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Microbial biodegradation of organic wastes containing surfactants in a continuous-flow reactor

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In a continuous flow bioreactor seeded with microbes from municipal activated sludge, complete organic carbon oxidation of simulated graywater (wastewater produced in human residences, excluding toilet wastes) was achieved at dilution rates up to 0.36 h^{-1} in the presence of 64.1 μM linear alkylbenzenesulfonate (LAS) L^{-1}. At LAS concentrations of 187 μM, the system functioned only at dilution rates up to 0.23 h^{-1}, and the biomass yield was two-fold lower. There were physiological changes in the microbial communities under different operating conditions, as measured by specific contents of ATP and extracellular hydrolases as well as the respiratory potential of the biomass. LAS inhibited the activity of LAS-degrading microbes at >150 μM LAS, and the activity of other microbes at >75 μM LAS. Chemical analysis of graywater indicated that samples consisted primarily of biological polymers (proteins and polysaccharides) and lower concentrations of surfactants. Biological remediation of graywater is possible, although treatment efficiency is influenced by the operating conditions and wastestream composition.

Keywords: biodegradation; chemostat; physiological state; graywater; LAS

Introduction

Graywater is wastewater produced from galley, scullery, laundry, shower, and lavatory sinks: it includes all wastewater produced in human residences, excluding toilet wastes [13]. The flow in households has been estimated from 96 to 172 L per person per day [6]. In arid regions, there has been an interest in using recycled graywater for irrigation and toilet flushing.

The fate of graywater is also an issue on board ships. Graywater accounts for two-thirds of shipboard, non-oily wastewater. As environmental regulations governing overboard disposal become more restrictive, shipboard treatments will be needed that can rapidly and reliably reduce the organic load. To date, treatment solutions rely on physical separation and waste concentration rather than true remediation. Biotechnological approaches have yet to be applied. We are investigating the utility of bioreactors as a component of a treatment process. Shipboard treatment must satisfy different criteria from land-based systems. Relatively large volumes of wastewater are generated per person per day, about 7–50 L from showers, 36 L from the galley, and 22 L from laundry on US Navy ships (J Benson, NSWC, Annapolis, MD, personal communication). However, the space available for holding tanks or other marine sanitation devices is limited. In order to carry out treatment in a small space, a shipboard biotreatment system must have a small hydraulic residence time (HRT; flow rate/reactor volume) for the wastewater.

We present data on the biodegradation of a simulated graywater by microbes in a continuous flow reactor. Although this exact reactor system is not contemplated for shipboard use, it did allow us to answer two relevant questions: (1) what is the minimum HRT in which the removal of soluble organic matter is maintained below an acceptable level; and (2) is this minimum HRT affected by the organic composition of the graywater input?

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Methods

Analytical methods

Graywater samples were provided by John Benson, Naval Surface Warfare Center (NSWC), Carderock Division, Annapolis, MD, USA). The graywater was collected by a commercial hauler from the US Naval Academy (USNA), Annapolis, MD, and composite samples of USNA graywater and galley water were collected, frozen, and shipped on ice to our laboratory. Shower water was collected at a residential home fitted with a low flow shower head as used on US Navy ships. Anionic surfactant concentrations were assayed as methylene blue active substances (MBAS) [1]. MBAS and chemical oxygen demand (COD) [1] were assayed on whole samples. Particulate and soluble components were separated by filtration through Whatman GF/C glass fiber filters. The particulate and soluble fractions were assayed for total carbohydrate by the anthrone method [11] and for protein [18]. We determined experimentally that LAS concentrations up to 70 μM had <10% effect upon the protein assay (data not shown). LAS was extracted and measured by HPLC chromatography [20]. For each of these assays, triplicate subsamples were analyzed, and the means are reported. Elemental analysis was performed using an inductively-coupled-plasma atomic emission spectrometer (Perkin Elmer Plasma 400, Norwalk, CT, USA), after sample digestion in nitric acid and hydrogen peroxide at 180°C.

Residual substrate concentrations in the bioreactors were measured after centrifugation of a culture sample at $10\,000 \times g$ for 10 min at 4°C. Microbial biomass in the reactors was monitored by measuring the optical density (OD) in a 1-cm cell at 600 nm on a Gilford 250 spectropho-

tometer (Gilford Instrument Laboratories, Oberlin, OH, USA). We determined experimentally that an optical density of 1.0 corresponded to 250 μg bacterial protein ml^{-1} in these reactors.

Cellular protein and total carbohydrate were measured as described by Herbert *et al* [10]. ATP was extracted from cells and analyzed as described in Cook *et al* [5]. Whole culture assays of amylase and protease activities were performed according to the methods of Bernfeld [3] and Rinderknecht *et al* [22] respectively. One unit of amylase is defined as the amount that produced one mg of product (maltose) per h. One unit of protease is defined as the amount that hydrolyzes hide azure at a rate to produce an OD change of 1.0 per h. The reported values are the means of triplicate determinations.

Oxygen consumption by washed resting cell suspensions was measured with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). A total volume of 1.5 ml of washed resting cells ($OD_{600} = 0.4$) was incubated at 28°C, and the rate of endogenous respiration was monitored. Test substrate was added and oxygen consumption was monitored over the next 5 min. For the measurement of potential respiration rates, the concentrations of added substrates were 2 mM maltose, 3 mM glucose, or 2 g casamino acids L^{-1}. These concentrations were sufficient to produce the maximum (saturated) rates of oxygen consumption. For LAS, it was necessary to determine the rate-saturating concentration for individual experiments; this concentration varied between 60 and 150 μM. The reported values are the means of triplicate analyses of subsamples.

Continuous cultures

Continuous-flow bioreactors were maintained in 580-ml vessels (CYTOLIFT glass airlift bioreactor, Kontes, Vineland, NJ, USA). The reactor was maintained at 28°C by circulating water through the water jacket of the reactor. The rate of sterile medium addition was controlled by a Gilson Minipuls 2 peristaltic pump (Gilson Inc, Middleton, WI, USA). Culture aeration and agitation occurred by passage of 8 L min^{-1} of sterile, humidified air through the culture.

The medium feeds to the continuous culture all contained the following components: 9.3 mM NH_4Cl, 3 mM NaCl, 2 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.25 mM $MgSO_4$, 5 μM $CaCl_2$, 14 μM $FeCl_3$, 23 μM disodium ethylenediaminetetraacetate, and 26 μM sodium citrate. The pH was 7.5. The media contained 87 mg of commercial laundry detergent and 1 ml of SL7 trace element solution [4] per L. The organic substrates per L of medium were as follows: medium I, 250 mg laundry starch and 17 mg of linear alkylbenzene sulfonate (LAS) (Sigma Chemicals, 80% by weight); medium II, 400 mg starch, 150 mg gelatin, and 70 mg LAS; medium III, 400 mg starch, 150 mg gelatin, and 17 mg LAS. The commercial laundry detergent contained 10% LAS by weight. Therefore, the actual concentrations of LAS in the three media were 64.1, 187, and 64.1 μM, respectively.

To recognize steady-state, the culture turbidity at 600 nm was monitored. After the turbidity had reached a constant value, the culture was maintained for an additional four hydraulic residence times (to ensure that a physiological steady state was achieved) prior to removal of samples for steady-state analyses.

Results

Chemical analysis of graywater

Although data exist on the amount of organic carbon in graywater (usually reported as BOD or COD), we required information on the types of organic substrates present, in order to prepare a reproducible simulated graywater for our laboratory experiments. We analyzed several graywater samples to provide some information as a basis for formulating our feed medium. Most organic matter in the USNA graywater consisted of biopolymers: polysaccharides (72%) and proteins (28%) (Table 1). The majority of these polymers were in the dissolved rather than the particulate phase. The average chain length of the polysaccharides was rather small; from the relative amounts of reducing sugar detected in the USNA graywater sample with or without hydrolysis of glycosidic bonds, we estimate an average of 34 residues. Anionic surfactants comprised a small proportion (<1%) of the organics in these samples.

We also analyzed the inorganic components of the USNA graywater sample. The concentrations (mg L^{-1}) were: boron (0.05), calcium (14), copper (0.01), iron (0.74), magnesium (5.3), manganese (0.02), molybdenum (0.0), phosphorus (18.4), potassium (7.7), sodium (95.0), and zinc (0.05). The conclusions drawn from these organic and inorganic analyses were that the graywater is highly susceptible to rapid microbial biodegradation.

After the bioreactor studies reported here were completed, we analyzed a set of 30 graywater samples collected at daily intervals from the US Naval Academy during the period February 21–April 4 1996. The amounts (mean ± s.d.) of carbohydrate and protein were 45 ± 20 and 89 ± 36 mg L^{-1}, respectively. The LAS concentrations were 23 ± 9 mg L^{-1} (66 ± 26 μM), substantially higher than in the USNA graywater samples that were analyzed earlier.

Studies involving bioreactors—continuous culture studies

Studies which employed bioreactors were designed to answer two questions: (1) what is the maximum rate at which microbial communities can metabolize the organics found in graywater; and (2) does the concentration of potentially toxic molecules such as LAS affect this maximum rate?

The inoculum came from activated sludge of the West Lafayette, IN municipal wastewater treatment plant. In medium I, 88% of the organic carbon was polysaccharide and 12% was LAS surfactant (calculated as mol C). As the dilution rate (D) to the continuous culture was increased, the concentrations of organic substrates in the culture supernatant remained low (that is, chemical oxygen demand (COD) <30 ppm, and total carbohydrate concentrations <5 mg L^{-1}) up to dilution rate = 0.36 h^{-1} (HRT = 2.75 h) (Table 2). Dilution rates greater than 0.36 h^{-1} approach the critical dilution rate for the culture as seen by increased steady-state substrate concentrations and decreased biomass (Figure 1a) in the cultures. The steady-state biomass level

Table 1 Analysis of organic substrates in wastewater samples. Values are means ± s.d. ($n = 3$)

Sample	COD (mg L^{-1})	MBAS (mg L^{-1})	Particulate		Soluble	
			Carbohydrate (mg L^{-1})	Protein (mg L^{-1})	Carbohydrate (mg L^{-1})	Protein (mg L^{-1})
USNA graywater	900 ± 10	0.2	19 ± 0.5	27 ± 7	340 ± 5	106 ± 3
Galley water	160 ± 26	0.2	23 ± 4	3 ± 0.5	280 ± 2	40 ± 6
Shower water	730 ± 46	1.2	16 ± 1	40 ± 8	5 ± 2	46 ± 9

Table 2 Chemical analyses of supernatants from steady state continuous cultures fed 250 mg starch and 60 μmol LAS per liter (medium I). Values are means ± s.d. ($n = 3$)

Dilution rate (h^{-1})	COD (mg L^{-1})	Carbohydrate (mg L^{-1})
0.036	8 ± 1	2 ± 0.3
0.091	5 ± 2	2 ± 0.2
0.145	5 ± 0.3	2 ± 0.9
0.218	9 ± 4	3 ± 0.4
0.364	9 ± 1	3 ± 0.2
0.455	50 ± 10	98 ± 0.3
0.545	295 ± 3	150 ± 1.0

was approximately constant between $D = 0.036$ and 0.36 h^{-1}. That is, no apparent effects of maintenance energy demands on carbon flow were evident even at $D < 10\%$ of the population's maximum growth rate. The mean biomass yield over this range was 0.4 mg cell protein (mg substrate C)$^{-1}$.

As D was varied, so did the physiological status of the microbial community (Figure 1b). There was a six-fold decrease in amylase activity (normalized to the amount of cellular protein) as conditions shifted from severely organic carbon-limited (low D) to less-limiting conditions (higher D). Furthermore the ratio of cellular ATP : protein increased. Most estimates of the ATP content in growing cells are in the range of 5–7 nmol mg^{-1} dry weight [15]. If protein is about 50% of cell dry weight, the ATP values in our bioreactors were generally within this range.

A second set of continuous culture runs involved a feed medium containing not only starch, but also protein and a higher LAS concentration, 187 μM. With feed medium II, the microbial community was unable to completely degrade the organic compounds in the feed at $D = 0.36\ h^{-1}$ (Table 3). However, at $D = 0.23\ h^{-1}$ residual substrate concentrations were similar to those found at $D = 0.23\ h^{-1}$ with medium I. The incomplete degradation of organic substrates at $D = 0.36\ h^{-1}$ was primarily due to a failure to degrade the polysaccharide in the feed—the residual concentrations of protein and LAS were significantly below the feed concentrations. The steady-state biomass yield with this feed medium was substantially less than in Medium I. At $D = 0.036$ and $0.23\ h^{-1}$, the yields were 0.19 and 0.15 mg cell protein (mg substrate C)$^{-1}$.

We attribute these differences to increased concentrations of LAS in feed medium II. Continuous cultures ($D = 0.11$–$0.22\ h^{-1}$) fed medium III, with the same concentrations of starch and gelatin (400 and 150 mg L^{-1}) but lower LAS concentration (64.1 μM) had steady-state biomass yields of 0.52 mg cell protein (mg substrate C)$^{-1}$. We attempted to run reactors with a similar feed that contained 345 μM LAS several times at $D = 0.036\ h^{-1}$, but these were unstable and failed within 7 days.

As found with medium I, the physiological state of the

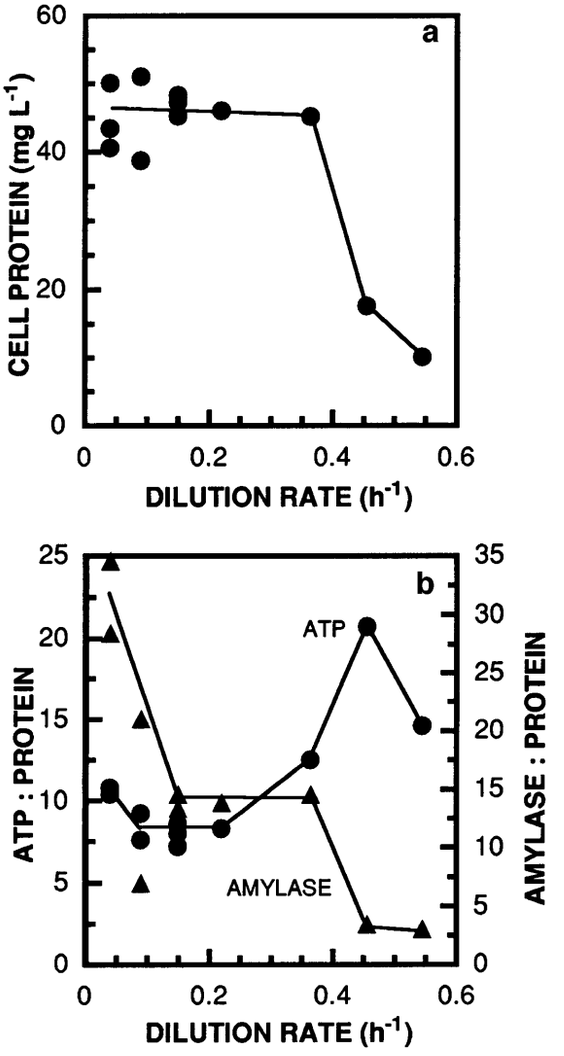

Figure 1 Changes in (a) steady-state biomass level (expressed as particulate protein) and (b) two physiological properties, nmol ATP : mg protein (●) and units amylase : mg protein (▲) as a function of dilution rate in bioreactors fed medium I.

Table 3 Chemical and physiological characteristics of steady-state continuous culture fed 400 mg starch, 150 mg gelatin, and 65 mg LAS per liter (medium II). Values are means ± standard errors

Dilution rate (h^{-1})	Cell protein (mg L^{-1})	COD (mg L^{-1})	Carbohydrate (mg L^{-1})	Soluble protein (mg L^{-1})	LAS (μM)	ATP : protein (nmol mg^{-1})	Amylase : protein (U mg^{-1})	Protease : protein (U mg^{-1})
0.036	53 ± 5	0	4.3 ± 1	4 ± 1.8	<3	13 ± 0.7	3.8	0.20
0.233	40 ± 0.1	4 ± 1.5	4 ± 1.5	3 ± 1.5	6	18 ± 0.5	4.5	0.16
0.364	20 ± 2.4	116 ± 5	250 ± 2	1 ± 0.5	6	24 ± 2.4	4.0	0.02

microbes that degraded medium II differed as a function of D (Table 3). The ATP : protein ratio increased from 13 to 24 nmol mg^{-1} cell protein as D increased from 0.036 to 0.36 h^{-1}. In contrast, the amylase specific activities remained at low levels, even under the carbon-limited conditions at low D. Extracellular protease activities were also similar at all D tested.

Kinetic analyses

The rates of microbial metabolism of specific organic substrates were quantified by measuring the rate of oxygen consumption. Resting cell suspensions were prepared from steady-state cultures fed medium II. Respiration measurements were made using monomers derived from the biopolymers in the feed (maltose and glucose from starch, and amino acids from protein) and also the surfactant LAS. The rates of oxygen consumption were functions of substrate concentration (Figure 2). Apparent half-saturation parameters (K_m) were 20, 25, and 40 μM for maltose, glucose, and LAS respectively. The respiration rate on an amino acid mixture (casamino acids) also was a nonlinear function of concentration, with an apparent K_m of 75 mg L^{-1}.

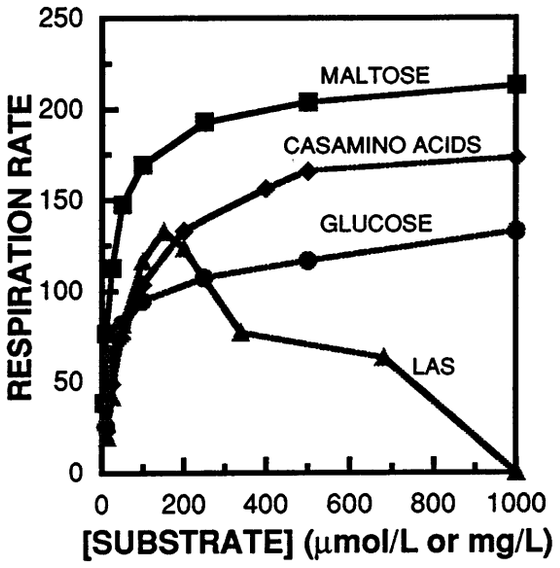

Figure 2 Respiration rates (μg O_2 mg^{-1} protein h^{-1}) of resting cell suspensions taken from a steady-state continuous culture (D = 0.036 h^{-1}) grown in medium II. Different concentrations of (■) maltose, (●) glucose, (◆) casamino acids or (▲) LAS were added, and the net respiration rate (minus the endogenous respiration rate) was calculated. Concentrations for maltose, glucose, and LAS are in μmol L^{-1}, and for the amino acid mixture (casamino acids) in mg L^{-1}.

The maximum rate of oxygen consumption was determined by adding a saturating concentration of substrate to resting cells (Table 4). These rates of metabolic potential were highest for microbial communities grown at the lowest D (and hence the most severe carbon limitation). If a saturating concentration of substrate is added, then the rate should be proportional to the number of catalysts (microbes) in the community capable of metabolizing that substrate. The three organic substrates (starch, gelatin, and LAS) comprised 59, 26, and 15% of the moles of C in medium feed II. The total potential respiration rates contributed by the monomers derived from these substrates (maltose, casamino acids, and LAS) were roughly proportional to the relative amount of substrate organic C in the feed (Table 4).

Although LAS was metabolized at low concentrations by microbes in the mixed population, it inhibited the LAS-degrading microbes at concentrations >150 μM (Figure 2). It also inhibited microbes degrading other substrates—glucose metabolism was inhibited at LAS concentrations >75 μM (Figure 3).

Discussion

Our objective was to determine whether microbiological remediation of graywater is feasible on ships. A major constraint is that the rate of wastewater generation is large in comparison to the volume available for holding and treatment. Therefore, the process must be capable of operation at HRT of less than a few hours.

Our chemical characterization suggested that graywater is highly susceptible to rapid microbial metabolism. Our survey was not exhaustive, but did indicate that graywater consisted primarily of biopolymers, with a small fraction of surfactants. The inorganic constituents of the samples

Table 4 Respiration rates at saturating substrate concentrations of resting cell suspensions taken from steady-state continuous cultures fed with medium II. Relative activities on each substrate are indicated in parentheses as the percentage of the summed respiration rates attributed to that substrate

Dilution rate (h^{-1})	Respiration rate (μg O_2 (mg protein)$^{-1}$ h^{-1})		
	Maltose	Amino acids	LAS
0.036	213 (41%)	173 (33%)	133 (26%)
0.23	81 (41%)	81 (41%)	36 (18%)
0.36	45 (54%)	22 (27%)	16 (19%)

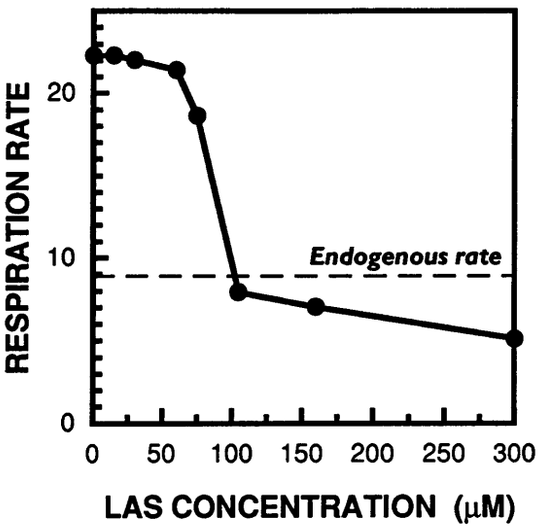

Figure 3 Inhibition of glucose respiration by LAS. A resting cell suspension taken from a continuous culture fed medium II ($D = 0.036\ h^{-1}$) was given 2 mM glucose to stimulate respiration. Different concentrations of LAS were added, and the change in respiration rate ($mg\ O_2\ L^{-1}\ h^{-1}$) was recorded.

were compatible with microbial activity. Pilot-scale studies with USNA graywater have demonstrated >95% reduction in BOD via biotreatment (J Benson, personal communication).

Simulated graywater feeds were rapidly metabolized in continuous flow bioreactors. These feeds generated C-limited conditions in the reactor, because the C : N : P ratios (by weight) in medium feeds I, II, and III were 7.3 : 8.4 : 1, 18 : 10 : 1, and 16.4 : 10 : 1, respectively. With a feed medium consisting of starch and 64.1 μM LAS, more than 98% of the anthrone-reactive material (carbohydrates) was metabolized at D up to $0.36\ h^{-1}$ (HRT = 2.75 h). The results for $D > 0.36\ h^{-1}$ were in accord with continuous culture theory; as D approaches the maximum growth rate of the microbes, substrate concentrations rise and biomass levels decline [10]. When the feedstock was changed to include higher concentrations of LAS, system performance declined. We could not obtain stable operation of continuous cultures even at $D = 0.036\ h^{-1}$ with feeds containing 345 μM LAS, and with 187 μM LAS, <50% of the polysaccharide in the feed was metabolized at $D = 0.36\ h^{-1}$ (Table 3). Note that these surfactant concentrations were much higher than those found in the graywater samples.

Higher feed concentrations of LAS led to a decreased microbial yield. In Media I and III (64.1 μM LAS), the yields (mg cell protein (mg organic C)$^{-1}$) were more than two-fold greater than yields in medium II with 187 μM LAS. The physiological mechanism responsible for this effect is unclear, because the steady-state LAS concentrations in cultures fed medium II were low (Table 3). Perhaps inhibitory metabolic products accumulated which we did not detect. For enteric bacteria, resistance to detergents requires energy consumption; this consequently reduces the growth yield [2]. The yield values in media with 64.1 μM LAS were similar to those reported for *Escherichia coli* growing on organic acids or amino acids, but approximately half that reported for aerobic growth on carbohydrates [9].

The physiological states of the microbes varied as a function of D and feed composition. A decrease in D imposed a more severe organic carbon limitation. This restriction resulted in a lower energetic status, expressed as a decrease in ATP : protein. Microbes often physiologically adapt to nutrient limitation by increasing the levels of proteins essential for use of the rate-limiting substrate [19,25]. This pattern was observed in the continuous cultures fed medium I. Amylase, required for the initial hydrolysis of the starch substrate, had its highest activity under the most severe nutrient limitation (Figure 1). This pattern was not observed in medium II (with a higher LAS concentration) for either amylase or protease. However, the respiratory potentials measured in resting cell suspensions were highest under the most severe nutrient limitations (Table 4), which suggests an adaptation to C-limited conditions.

Note that our respiration measurements were not the actual *in situ* rates in the reactor, but rather the potential achieved when cells taken from the reactor were provided a non-limiting substrate concentration. Thus, our assay assumed that 'respiratory control' did not occur as in mitochondria [7], and that energy was dissipated as fast as it was generated either by use for growth or via overflow metabolism [24]. Respiratory control is usually absent in bacteria. We further assumed that the response to specific substrates reflected the activity of those microbes that were metabolizing a specific substrate in the reactor. That is, the response to maltose, casamino acids, and LAS reflected the capacity to metabolize carbon derived from starch, gelatin, and LAS respectively.

We derive two conclusions from these respiratory potentials: (1) the metabolic diversity of the microbial community was a consequence of the organic constituents in the feed medium. For example, LAS was 16% of the mol organic C in feed II, and 18–26% of the total respiratory potential was due to LAS catabolism (Table 4); (2) as the intensity of nutrient limitation increased, the respiratory potential increased. This can be viewed as another example of the physiological adaptation in which synthesis of catabolic enzymes is increased when substrate becomes limiting [19]. For example, an increase in cytochrome content and respiratory potential was found in *Beneckea natriegens* grown at a reduced oxygen tension [17]. In contrast, respiratory potential decreased in two strains of *Klebsiella aerogenes* as D decreased in organic substrate-limited continuous cultures [8,21].

LAS concentration was a critical factor for operation of this system. LAS has long been known to be biodegradable [16]; degradation entails omega or beta oxidation of the alkyl chain, cleavage of the benzene ring by oxygenases, and desulphonation [26]. However, slow degradation has often been reported [12,23]. Larson *et al* [16] reported a half-life of 1.5–2.2 days for 14.4 μM LAS in activated sludge; however, they also found >90% mineralization of 14.4 μM LAS fed to a laboratory-scale continuous activated-sludge system with a HRT of 6 h.

High concentrations of LAS inhibited the microbes degrading LAS, and lower LAS concentrations inhibited the other microbes in the reactor. Therefore, the microbial community depended upon the LAS-degrading population to maintain a non-inhibitory LAS concentration. Appar-

ently they were unable to do so at LAS feed concentrations of 347 μM because the system was unstable and failed within 7 days. Jimenez *et al* [14] obtained biodegradation of LAS at concentrations below 115 μM but reported problems in stable operation at higher concentrations. In contrast, Sigoillot and Nguyen [23] reported on cultures enriched at LAS concentrations up to 2 mM. Aspedon and Nickerson [2] reported that growth in sodium dodecyl sulfate imposed an energy burden (and consequently a reduced yield) in enteric bacteria. We noted this effect at LAS concentrations of 187 but not 64.1 μM.

In summary, the graywater samples we analyzed were comprised of organic compounds susceptible to microbiological remediation. In a continuous flow system, organic carbon oxidation was achieved at HRT as short as 2.75 h, although the surfactant loads in the simulated graywater affected this value. Operating conditions affected the physiological state of the microbial communities. In order to achieve faster and more stable operation of this system, we are investigating a continuous flow reactor in which there is 100% recycling of biomass in the system.

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