate of the oxidative process is dependent upon the amount of substrate accumulated by the organism having a transport system with activity defined by  $K_t$ , then one would expect  $K_{\text{ind}}$  and  $K_t$  to be similar because as saturation reduces transport rate, the build-up of internal inducers is throttled. Concentrations of toluene sufficient to initiate induction ( $0.7 \mu\text{g l}^{-1}$ ) are very small by comparison with Michaelis

constants for transport (Button 1985), and even smaller concentrations may be effective over longer periods.

Induction is often hastened by the availability of amino acids for enzyme production (Wiseman 1975) as we also have observed in studies with toluene and *Pseudomonas* sp. strain T2. Bottle effect observed has been suggested to be the result of accelerated decomposition of particulate material due to changes in conditions (S. Watson pers. comm.) which could facilitate more rapid induction in our experiments than in the environment from which the system was removed.

Toluene induction loss (upon removal of substrate) can be a slow process. More than 200 generations of growth without exposure to toluene were required before the toluene affinity of *Pseudomonas* sp. strain T2 was reduced (> 15,000-fold) to minimal values (our laboratory unpubl.). In the marine system such a reduction might take months or years.

These data establish the fact that toluene at concentrations near those measured in the environment (Schwartzbach et al. 1978) are sufficient to effect an increase in the ability of the microflora of seawater to metabolize it, and the process (at least in the system observed) involves a major fraction of the total microbial biomass.

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