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MOLECULAR WEIGHT DISTRIBUTION OF SOLUBLE MICROBIAL PRODUCTS IN BIOLOGICAL SYSTEMS

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ABSTRACT

The molecular weight, MW, distribution of soluble microbial products, SMP, was examined. Phenol, an inhibitory substrate, and glucose, a non-inhibitory substrate, were degraded using acclimated cultures of bacteria. Three distinct regions were found to exist, Region I: Original substrate present, Region II: Biodegradable SMP present, and Region III: Endogenous respiration. Phenol degradation resulted in more SMP than glucose, about 25 percent versus 3 percent as residual SMP at the end of Region I, and 3 percent versus 1 percent at the end of Region II, respectively. In Region III, the production of SMP due to endogenous decay, SMP_E, was proportional to the rate of cell degradation. The rate coefficient for SMP_E production for cells grown on phenol was higher than for glucose, 0.005 mg SMP_E per mg cell carbon per day for phenol versus 0.002 mg per mg per day for glucose. Although differences existed in the magnitude of SMP produced, the MW distributions for phenol and glucose were similar in each region. While in Region I most of the SMP consisted of the lowest MW (<1 K daltons) compounds, 90 percent for phenol and 75 percent for glucose, at the end of Region II only 41 percent of the SMP for phenol and 56 percent for glucose were in the <1 K fraction. Finally, for endogenous decay products, 48 and 50 percent of the SMP_E were in the highest MW fraction >100 K. Copyright © 1996 IAWQ. Published by Elsevier Science Ltd

KEY WORDS

Glucose degradation; molecular weight distribution; phenol degradation; soluble microbial products.

INTRODUCTION

The bulk of soluble organic carbon in biological wastewater effluents consists of soluble organic compounds that have a microbial origin, usually referred to as soluble microbial products, SMP, rather than the original organic substrates. Soluble microbial products result from intermediates or end products of substrate degradation and endogenous cell decomposition, and are composed of a wide range of organic compounds that differ in structure and molecular weight, MW, (MWs in the 10^3 to 10^8 daltons and sizes smaller than 0.45 μm). From a biological standpoint, SMPs consist of compounds which are both biodegradable and non-biodegradable within the time-frame of the experiments performed by researchers. In wastewater effluents,

the nonbiodegradable portion is composed of compounds present in the raw wastewater plus those nonbiodegradable compounds produced by the microorganisms. The biodegradable portion is composed of the fractions of the degradable influent soluble organic compounds that have not been degraded, and of the residual soluble biodegradable compounds produced by the microorganisms.

SMPs are becoming increasingly important because they affect the quality of the effluents from biological treatment processes. In addition, there is increasing concern that effluent toxicity may actually be created in the biological treatment process itself. In other words, soluble microbial products may be more toxic than the original organic compounds present in the wastewater (Eckenfelder, 1988).

Ultrafiltration and gel permeation chromatography have been used previously to find effluent MW distributions. In spite of the differences in techniques that make definitive comparisons difficult, it has been substantiated that a broad spectrum of MW organics exist in biological effluents. Various studies have concluded the following (Grady et al., 1984; Chudoba, 1985; Amy et al., 1987; Namkung and Rittmann, 1986):

- Many biological effluents have considerably more high MW compounds than the influent.
- A broad spectrum of MWs exists in biological effluents (<500 to >50,000 daltons).
- The approximate MW distribution of secondary effluents generally exhibited bimodal patterns, while the primary effluent followed skewed non-normal distributions with predominance of the very low MW fraction (<500 daltons).

This work examines the MW distribution of soluble microbial products in activated sludge arising from specific substrates under controlled laboratory conditions. ^{14}C-labeled compounds were used to determine the MW distributions to eliminate interferences from cosubstrates and filtration membranes, and to distinguish between short term substrate related compounds and long term endogenous decay compounds.

MATERIALS AND METHODS

Substrates and Analytical Techniques

Phenol and glucose were chosen as the substrates. Universally labeled ^{14}C-phenol and glucose with source specific activities of 110 to 121 mCi/mmole and 5.5 to 7.0 mCi/mmole, respectively, were used as tracers.

Phenol was determined by the "Direct Photometric Method" using a Spectronic 20 (Bausch & Lomb, Inc.). All procedures were in accordance with *Standard Methods* (American Public Health Association, 1985). Samples were filtered using disposable 0.45 μm syringe filters (Acrodisc No. 4218, Gelman Sciences Inc.). Samples not processed immediately were acidified to pH <4.0 using concentrated H_3PO_4, and then refrigerated at 4°C. Glucose was determined using the D-glucose W-method. Boehringer Test Combination Kits were used. Procedures followed the manufacturer instructions (Boehringer Manheim, Inc., Germany, No. 716-251). Samples were filtered in the same manner as the phenol and processed immediately.

Isotope activity, ^{14}C, was determined using a Beckman LS 3801 Liquid Scintillation Counter. Counting was done in a 400 to 670 window (70 percent of total counts). Counts were used directly without correction due to the uniformity in the H#'s and the small amount of quench encountered (maximum H# was 150). A sample (1 or 2 mL) was placed into a standard plastic scintillation vial, two drops of concentrated HCl were added, followed by swirling, and then sample was vented at least three times in 24 hours. This procedure separated any residual $^{14}CO_2$ from the organics. After this, 15 mL of liquid scintillation cocktail was added, and after 24 hours, the sample was counted. A conversion factor, C_f [(cpm/mL)/(mg C/l)], calculated as the ratio between the total initial ^{14}C activity and the initial substrate carbon concentration, $S_{OC}(0)$ [mg C/l], or $C_f = C_{cpm/mL}(0)/S_{OC}(0)$, was used to compute the total organic carbon concentrations. The scintillation

counting technique using ^{14}C-labeled compounds gave very good resolution, accurate to <0.3 percent of the original organic carbon concentration.

MW distributions were determined using a 400 mL ultrafiltration stirred cell (Spectra/Por, Spectrum) using 76-mm Millipore Disc Ultrafiltration Membranes. Three membranes with nominal MWs of 100,000 (100 K), 10,000 (10 K), and 1,000 (1 K) daltons were used in succession with the highest MW first and lowest MW last. Pure nitrogen was used to pressurize the cell. The pressure in the ultrafilter was kept constant at 30 psig (207 kPa or 2.04 atm). Samples taken after each of the filters were analyzed for ^{14}C to determine specific total organic carbon, TOC. This eliminated interference from the original cells or the molecular sieve filters.

Experimental Reactors

Sludge was acclimated to each substrate in 20 liter fill and draw reactors using a sludge age of 5 days. Sufficient nutrients were supplied to ensure that carbon was the limiting substrate in the sludge generator. Nitrogen [$(NH_4)_2SO_4$, 72 mg/L]; iron ($FeCl_3 \cdot 6H_2O$, 0.8 mg/L), and magnesium ($MgSO_4 \cdot 7H_2O$, 29 mg/L) were supplied in sufficient excess quantities as well as phosphate (KH_2PO_4, 857 mg/L and K_2HPO_4, 1,550 mg/L) that was used as a pH buffer as well. All components were dissolved in tap water to supply calcium and other trace minerals.

Experiments were performed in batch well-mixed reactors. These reactors consisted of a 2.5 liter glass jar which was sealed air-tight with a large Plexiglas top having a rubber O-ring around the edges. Air flow was monitored going into the system, and the air was humidified with a gas-wash bottle to minimize evaporative losses in the reactor. The off-gas was passed through two successive 500 mL gas-wash bottles containing NaOH or KOH solution (pH > 12) to act as CO_2 traps. It was found over the course of study that most of the CO_2, approximately 90 percent, was collected in the first trap, so that a third trap was not needed.

The same apparatus was used for the phenol and glucose tests, as well as for aerobic cell degradation (endogenous decay) experiments. At the end of the tests, the mixed liquor was centrifuged at 3,000 rpm (767 g) for two minutes, decanted, and the cells resuspended in washing solution (same as nutrient solution). The centrifugation-decantation-resuspension cycle was repeated three times. While washing the cells from the previous tests, the experimental apparatus was disassembled, the reactor and CO_2 traps carefully cleaned, and reassembled to receive the washed cells. Then, the ^{14}C marked cells underwent the endogenous metabolism process for an evaluation of the cell decay related soluble organics.

RESULTS AND DISCUSSION

Soluble microbial products may arise from various bacterial phases and may be grouped into two basic categories (Boero et al., 1991):

1. Substrate Utilization Associated Products, SMP_S - SMPs resulting from intermediates or end products of substrate degradation, cell metabolism, or cell growth.

2. Biomass Associated Products, SMP_E - SMPs as a result of endogenous respiration--i.e., cell lysis and decay.

Then, in a batch reactor, different SMPs will accumulate in time depending upon the original source of the organic material. These source materials may include the original substrate(s), S, the biodegradable soluble microbial products, SMP_{SD} and the biomass itself, X. The reaction stoichiometries may be summarized as follows:

Substrate Degradation:

$$\text{Substrate, S} + \text{Cells} \xrightarrow{O_2} Y_{X,S}\ X + Y_{SMPS}\ SMPS + Y_{CO2,S}\ CO_2 \quad (1)$$

Soluble Microbial Product Degradation:

$$SMP_{SD} + \text{Cells} \xrightarrow{O_2} Y_{X,SMPS}\ X + Y_{CO2,SMPS}\ CO_2 \quad (2)$$

Endogenous Decomposition:

$$X \xrightarrow{O_2} Y_{SMPE,X}\ SMP_E + Y_{CO2,X}\ CO_2 \quad (3)$$

where:

$Y_{CO2,S}$	=	CO_2 per unit mass of substrate degraded
Y_{SMPS}	=	SMP_S per unit mass of substrate degraded
$Y_{X,S}$	=	biomass yield coefficient from substrate degradation
$Y_{CO2,SMPS}$	=	CO_2 per unit mass of SMP_S degraded
$Y_{X,SMPS}$	=	biomass yield coefficient from SMP_S degradation
$Y_{C02,X}$	=	CO_2 per unit mass of cells degraded
$Y_{SMPE,X}$	=	SMP_E per unit mass of cells degraded

The degradation process, in terms of the substrate(s), may be broken down into a region where the original substrate(s) is present, Region I [Equations (1), (2), and (3) apply], a region where the original substrate(s) is no longer present but S_{MPSD} are available, Region II [Equations (2) and (3) apply], and an endogenous region where only the biomass itself is available as a food and energy source, Region III [only Equation (3) applies]. The time for the substrate to become totally depleted--i.e., the substrate degradation time, t_d, is the idealized limit between Regions I and II.

SMP studies were performed for phenol and glucose individually. Samples were collected from the batch reactor within minutes after the original substrate was completely removed from solution (after about 3.2 to 3.5 hours)--i.e., representative of Region I, and after about 43 hours--i.e., representative of Region II. A typical set of data for the original substrates (phenol and glucose) and residual soluble organic carbon are presented in Figure 1. The data for both phenol and glucose indicate a rapid uptake of the original substrates, t_d = 3.5 hours and 3.2 hours, respectively, and at the end of Region I ($t = t_d$) a substantial amount of substrate related SMP exists, 25 percent and 3 percent of the initial carbon concentration, respectively. However, in Region II, most of the initial SMPs appeared to be biodegradable were removed from solution in the early stages, quickly leaving a non-biodegradable residual SMP that remained fairly constant over Region II.

A complete mass balance of the total carbon originating from the initial substrate was obtained using the labeled carbon. The distribution of carbon at the test points indicated in Figure 1 indicate that the utilization of carbon during these tests was as follows:

- In Region I, 26 percent of the phenol and 43 percent of the glucose were converted to CO_2 for energy; 25 and 3 percent were converted to SMPs, and 49 and 54 percent were incorporated to the biomass for phenol and glucose respectively. Biomass was determined based on filtered solids ^{14}C, so that biomass represented: new biomass, adsorbed organics, or stored products.

- In Region II, more CO_2 was produced (finally about 60 percent for both phenol and glucose) and the fraction of the carbon converted to biomass was 37 percent for phenol and glucose. Also, it is shown in Region II that the SMPs decreased from 25 to 3 percent for

phenol and from 3 to 1 percent for glucose, indicating that the initial SMPs were mostly biodegradable. However, a significant residual did exist, and it is interesting to note that the inhibitory substrate, phenol, exhibited three times the residual non-biodegradable SMP as the non-inhibitory substrate, glucose.

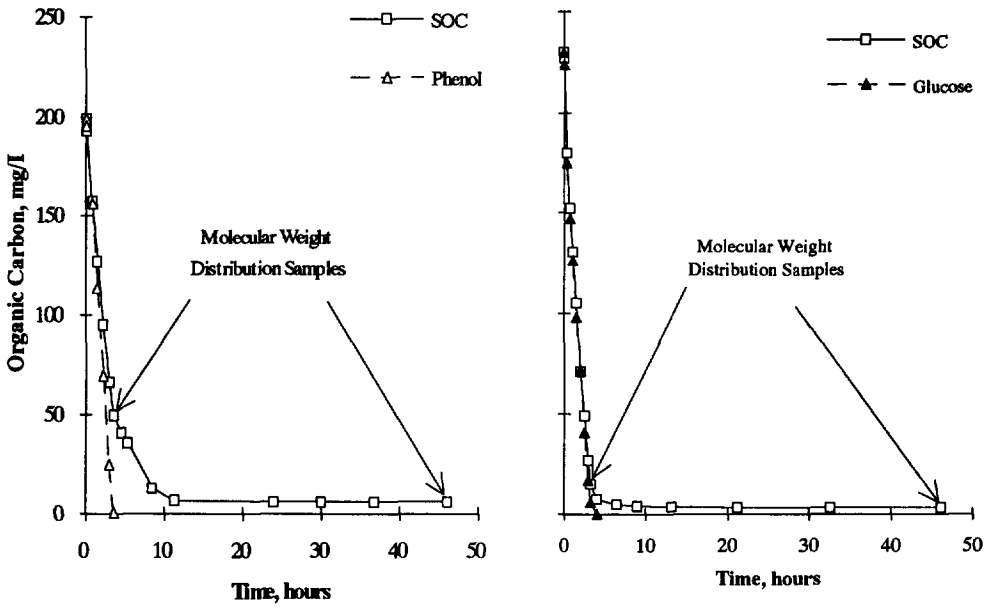

Fig. 1. Typical data for the behavior of the initial substrate and residual soluble substrate related TOC in Region I and Region II.

Finally, after 48 hours, the cells were washed to remove any remaining SMPs, and then allowed to react under endogenous conditions to evaluate the cell associated SMPs--i.e., Region III. The total soluble organic carbon, SMP_E, and the biomass on a carbon basis, X_C, as a function of time are shown in Figure 2 for cells derived from phenol and those derived from glucose. The data show a very slow deterioration of the cells and limited production of SMP during completely endogenous behavior (no carbon sources available outside of the biomass itself). The slow rate of SMP_E production from the biomass means that this source of SMP is insignificant compared to the substrate associated SMP produced in Region I and at the beginning of Region II. Therefore, the MW distributions for these regions were considered to be free of interference from the endogenous decay products. However, at the end of Region II some endogenous SMP by-products might contribute to the MW distribution.

Boero et al. (1991) and Rittmann, et al. (1987) suggested that the production of SMP_E may be proportional to the endogenous cell degradation rate--i.e., $dSMP_E/dt = Y_{SMPE,X}\,[-dX_C/dt]$, or assuming a first order cell decay rate, $dSMP_E/dt = Y_{SMPE,X}\,k_d\,X_C$, where X_C is the biomass concentration on a carbon basis, mg/L, and k_d is the endogenous cell decay rate constant, d^{-1}. Defining the net endogenous SMP production rate constant, k_{SMPE}, as the product $Y_{SMPE,X}\,k_d$, Equation (4) can be written.

$$d_{SMPE}/dt = k_{SMPE}\,X_C \tag{4}$$

Thus, k_{SMPE} may be found using the following equation derived by integration of Equation (4):

$$SMP_E - SMP_E(0) = k_{SMPE} \int X_C \, dt \quad (5)$$

where $\int X_C \, dt$ may be evaluated numerically using the summation of the trapezoids. This technique has been shown to be exceptionally accurate for this type of analysis (Bowers et al., 1988). Then, k_{SMPE} may be found from the slope of a plot of SMP_E versus $\int X_C \, dt$. The data verified the first order dependence of SMP_E on the microorganism concentration (expressed in terms of cellular carbon, as ^{14}C). The k_{SMPE} values are reported as 0.00524/day for phenol and 0.00199/day for glucose. These constants indicate a difference in the decay of cellular products from two types of cells--i.e., $k_{SMPE,phenol}/k_{SMPE,glucose}$ = 2.6. This may be a result of differences in the types of cellular materials or that the cells tend to accumulate more storage products on one substrate--e.g., phenol, that tends to be more readily broken down and released upon the onset of endogenous respiration.

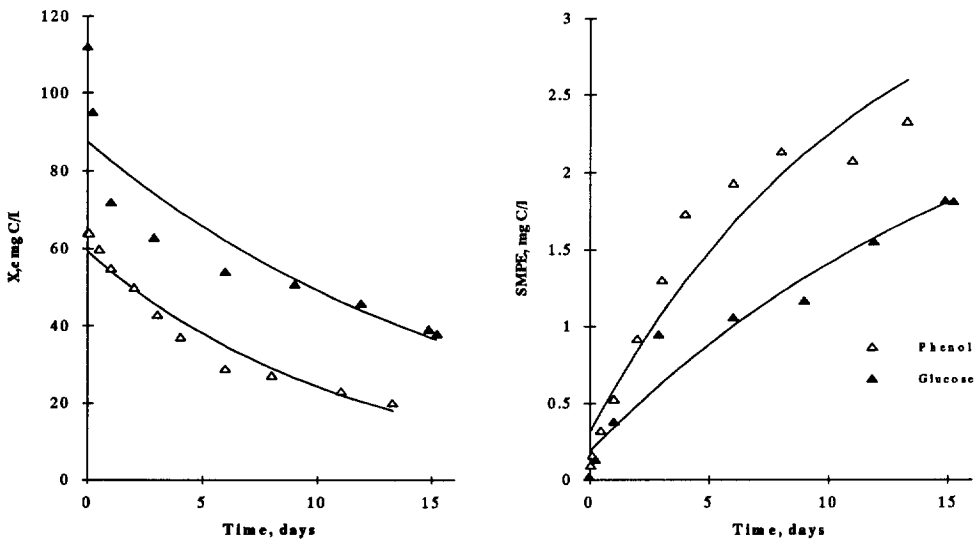

Fig. 2. Variation of endogenous SMP and biomass with time.

Typical examples of results of the MW distribution determinations at each of the test points indicated in Figure 1 and at the end of the endogenous experiments are shown in Figure 3--i.e., Regions I, II, and III. The apparent MW distributions from the phenol test at 3.5 hours, 46 hours and 15 days (13 days after washing the cells) presented in Figure 3 show that at 3.5 hours the lowest MW fraction (<1 K) was by far the most important (91 percent of the soluble organic carbon). The 46-hour distribution exhibited a bimodal shape with a minimum in the 10 to 100 K fraction, and a maximum in the <1 K fraction. From an initial substrate totally in the < 1 K MW fraction, almost 60 percent of the SMP existed as the higher MW material (>1 K) after 46 hours. The MW distribution performed on the 15-day endogenous soluble sample showed an overwhelming presence of high MW products (more than 85 percent of SMP_E >1 K). The apparent MW distributions from the glucose test at 3.3 hours, 46.2 hours, and 17 days (15 days after washing the cells) presented in Figure 3 show that for the distribution performed at 3.3 hours the <1 K fraction was by far the most important (76 percent of the soluble organic carbon). The 46.2-hour distribution showed a bimodal shape with the minimum in the 10 to 100 K fraction, and the maximum in the <1 K fraction. From the initial substrate (all < 1 K) 44 percent was converted to higher MW material after 46.2 hours. The MW distribution performed on the 17-day endogenous soluble sample showed a dominant amount of high MW products (75 percent >1 K).

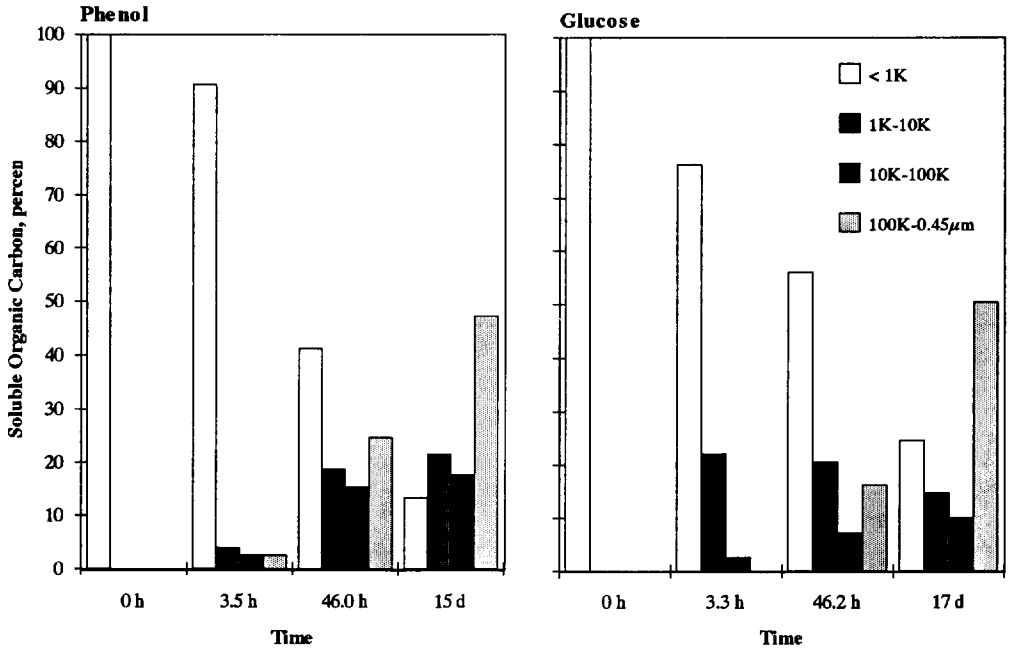

Fig. 3. Molecular weight distributions of the initial substrate, Region I SMP, Region II SMP, and Region III SMP. Cells washed at 48 hours.

Due to variability and uncertainty that is inherent to biological data, these tests were repeated three times for each substrate with the MW fractions being evaluated for Region I ($t < td$), Region II ($t > td$, i.e., 30 to 46 hours), and Region III (more than 2 days after washing cells). A summary of all the results is represented by the averages reported in Table 1. The results show that utilization of phenol resulted in more SMPs than glucose, e.g., 5.7 percent versus 1.7 percent respectively for Region II. Also, for the substrate associated SMPs produced, the ones produced from phenol were in the higher MW region, e.g., 20.8 percent versus 6.7 percent in the >100 K fraction, respectively for Region II.

SUMMARY AND CONCLUSIONS

Soluble microbial product formation was studied using ^{14}C labeled substrate coupled with measurements of the initial substrate. This permitted tracking of the initial substrate and a complete mass balance on carbon while it was transformed into CO_2, cell mass and SMPs. Cell washing and resuspension was used to separate out carbon generated from endogenous decay.

The generation of SMP was observed in three distinct regions based upon the direct transformation of the original substrate (Region I), the polymerization of the lower MW SMPs (Region II), and the endogenous decay of cells (Region III). On the average, the trends were similar for both substrates (glucose and phenol) and indicated that the lower MW fraction (<1 K) SMPs dominated Region I but decreased with time as these compounds were polymerized to form the higher MW fractions. However, the phenol associated SMPs tended to be of a higher MW than the glucose associated SMPs.

The endogenous decay of cells to form SMP_E was a first order process. The corresponding rate of SMP_E production was significantly higher for cells grown on phenol than glucose, i.e., phenol = 2.6 × glucose.

TABLE 1 SUMMARY OF MOLECULAR WEIGHT DISTRIBUTION DATA[a]

Substrate/ Region	Time	Molecular Weight Fractions[b] <1 K	1 K to 10 K	10 K to 100 K	100 K to 0.45 μm	Total
Glucose, t_d = 4.53 h						
Region I	3.3 h	0.760	0.220	0.030	0.000	1.0
	(3.3 h)[c]	(0.016)	(0.004)	(0.001)	(0.000)	(0.021)
Region II[d]	8 to 46.2 h	0.660	0.220	0.057	0.067	1.0
	(25.9 h)[c]	(0.011)	(0.003)	(0.001)	(0.001)	(0.017)
Region III[e]	16.3 to 19 h	0.297	0.187	0.223	0.297	1.0
	(17.43 d)[c]	(0.002)	(0.001)	(0.002)	(0.002)	(0.007)
Phenol, t_d = 3.87 h						
Region I	2.8 to 3.5 h	0.860	0.020	0.060	0.065	1.0
	(3.2 h)[c]	(0.150)	(0.005)	(0.010)	(0.010)	(0.175)
Region II[d]	7 to 46 h	0.418	0.213	0.168	0.208	1.0
	(22.3 h)[c]	(0.022)	(0.013)	(0.009)	(0.012)	(0.057)
Region III[e]	15 to 35 d	0.185	0.350	0.170	0.300	1.0
	(25.0 d)[c]	(0.001)	(0.002)	(0.002)	(0.003)	(0.008)

[a]Average of three glucose and three phenol tests.
[b]Listed as average fractions of residual soluble organic carbon or SMP. Values in parentheses represent average fractions of initial carbon as SMP (values do not total 1.0 reflecting carbon lost as CO_2 and accumulated as biomass).
[c]Value in parenthesis represents the average time.
[d]Before washing cells.
[e]After cell washing.

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