

# Characterization of Soluble Microbial Products (SMP) Derived From Glucose and Phenol in Dual Substrate Activated Sludge Bioreactors

Benjamin S. Magbanua, Jr.,<sup>1</sup> Alan R. Bowers<sup>2</sup>

<sup>1</sup>Department of Civil Engineering, Mississippi State University, Mississippi State, MS 39762; telephone: 662 325 9777; fax: 662 325 7189; e-mail: magbanua@engr.msstate.edu

<sup>2</sup>Department of Civil and Environmental Engineering, Vanderbilt University, Nashville, Tennessee

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**Abstract:** Soluble microbial products (SMP) generated by activated sludge cultures receiving a mixed feed of phenol and glucose were characterized with respect to molecular weight (MW) distribution, octanol-water partition coefficient ( $K_{ow}$ ), and Microtox<sup>®</sup> toxicity. Short-term batch reactor tests using <sup>14</sup>C-labeled substrates were performed to collect SMP derived from each substrate, while long-term tests were performed with SMP accumulated over multiple feed cycles using fed-batch reactors receiving non-labeled substrates. Yield of SMP in the batch tests, 10%–20% for phenol and 2%–5% for glucose, differed for each substrate and was independent of initial concentration. The MW distribution (MWD) of SMP was independent of feed composition, and was bimodal in the <1 kDa and 10–100 kDa MW ranges for phenol-derived SMP and predominantly <1 kDa for glucose-derived SMP. In the non-labeled tests, the fraction of SMP of MW >100 kDa increased with the proportion of glucose in the feed. The  $K_{ow}$  of phenol-derived SMP was higher compared to glucose-derived SMP, indicating that the phenol-derived SMP were more hydrophobic. This was particularly true at an acidic pH, where the  $K_{ow}$  was  $4.2 \pm 1.0$  for phenol-derived SMP versus  $0.13 \pm 0.13$  for glucose-derived SMP. Toxicity testing indicated that phenol-derived SMP, exerting a mean Microtox<sup>®</sup> inhibition of 1%, were less toxic than phenol itself, and showed little correlation between toxicity and concentration. However, glucose-derived SMP were generally more toxic than glucose itself (a non-toxic substrate), and the toxicity increased linearly with the concentration of SMP. © 2006 Wiley Periodicals, Inc.

**Keywords:** biotreatment; wastewater; <sup>14</sup>C tracer experiments; toxicity; octanol-water partition coefficient; molecular weight distribution

## INTRODUCTION

Biological processes have been widely used for the treatment of municipal and industrial wastewater. Under aerobic conditions, substrate carbon (C) is converted primarily into biomass and carbon dioxide. A fraction of the substrate, however, is converted into soluble microbial

products (SMP), which accounts for the bulk of the soluble organic carbon (SOC) in biotreatment effluents (Baskir and Hansford, 1980; Boero et al., 1991; Chudoba, 1967; Daigger and Grady, 1977; Grady and Williams, 1975) and can consequently thwart compliance with discharge requirements. Furthermore, the SMP would be a major determinant of the chemical properties of the effluent. In particular, SMP toxicity has been a growing concern, with some research suggesting that toxicity can be generated in the biotreatment process. For example, Eckenfelder (1988) reported that the aquatic toxicity of some industrial wastewaters after biotreatment was higher than the untreated wastes. Similarly, Rappaport et al. (1979) found using the Ames test that secondary effluents exhibited greater mutagenicity than primary effluents. The SMP may also exert toxicity on the activated sludge community: Chudoba (1985) reported that some SMP are inhibitory to nitrification, while Washington et al. (1969) reported that high molecular weight (MW) (>700 Da) SMP interfere with activated sludge metabolism.

This research examined the properties of SMP derived from phenol and glucose in activated sludge reactors under dual-substrate conditions. Phenol is an inhibitory substrate and an important industrial pollutant, while glucose is readily biodegradable and non-hazardous. Both contain six carbon atoms, and glucose is hydrophilic while phenol is moderately hydrophobic. By using <sup>14</sup>C-labeled and non-labeled substrates, it was possible to distinguish between the SMP derived from each substrate. Consequently, some insight could be drawn regarding the effects of substrate interaction and feed composition on the generation and properties of the SMP derived from each substrate.

## METHODOLOGY

### Biomass Generation

Activated sludge was collected from the return biosolids line of the Central Wastewater Treatment Plant, Metro Water

Correspondence to: B. S. Magbanua, Jr.

Services, Nashville, TN. Biomass acclimation and maintenance were performed in a 15 L sequencing batch reactor (SBR) provided with a 2-in propeller-type impeller rotating at ~750 rpm and aerated through diffuser stones at 5–10 L/min. The pH was maintained at 6.5–7.5, the dissolved oxygen (DO) concentration at >2 mg/L, and the salinity at ~4 parts per thousand. The biomass was acclimated to a feed that contained 49% of the substrate C as phenol and 51% as glucose, by gradually increasing the volumetric substrate loading while reducing the solids retention time (SRT) from ~30 d to 3–5 d. The acclimated biomass was fed daily by wasting 3 L of mixed liquor, settling for ~20 min under quiescent conditions, decanting 2 L of supernatant, then instantaneously adding, on a reactor volume basis, 250 mg/L phenol, 500 mg/L glucose, 187 mg/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 77 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 mg/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 73 mg/L KH<sub>2</sub>PO<sub>4</sub>, 133 mg/L K<sub>2</sub>HPO<sub>4</sub>, and sufficient tap water to restore the volume to 15 L. This semi-batch procedure provided a hydraulic residence time of 5 d and a SRT of 3–5 d, and resulted in a non-filamentous sludge similar to biomass generated in field reactors. SBR biomass was harvested for <sup>14</sup>C radiolabeled and non-labeled tests, by centrifuging (3,000 rpm, 20 min) 3 L of mixed liquor (the wastage on a given day), then washing the centrate 3× with buffered tap water (labeled tests: 73 mg/L KH<sub>2</sub>PO<sub>4</sub>, 133 mg/L K<sub>2</sub>HPO<sub>4</sub>; non-labeled tests: 365 mg/L KH<sub>2</sub>PO<sub>4</sub>, 665 mg/L K<sub>2</sub>HPO<sub>4</sub>) and resuspending in ~4 L of buffered tap water. The total (TSS) and volatile (VSS) suspended solids concentrations of the resuspended biomass were determined before use.

### Radiolabeled Batch Tests

<sup>14</sup>C-labeled batch tests with varying initial substrate compositions (Table I) were conducted in 2.5 L vessels, each provided with a magnetic stirrer and aerated through a glass frit with ~90 mL/min of filtered humidified air. Inorganic nutrients (187 mg/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 77 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 mg/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 73 mg/L KH<sub>2</sub>PO<sub>4</sub>, 133 mg/L K<sub>2</sub>HPO<sub>4</sub>) were supplied in quantities sufficient to ensure C-limited growth. Dual substrate tests were run in pairs, with each batch reactor operated under conditions identical in all respects except for the identity of the labeled substrate, that is, with one reactor fed labeled glucose and non-labeled phenol and the second fed labeled phenol and non-labeled glucose, at the same initial substrate

**Table I.** Substrate concentrations (based on reactor volume) used during <sup>14</sup>C-labeled batch reactor tests.

% Substrate C from phenol	Phenol		Glucose	
	mg/L	mg/L C	mg/L	mg/L C
100	100	77	0	0
65	67	51	67	27
49	50	38	100	40
33	33	26	133	53
0	0	0	200	80

concentrations. This permitted discrimination of SMPs derived from each substrate.

The tests were conducted by placing 1.5 L of biomass in each batch reactor, initiating aeration and mixing, and injecting the feed. Substrate concentration in the batch reactors was monitored for the first 2 h, by which time the substrate was exhausted. Samples (250 mL) for SMP characterization were collected from the reactors 2, 6, 12, 24, and 48 h after feed injection, acidified with *o*-phosphoric acid, and centrifuged (3,000 rpm, 20 min). The supernatant was filtered (0.45 μm) and the filtrate fractionated by MW using ultrafiltration (UF). Each MW fraction was evaluated for <sup>14</sup>C activity and octanol-water partition coefficient (*K*<sub>ow</sub>). The labeled tests were terminated after 48 h.

### Long-Term Cumulative Tests

Long-term cumulative tests using non-labeled substrates were performed in aerated fed-batch reactors using 1.5 L of resuspended biomass. The reactors were each supplied with phenol and glucose in different proportions (Table II), and were fed 25 times at 3–6 h intervals, cumulatively receiving 3,850–4,000 mg/L (based on test reactor volume) substrate C over a 10–12 d period. The medium had sufficient nutrients and buffer capacity (374 mg/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 154 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 4 mg/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 365 mg/L KH<sub>2</sub>PO<sub>4</sub>, 665 mg/L K<sub>2</sub>HPO<sub>4</sub>) to ensure C-limited growth and to maintain a suitable pH. Immediately prior to each feeding, samples were collected from the reactors, acidified using *o*-phosphoric acid, filtered (0.45 μm), and analyzed for residual substrate, SOC, and toxicity. At the end of the test, the reactor contents were acidified using *o*-phosphoric acid and centrifuged (3,000 rpm, 20 min), the supernatant filtered (0.45 μm) and the filtrate fractionated by MW. Each MW fraction was analyzed for SOC and toxicity.

### SMP Characterization

The concentration of SMP in whole or fractionated (by MW or by extraction into octanol) samples was determined based on <sup>14</sup>C or SOC concentration, and corrected for residual substrate concentration when necessary.

Fractionation of samples according to MW was performed by UF in a stirred cell using UF membranes with nominal

**Table II.** Substrate concentrations (based on reactor volume) used during non-labeled fed-batch reactor tests.

% Substrate C from phenol	Phenol		Glucose	
	mg/L	mg/L C	mg/L	mg/L C
100	200	154	0	0
65	134	102	134	54
49	100	76	200	80
33	66	52	266	106
0	0	0	400	160

MW limits of 100, 10, and 1 kDa. Membranes were washed with 200–240 mL of distilled water under 40–50 psig N<sub>2</sub> pressure prior to sample loading. The UF cell was then loaded with sample and pressurized with N<sub>2</sub> to 40–50 psig. The first 20–25 mL of permeate was discarded, and the remainder collected for analysis and further fractionation.

The octanol-water partition coefficient,  $K_{ow}$ , was determined under acidic and neutral conditions using a procedure adopted from other researchers (Chiou et al., 1977; Lee et al., 1990; Madhun et al., 1986). Filtered and MW-fractionated samples (10 mL) from the labeled batch tests were placed in 50 mL glass centrifuge tubes with threaded closures and purged of inorganic C by adding 1 mL of 10N HCl and venting three times over 24 h (Boero et al., 1991), then neutralized if necessary with 5 mL phosphate buffer (55 g/L KH<sub>2</sub>PO<sub>4</sub>, 100 g/L K<sub>2</sub>HPO<sub>4</sub>) and 1 mL of 10N NaOH. *n*-Octanol (10 mL) was added, and the tubes were capped and shaken ~30 min. The samples were then centrifuged (4,000 rpm, 30 min) and an aliquot was collected from each layer to measure <sup>14</sup>C activity and calculate  $K_{ow}$ .

Toxicity was evaluated using the Microtox<sup>®</sup> Assay (AZUR Environmental, Carlsbad, CA), where acute toxicity is quantified by measuring the reduction in light output of a reconstituted culture of the bioluminescent marine bacteria *Vibrio fischeri* after exposure to a sample. A modification of the standard test procedure, which is designed for relatively powerful toxicants, was used. Filtered and MW-fractionated samples (900 µL) from the unlabeled batch tests were pipetted into test cuvettes, along with 90 µL of osmotic adjusting solution to provide the salinity level (12%) optimum for the test organisms, while controls consisted of 990 µL of Microtox<sup>®</sup> diluent (reconstituting solution). Triplicate cuvettes containing samples and controls were cooled for 5 min to 20°C (the recommended temperature of the test), inoculated with 10 µL of reconstituted Microtox<sup>®</sup> bacteria, mixed, and their light output measured 5 min thereafter.

Toxicity was quantified in terms of the inhibition ratio  $\Gamma$ , the ratio of activity lost to residual activity after exposure to toxicant. This is the parameter recommended by the manufacturer for the evaluation of dose-response relationships in the Microtox<sup>®</sup> assay (AZUR Environmental, 1995). For the Microtox<sup>®</sup> test, activity is measured in terms of the light intensity,  $I$ , hence:

$$\Gamma_t = \frac{I_C - I_S}{I_S} = \frac{I_C}{I_S} - 1 \quad (1)$$

where  $\Gamma_t$  = inhibition ratio at time  $t$  (min),  $I_i$  = mean light output of triplicate cuvettes  $i$  at time  $t$ , subscript S denotes a sample, and subscript C denotes a control. The inhibition ratio is related to the fractional inhibition  $\Delta$ , which is widely used to report the degree of inhibition, as follows:

$$\Delta_t = \frac{I_C - I_S}{I_C} = 1 - \frac{I_S}{I_C} \quad (2)$$

where  $\Delta_t$  = fractional inhibition at time  $t$  (min).  $\Delta_t$  is related to  $\Gamma_t$  by:

$$\Delta_t = \frac{\Gamma_t}{\Gamma_t + 1} \quad (3)$$

Microtox<sup>®</sup> test results are commonly analyzed by plotting  $\Gamma_t$  against toxicant concentration and graphically estimating the concentration at which a  $\Gamma_t$  of 1.0 (50% inhibition) would occur; this concentration is then reported as the EC<sub>50</sub>.

### Analytical Methods

<sup>14</sup>C activity was determined using a liquid scintillation (LS) counter (Beckman Instruments, Fullerton, CA). The counting window was restricted to 400–670 to minimize the effects of chemiluminescence. Quench, a measure of the efficiency of detection of radioactive events, was quantified automatically as the Horrocks number (H#), to permit direct determination of <sup>14</sup>C activity in disintegrations/min (dpm) through the use of a quench curve developed using quenched <sup>14</sup>C standards. Samples were placed in 20 mL borosilicate glass LS vials and combined with the appropriate LS fluid: 12 mL of ScintiVerse (Fisher Scientific, Pittsburgh, PA) or ReadySafe (Fisher Scientific) for 2–4 mL of UF-fractionated sample, 12 mL of ScintiVerse or ReadySafe for 2–4 mL aqueous  $K_{ow}$  aliquot or 12 mL of ScintiLene (Fisher Scientific) for 2–4 mL of organic  $K_{ow}$  aliquot. Each sample was counted for up to 20 min, or until a sufficient number of radioactive events was detected so that the reported activity had a 95% confidence interval  $\leq 1\%$ .

SOC was determined based on the combustion-infrared method, *Standard Methods* Part 505A (Clesceri et al., 1998), using an organic carbon analyzer (Shimadzu, Kyoto, Japan). Standards were prepared using potassium hydrogen phthalate. Samples were analyzed in triplicate, with replicate samples having a coefficient of variation typically  $< 5\%$  and in all cases  $< 10\%$ .

Phenol was measured spectrophotometrically, based on rapid condensation with 4-aminoantipyrene followed by oxidation with potassium ferricyanide under alkaline conditions to yield a red antipyrene dye (Yang and Humphrey, 1975). Glucose was quantified using an analysis kit (Boehringer, Mannheim, Germany) based on the spectrophotometric detection of NADPH generated during the enzymatic dehydrogenation of glucose-6-phosphate. TSS and VSS were determined according to *Standard Methods* Parts 209D and 209E (Clesceri et al., 1998).

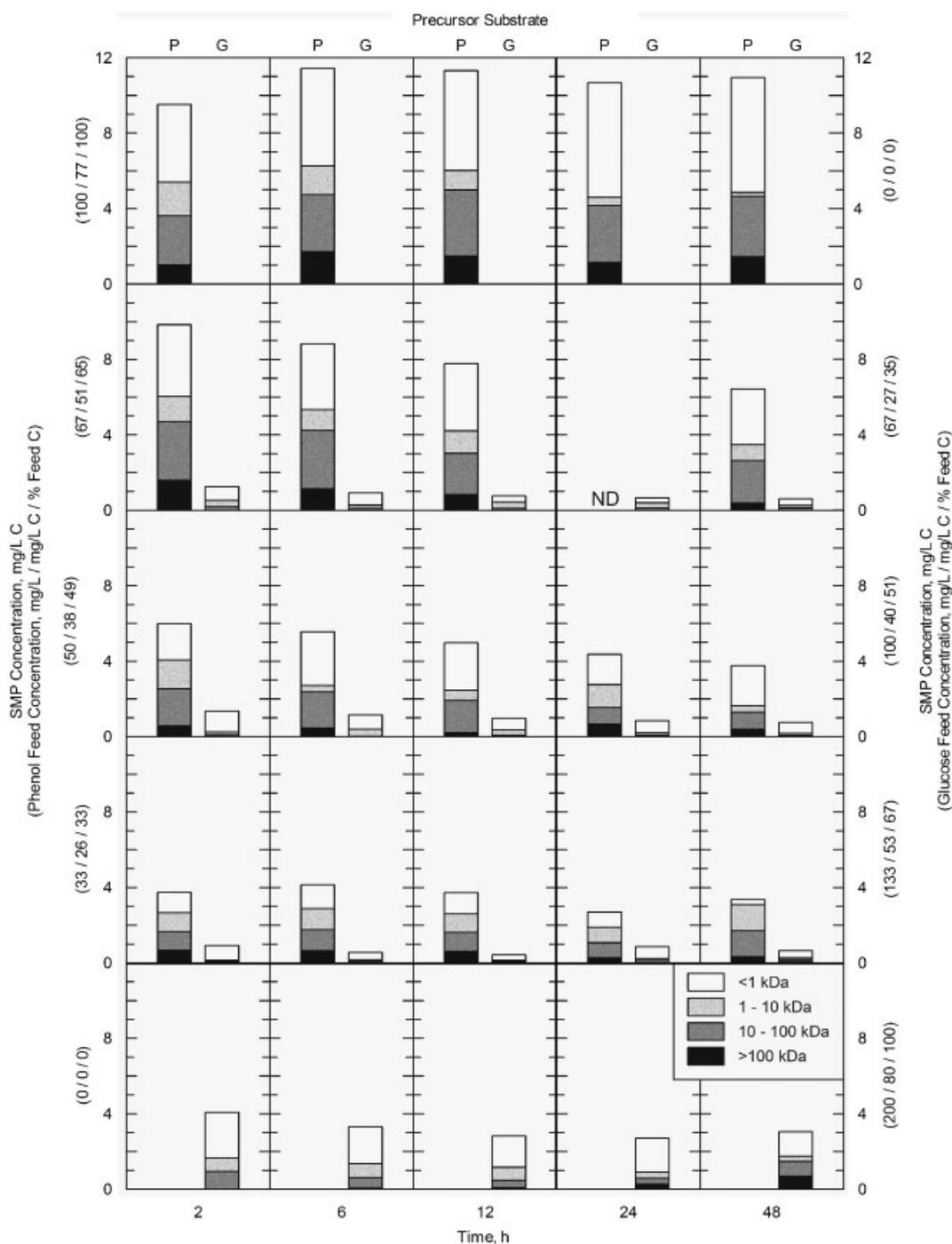
Statistical analysis of the observed SMP properties was conducted using the MEANS, analysis of variance (ANOVA), and general linear models (GLM) procedures in SAS (The SAS Institute, Cary, NC). A confidence level of  $P \leq 0.05$  was taken to indicate a statistically significant correlation.

## RESULTS

### Molecular Weight Distribution

Samples collected from the radiolabeled batch reactors at 2, 4, 12, 24, and 48 h after feed injection were fractionated by UF and the  $^{14}\text{C}$  activity measured to determine the MW distribution (MWD), on a C basis, of the SMP derived from each substrate (Fig. 1). With phenol (77 mg/L C) as sole

substrate, the SMP concentration was 9.5–11.5 mg/L C (12%–15% of the initial substrate C). The phenol-derived SMP exhibited a bimodal MWD, with 43%–56% in the <1 kDa range and 26%–32% in the 10–100 kDa range, together representing 70%–85% of the total SMP. The <1 kDa fraction increased while the 1–10 kDa fraction decreased over time. With glucose (80 mg/L C) as sole substrate, the SMP concentration was 2.7–4.0 mg/L as C, 3%–5% of the feed C. The glucose-derived SMP were predominantly



**Figure 1.** Molecular weight distribution of phenol-derived and glucose-derived SMP during the radiolabeled batch single-dose tests. Precursor substrates: P, phenol; G, glucose. ND, no data collected.

in the <1 kDa range, 43%–66% of the total SMP. The >100 kDa fraction increased while the <1 kDa fraction decreased during the test. In dual-substrate radiolabeled tests, the MWD of the phenol-derived SMP was bimodal, with 55%–86% having MW <1 kDa or 10–100 kDa. For the glucose-derived SMP, 40%–82% had MW <1 kDa and 0%–5% had MW >100 kDa. Overall, the SMP yield, on a C basis, was 10%–20% for phenol and 2%–5% for glucose. Statistical analysis indicated that the yield (mg SMP/mg substrate) of SMP varied over time ( $P = 0.0001$ ) and differed for each substrate ( $P = 0.0001$ ), but was independent of the substrate's initial concentration, that is there was no observed correlation between yield and initial substrate concentration. Each substrate produced SMP with a distinct MWD ( $P < 0.008$ ), but the relative proportion of only the >100 kDa SMP fraction was affected by feed composition.

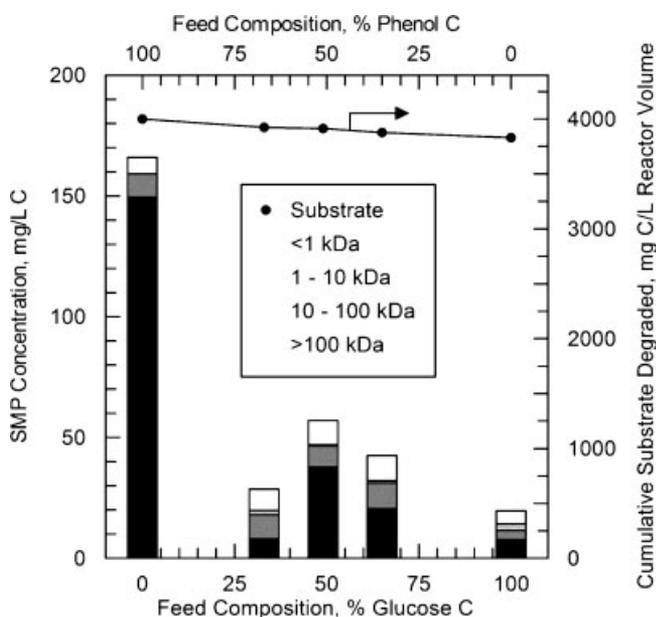
Among the non-labeled intermittent feed reactors, SMP accumulation (Fig. 2) was greatest when glucose was the sole substrate, even though all five reactors received approximately the same quantity of substrate C. SMP yield and concentration generally decreased as the proportion of phenol in the feed increased, although results obtained with a feed of 66% phenol C and 34% glucose C did not appear to fit the trend. The conversion of substrate to SMP ranged from 0.5% to 4.0% (20–166 mg/L C), lower than that observed in the labeled batch tests, indicating that the SMP undergo biodegradation over time, or that a biochemical feedback mechanism controls production of SMP. SMP accumulated in the non-labeled test generally contained a higher fraction of SMP in the >100 kDa MW range (Fig. 2) compared to the labeled test. In particular, the SMP that accumulated when glucose was the sole substrate had a considerably higher fraction in the >100 kDa MW range, while the quantities of

all other MW fractions appeared comparable among the five reactors.

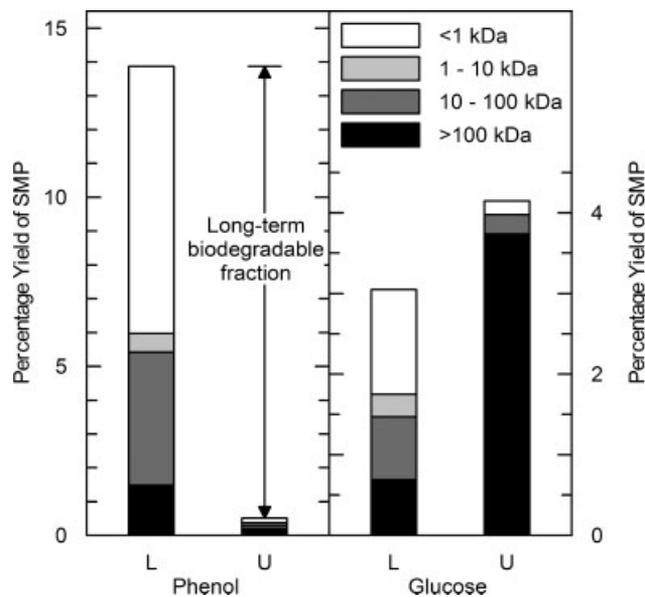
Results of the radiolabeled and unlabeled tests were used to compare short-term single-dose SMP production (at 48 h) and long-term SMP accumulation through multiple feed doses, with either glucose or phenol as sole substrate (Fig. 3). The yield of phenol-derived SMP over the short term (13.9%) was much higher compared to the long-term yield (0.5%), indicating a loss of SMP due to biodegradation. The short-term yield of glucose-derived SMP (3.1%), on the other hand, was comparable to the long-term yield (4.2%), indicating that these SMP were relatively non-biodegradable. Furthermore, glucose-derived SMP in the >100 kDa MW fraction accumulated to a much greater extent than the other MW fractions.

### Octanol-Water Partition Coefficient

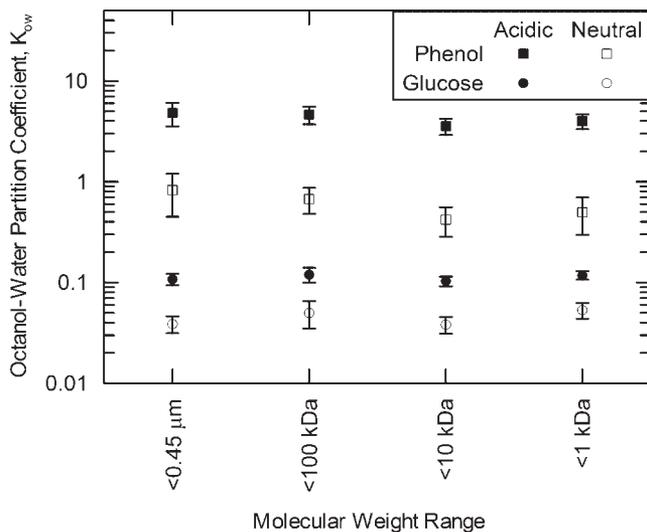
The  $K_{ow}$  was determined under acidic and neutral pH conditions for MW-fractionated samples obtained from the labeled batch test (Fig. 4). The phenol-derived SMP had a significantly higher  $K_{ow}$  than the glucose-derived SMP ( $P = 0.001$ ), and significantly higher  $K_{ow}$  values were observed under acidic than under neutral pH conditions ( $P = 0.001$ ). The  $K_{ow}$  under acidic conditions was  $3.6 \pm 0.7$  to  $4.8 \pm 1.3$  ( $\log K_{ow}$  of 0.55 to 0.68) for phenol-derived SMP and  $0.10 \pm 0.01$  to  $0.12 \pm 0.02$  ( $\log K_{ow}$  of  $-0.99$  to  $-0.94$ ) for glucose-derived SMP. At a neutral pH, the  $K_{ow}$  was  $0.42 \pm 0.14$  to  $0.83 \pm 0.38$  ( $\log K_{ow}$  of  $-0.37$  to  $-0.08$ ) for phenol and  $0.04 \pm 0.01$  to  $0.05 \pm 0.01$  ( $\log K_{ow}$  of  $-1.42$  to  $-1.28$ ) for glucose. Compounds with  $K_{ow}$  values <1 are hydrophilic in nature, while those with  $K_{ow} > 1$  are hydrophobic. Thus, the  $K_{ow}$  values obtained indicate that



**Figure 2.** Molecular weight distribution of SMP collected after the long-term cumulative tests using non-labeled substrate.



**Figure 3.** Percentage yield and molecular weight distribution of SMP on phenol and glucose as sole substrate, in the radiolabeled 48 h single dose batch test (L) and the long-term cumulative feeding tests with unlabeled substrate (U).



**Figure 4.** Octanol-water partition coefficient of phenol-derived and glucose-derived SMP.

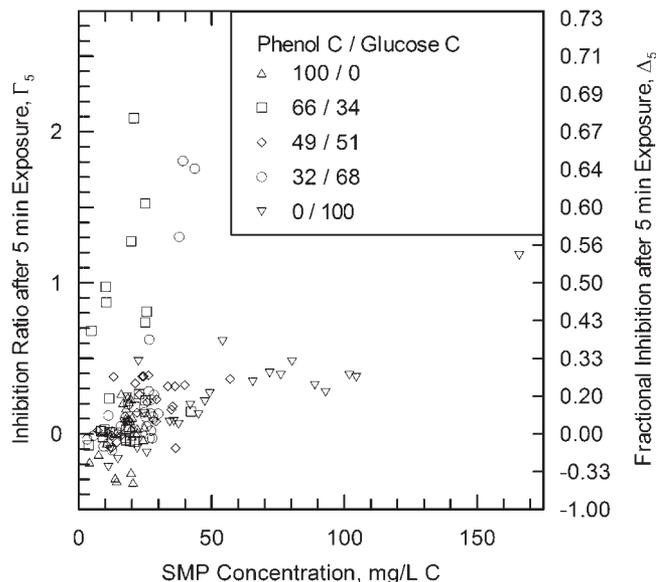
the glucose-derived SMP were hydrophilic under acidic and neutral conditions. The phenol-derived SMP, on the other hand, were hydrophilic at a neutral pH and slightly hydrophobic ( $K_{ow}$  values  $>1$ , but still relatively low) under acidic conditions. The reported  $K_{ow}$  and  $\log K_{ow}$  for phenol (Fujita et al., 1964; Hansch et al., 1995) are 28.8 and 1.46, respectively, indicating that it is slightly hydrophobic, more so than the SMP derived from it. In contrast, the  $K_{ow}$  and  $\log K_{ow}$  for glucose (Sangster, 1989) are 0.0058 and  $-3.24$ , respectively, indicating that it is strongly hydrophilic, even more so than the SMP derived from it. The  $K_{ow}$  values clearly indicate that the SMP from each substrate had distinct chemical compositions and contain different functional groups that behave differently in response to changes in the pH. The  $K_{ow}$  values did not differ significantly between MW fractions.

### Toxicity

$\Gamma_5$  was evaluated both for whole samples collected over the duration of the unlabeled cumulative feed test (Fig. 5) and for MW-fractionated samples collected at the end of that test. At 90% sample volume, of 130 samples tested, the maximum toxicity observed was  $\Gamma_5 = 2.090$  (68% inhibition), and only 5% of the samples had  $\Gamma_5 > 1.0$  (50% inhibition). SMP toxicity was therefore relatively low. In fact, values of  $\Gamma_5 \leq 0.0$ , indicating that the SMP was innocuous or even stimulatory to the Microtox<sup>®</sup> organisms, were observed for a number of samples. Statistical analysis indicated that toxicity was correlated to SMP concentration ( $P = 0.009$ ) but independent of feed composition. Among the fractionated samples, no statistical correlation was found between toxicity and MW range.

### DISCUSSION

Examination of the properties of SMP and the factors that may influence them is driven by concerns over the potential

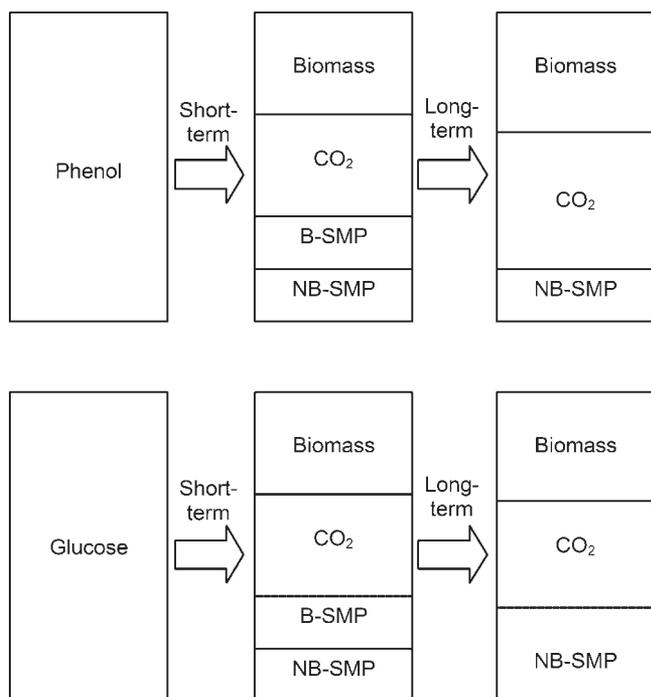


**Figure 5.** Inhibition of *Vibrio fischeri* (Microtox<sup>®</sup> test) by SMP.

impact of SMP on effluent properties. In this research, SMP were generated using  $^{14}\text{C}$ -labeled substrates under dual substrate conditions to evaluate the importance of substrate interaction as a determinant of SMP properties. SMP toxicity has been identified as an area of potential concern and was evaluated in this research. The MWD and  $K_{ow}$  of the SMP were likewise determined.

The yield of SMP derived from glucose and phenol, as well as the properties of those SMP, appeared unaffected by the relative quantities of phenol and glucose in the feed, hence substrate interaction did not appear to be a significant factor in this particular system. In the labeled tests, the yield of SMP on phenol (10%–20%) was higher compared to glucose (2%–5%), which was consistent with the findings of Boero et al. (1991, 1996). On the other hand, SMP yields observed in the long-term (non-labeled) tests were 0.5%–4.0%, suggesting that over the much longer duration of the non-labeled test, the SMP were degraded to a greater extent than in the short-term (labeled) tests. This is consistent with the results of Gaudy and Blachly (1985), who observed that SMP were degraded after extended reactor operation.

The SMP were characterized based on MWD, given the potential utility of this parameter as an indicator of SMP biodegradability, toxicity (Washington et al., 1969), solubility, and other properties. Fractionation of the SMP by UF revealed a broad spectrum of MW,  $<1$  kDa to  $>100$  kDa. Phenol-derived SMP produced after a single, sole-substrate dose exhibited a bimodal ( $<1$  kDa and 10–100 kDa) MWD. These SMP were for the most part biodegradable (Fig. 6), however, and did not accumulate after long-term, accumulative sole-substrate dosing. In contrast, the glucose-derived SMP produced after a single sole-substrate dose was predominantly in the low ( $<1$  kDa) MW range. After long-term accumulative sole-substrate dosing, however, the accumulated SMP consisted predominantly of high MW ( $>100$  kDa) compounds. This suggests (Fig. 6) that the high



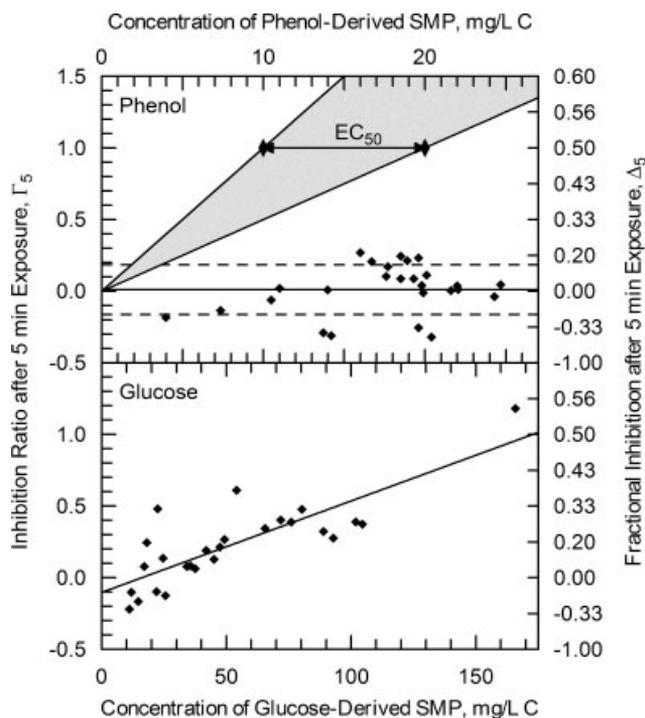
**Figure 6.** Conversion of substrate into biomass, CO<sub>2</sub>, biodegradable SMP (B-SMP), and non-biodegradable SMP (NB-SMP) over short-term (single-dose sole) and long-term (accumulative multiple-dose) operation. The size of each box is indicative of the relative quantity of each C fraction, but is not drawn to scale.

MW (>100 kDa) glucose-derived SMP were of limited biodegradability and consequently accumulated within the reactor. Furthermore, a portion of the lower MW (<100 kDa) SMP may undergo only partially mineralization, with the remainder converted into high MW (>100 kDa) compounds. It is also possible that phenol-derived SMP interfered with the production of high MW (>100 kDa) glucose-derived SMP and so reduced the accumulation of these compounds in the dual-substrate reactors; whether this is indeed the mechanism, however, is difficult to verify. Barker and Stuckey (1999) in reviewing the literature on SMP characterization, noted that MWD was dependent on the substrate and that high MW material became more prevalent with increasing SRT. High SRT systems are characterized by a low substrate concentration, so that cellular maintenance and decay processes are an important determinant of system behavior. The batch tests conducted in this study likewise resulted in a significant role for decay and maintenance because of the extended periods over which the biomass was deprived of exogenous substrate, consequently the predominance of high MW SMP in these systems is not surprising. Overall, the diversity of MW, the bimodal MWD, and the impact of operating conditions on MWD observed in this research are consistent with the literature on the generation and characterization of high-MW compounds in biotreatment systems.

Measurement of the  $K_{ow}$  of SMP derived from each substrate became possible through <sup>14</sup>C labeling. The  $K_{ow}$  was reported to be a good predictor of Microtox<sup>®</sup> response to a

group of structurally similar chemicals exerting non-reactive toxicity (Kamlet et al., 1986) and is related to the bio-concentration factor of a compound (Chiou, 1981; Selvakumar and Hsieh, 1988), therefore the  $K_{ow}$  could serve as a rough predictor of the environmental risk posed by a chemical. The SMP collected, however, exhibited  $K_{ow}$  values well below 10<sup>2</sup>, indicating a low potential for bioaccumulation and minimal environmental risk (Karickhoff and Brown, 1979). The phenol-derived SMP were more hydrophobic (higher  $K_{ow}$  values) under acidic conditions than the glucose-derived SMP, regardless of feed composition, indicating that each substrate resulted in SMP with distinct chemical properties and structure. Measured  $K_{ow}$  values were not affected by MW even though higher MW compounds would tend to be less soluble, that is, more hydrophobic. The higher  $K_{ow}$  values observed under acidic conditions are indicative of weakly acidic functional groups that at low pH would be protonated and tend to concentrate in the non-polar organic phase. This is consistent with the structure of humic substances, whose presence in SMP has been extensively documented (Manka and Rebhun, 1982; Manka et al., 1974; Parkin and McCarty, 1975; Rebhun and Manka, 1971).

The production of toxicity in the biotreatment process was examined, however the effluent samples exhibited relatively low toxicity, even when SMP was permitted to accumulate over an extended period after degradation of a substantial (up to 4,000 mg/L as C) quantity of substrate. The measurable toxicity was correlated to SMP concentration ( $P = 0.009$ ), which may have implications on the biotreatment of high-strength wastewaters. Microtox<sup>®</sup> test results for SMP generated through accumulative dosing with a single substrate feed are presented in Figure 7. These data illustrate a number of interesting points. First, the phenol-derived SMP exhibited less toxicity than the parent compound. Of the 26 SMP samples generated using phenol as sole substrate, 8 exhibited no toxicity. The reported Microtox<sup>®</sup> EC<sub>50</sub> of phenol ranges from 10 to 20 mg/L as C (13–26 mg/L as phenol) (AZUR Environmental, 1995; Blum and Speece, 1992; Ribo et al., 2001; Tchounwou et al., 2000), and on this basis the projected Microtox<sup>®</sup> dose-response to phenol would be bounded by the shaded area in Figure 7. In contrast, the response exhibited by the Microtox<sup>®</sup> organisms to phenol-derived SMPs indicated that they had a much lower toxicity than phenol itself: 10–20 mg/L phenol-derived SMP (as C) resulted in no more than 21% inhibition ( $\Gamma_5 = 0.27$ ). The mean inhibition ratio  $\Gamma_5$  exerted by the phenol-derived SMP was 0.01 (1% inhibition)  $\pm 0.17$ , and there was no correlation between  $\Gamma_5$  and SMP concentration. In contrast to phenol, glucose is essentially non-toxic to *V. fischerii* (Ramsay and Nguyen, 2002). However, except for a few low-concentration samples that were stimulatory, SMP obtained using glucose as sole substrate generally exhibited some degree of toxicity. Additionally, there was a strong correlation ( $P < 0.0001$ ,  $r^2 = 0.662$ ) between  $\Gamma_5$  and the glucose-derived SMP concentration. It was difficult to assess whether SMP toxicity in dual-substrate accumulative dosing tests was influenced by interaction between the SMP derived from



**Figure 7.** Inhibition of *Vibrio fischeri* (Microtox<sup>®</sup> test) by phenol-derived (top) and glucose-derived (bottom) SMP. The reported EC<sub>50</sub> range and the projected inhibition response to phenol (shaded area), the mean  $\pm$  standard deviation  $\Gamma_5$  exerted by phenol-derived SMP, and the linear fit between  $\Gamma_5$  and the concentration of glucose-derived SMP are also presented.

each substrate because the contribution of each substrate to the total SMP concentration could not be determined. The toxicity did not appear to be correlated to MW, although high MW (>700 Da) SMP have been reported to inhibit activated sludge metabolism (Washington et al., 1969).

## CONCLUSIONS

Collection and analysis of SMP generated in <sup>14</sup>C radiolabeled (short-term) and non-labeled (long-term cumulative) single- or dual-substrate batch reactor tests permitted some insight into the nature of SMP derived from phenol and glucose. Phenol-derived SMP were readily biodegradable, while glucose-derived SMP appeared to accumulate in the high MW (>100 kDa) range. The phenol-derived SMP were more hydrophobic than glucose-derived SMP, but their low  $K_{ow}$  indicated a low potential for bioaccumulation. Microtox<sup>®</sup> testing revealed that the phenol-derived SMP were less toxic than phenol while, in contrast, glucose-derived SMP were more toxic than glucose (a non-toxic substrate).

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