

## SORPTION OF HYDROPHOBIC ORGANIC CHEMICALS TO BACTERIA

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**Abstract**—The toxicity and time-dependent sorption of three hydrophobic organic chemicals to *Rhodococcus rhodochrous* bacteria were investigated. In experiments, environmentally relevant concentrations of pentachlorophenol (PCP), hexachlorobenzene (HCB), and dichlorobiphenyl (DPCB) were applied to living (both growing and nongrowing) bacteria as well as to dead bacteria. For PCP (an ionizing chemical), bacterial growth decreased, and bacterial death increased, as the PCP concentration increased. In sorption experiments with PCP, the partition coefficient was affected by the active uptake of PCP by living but not by dead bacteria, by the death of the living bacteria because of PCP toxicity, and by saturation of site-specific sorption as the PCP concentration increased. Hexachlorobenzene (a nonionizing chemical) did not affect bacterial growth or death at all HCB concentrations investigated. In sorption experiments with HCB, the partition coefficient depended on the rate of bacterial growth relative to the sorption rate. The sorption rate depended on the state of bacterial aggregation, and this changed with time. Results for DPCB (a nonionizing chemical with an equilibrium partition coefficient similar to that of HCB) were similar to those for HCB.

**Keywords**—Sorption Bacteria Organic chemicals Hydrophobic Biosorption

## INTRODUCTION

Bacteria are a major component of the aquatic environment and serve not only as chemical storage but also as a source/sink to the surrounding environment. Because they have the capacity to bioaccumulate hydrophobic organic chemicals (HOCs), they provide a fundamental route for HOC transport into food webs; they form the base of the microbial loop and play a major role in the recycling and uptake of contaminated organic matter. In oligotrophic ecosystems (nutrient-poor, low-organic-input systems), bacteria make up a large proportion of the biomass, whereas in eutrophic ecosystems (nutrient-rich, high-organic-input systems), the proportion of bacterial biomass is smaller [1]. In each case, the sorption of HOCs to bacteria is different. Therefore, it is important to distinguish between bacterial sorption in a relatively fast-growing, eutrophic ecosystem and that in a slower-growing, oligotrophic ecosystem.

In the present study, the toxicity and time-dependent sorption of three HOCs to *Rhodococcus rhodochrous* bacteria were investigated. In experiments, environmentally relevant concentrations of pentachlorophenol (PCP), hexachlorobenzene (HCB), and dichlorobiphenyl (DPCB) were applied to living (both growing and nongrowing) bacteria as well as to dead bacteria. In the sorption experiments, results are presented in terms of the partition coefficient ( $K_p$ ), which is defined as

$$K_p = \frac{C_B}{C_w} \quad (1)$$

where  $C_B$  (kg/kg) is the mass of the chemical sorbed to the bacteria divided by the mass of the bacteria and  $C_w$  (kg/L) is the mass of the chemical dissolved in the water divided by the volume of water.

Pentachlorophenol is a chlorinated insecticide and fungicide used primarily to protect timber from fungal rot and wood-

boring insects. It is an ionizing compound that is more than 95% ionized for pH greater than six. Because it is a substantial health threat with high carcinogenic potential, its use is in decline (e.g., Sweden, Germany, Finland), restricted (e.g., United States), or totally discontinued (e.g., Denmark) [2]. However, PCP is still a widely used and important pesticide in some developing countries because of its low cost and broad spectrum of uses as an insecticide, fungicide, molluskicide, defoliant, herbicide, and wood preservative. Even for those countries in which PCP use has been abandoned, it continues to be a persistent environmental contaminant because of its tendency to sorb to sediments and form stable metabolites.

Hexachlorobenzene and DPCB are nonionizing HOCs that are not readily biodegradable by most bacteria. As with most HOCs, HCB and DPCB have a strong persistence in coastal systems and food webs; they are characterized by low water solubility and high lipid solubility, coupled with a strong resistance to photochemical, biological, and chemical degradation. Hexachlorobenzene has been used as a pesticide and industrial chemical since 1933, when it was introduced as a commercial fungicide for wheat and an industrial raw material for synthetic rubber. Most of the toxic effects of HCB appear to be correlated with disruption of the endocrine system (<http://www.iarc.fr/>). Dichlorobiphenyl has been used as a heat-transfer and insulating fluid in cooling systems and electrical equipment as well as in sealants, rubber, paints, plastics, printing ink, and pesticides. Studies strongly suggest that polychlorinated biphenyls (PCBs) are probable human carcinogens (<http://www.epa.gov/opptintr/pcb/effects.html>). Production of all PCBs has been banned in several industrialized countries; the United States stopped producing PCBs in 1977.

Considerable work relevant to the present problem has been done on the sorption of HOCs to suspended sediments and, to a lesser extent, to phytoplankton. Studies on sediments (see, e.g., [3–6]) have demonstrated that HOC sorption to suspended sediments is a function of the amount of organic matter in the sediments and the hydrophobicity of the HOCs; both adsorp-

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tion and desorption can be quite slow, often days to weeks to months.

In the analyses of experiments concerning the time-dependent sorption of HOCs to sediments, a one-dimensional, time-dependent diffusion equation was sufficient to describe the time-dependent behavior of  $K_p$  and the time to equilibrium. This latter quantity was shown [3,7] to be given by

$$t = \frac{d^2}{24D} \quad (2)$$

where  $t$  is the time to equilibrium,  $d$  is the diameter of the sedimentary particle or floc, and  $D$  is an effective diffusion coefficient given by

$$D = \frac{D_m f}{1 + \left(\frac{1 - \phi}{\phi}\right) \rho_p K_p} \quad (3)$$

where  $D_m$  is the molecular diffusion coefficient,  $f$  is a correction for tortuosity,  $\phi$  is the porosity of the particle or floc, and  $\rho_p$  is the density of the solid particle.

Research concerning the sorption of HOCs by phytoplankton has shown that the time required for a highly hydrophobic compound to reach equilibrium also is slow, and much longer than the time for phytoplankton division; this makes the partition coefficient appear to be erroneously small and dependent on growth [8]. Stange and Swackhamer [9] observed that for cyanobacteria and phytoplankton, an initial rapid rate of accumulation of PCBs was followed by a slower accumulation rate; this was interpreted as an initial sorption of PCBs to the cell membrane exterior, followed by a slower diffusion through the cell membrane and into the cell matrix. However, those authors found that whereas this held true for less hydrophobic PCBs, the trend did not hold true for more hydrophobic PCBs ( $\log K_{ow} > 6$ ). Because of the larger compound size that is typical of highly hydrophobic molecules, the more hydrophobic PCBs had restricted cell membrane permeability. It also was demonstrated that the more hydrophobic compounds sorbed primarily to the cell membrane exterior, whereas the smaller, less hydrophobic compounds sorbed primarily to cytoplasm within the cell matrix.

In a broad study by Wallberg and Andersson [10], the difference between adsorption (sorption to the cell membrane surface) and absorption (sorption to within the cell interior) was investigated among three different size classes of marine microorganisms. Adsorption was observed in all three size fractions: The bacteria fraction (0.2–2  $\mu\text{m}$ ), the flagellate fraction (2–10  $\mu\text{m}$ ), and the microplankton/phytoplankton/protozoa fraction (>10  $\mu\text{m}$ ). Absorption did not occur in the bacteria and flagellate fractions, but the HOCs associated with the larger plankton/protozoa fraction gradually absorbed into the cell matrix.

Some experiments with phytoplankton have indicated that the amount of sorption does not differ between living and dead cells [11], but others have found that the accumulation to dead cells is greater than that to living cells [12,13]. Lederman and Rhee [14] attributed this greater accumulation to dead cells to the method of heat-killing. Heat-killing causes denaturation of cellular constituents and disintegration of subcellular organization, thus allowing interior cellular material to be exposed to the PCBs. This effectively circumvents the protective cell membrane barrier of living cells and allows the PCBs direct access to the organic matter in the cellular matrix, which ordinarily is not available in living cells.

Sikkema et al. [15] studied the toxicity of cyclic hydrocarbons to *Escherichia coli* and found that the majority of sorption was to the cell's cytoplasmic membrane. Berlanga et al. [16] investigated the routes that quinolones (antimicrobial agents used in the treatment of selected community infections) can take into the cell and discovered that highly hydrophobic quinolones could not pass through the cell membrane. However, less hydrophobic quinolones could pass through the cell membrane via porins, the lipid bilayer, or active displacement of the cell membrane exterior.

## MATERIALS AND METHODS

### Bacteria

The bacteria used for these experiments were *R. rhodochrous*, type strain ATCC 13808; these are gram-positive, aerobic bacteria that were isolated from a soil sample and subsequently cultured by Los Alamos National Laboratory (Los Alamos, NM, USA). They are prevalent in coastal marine and aquatic sediments. *Rhodococcus rhodochrous* generally are 1-  $\times$  3- $\mu\text{m}$ , rod-shaped bacteria that have a tendency to form aggregates as they multiply but also may fragment into cocci. They are hydrocarbon degraders that tolerate high concentrations of some HOCs [17]. The bacteria were grown in modified AM-1 salt medium [18], and after 4 d of incubation, the culture was estimated to be at the beginning of the stationary phase. This estimate was based on previous growth experiments. The mass of the bacterial culture was measured via a spectrophotometer and diluted to a concentration of  $0.5 \times 10^{-4}$  kg/L. The culture was then distributed via pipetting into individual 50-, 100-, or 240-ml (depending on the airspace needed in the experiment) Qorpak<sup>®</sup> (Bridgeville, PA, USA) amber bottles for the sorption experiments. Each individual bottle contained a total volume of 25 ml of solution, which consisted of bacteria (at a concentration of  $0.5 \times 10^{-4}$  kg/L), chemical, a saltwater nutrient medium, and in some cases, acetone. Sorption experiments were then conducted as a function of time; each data point in time corresponded with the contents of three individual bottles that were sampled and averaged.

The organic content of the bacteria was determined by filtering 100 ml of bacterial culture through dried and tared Millipore (Billerica, MA, USA) membrane filters (pore size, 0.45  $\mu\text{m}$ ). The filter was immediately oven-dried (65°C), and the cells were scraped from the filter and measured for organic carbon content by the University of California, Marine Science Institute analytical laboratory using an Exeter Analytical Control Equipment Corporation Model 240 XA Elemental Analyzer (Chelmsford, MA, USA).

### Dose response

The toxicities of the HOCs were determined by measuring bacterial viability and bacterial mass concentration at various HOC concentrations as a function of time. Bacterial viability was measured as colony-forming units (CFU) per liter using the plate-count method (<http://www.icc.or.at/methods3.php>). Triplicate experiments were conducted. It was determined that the plate-count method has a 30% error. Bacterial mass concentration was measured using a spectrophotometer (Thermo Electron, Spectronic<sup>®</sup> Instruments 20 Genesys<sup>™</sup> Rochester, NY, USA) at a wavelength of 600 nm. The spectrophotometer measures turbidity and was calibrated to a baseline of dry bacterial weight to obtain bacterial mass concentration as a function of turbidity. According to the results of control ex-

periments, the spectrophotometer measurements have an error of less than 5% at bacterial concentrations of less than  $2.5 \times 10^{-4}$  kg/L and an error of 15% at bacterial concentrations of greater than  $2.5 \times 10^{-4}$  kg/L.

### Sorption

Sorption experiments were conducted for each of three HOCs (PCP, HCB, and DPCB) using a method adapted from HOC-sediment sorption experiments [5]. Each HOC was  $^{14}\text{C}$ -radiolabeled (Sigma-Aldrich Chemicals, St. Louis, MO, USA). The PCP was 99% pure, with a specific activity of 11.9 mCi/mmol. The HCB was 97% pure, with a specific activity of 15.9 mCi/mmol. The 4,4'-DPCB was 97% pure, with a specific activity of 3.3 mCi/mmol. The HOCs were introduced with a cosolvent, which was allowed to evaporate and left the HOCs as a solid coating on the stock bottles. The stock bottles were then filled with a saltwater medium, and the HOCs were allowed to dissolve in the medium for at least two weeks. The stock solution was then filtered to remove all remaining solid particles that had not dissolved in the two-week period. The dissolved HOC was then added to experiments directly from the stock solution. The HOCs sorbed to the bacteria and HOCs dissolved in the water were separated by vacuum filtration. Glass-fiber filters (pore size 1.0  $\mu\text{m}$ ; Pall Gelman, East Hills, NY, USA) were used with less than 7 psi vacuum. Measurements of the radioactivity on the filter and in the filtrate were quantified using a Beckman Coulter LS 6500 Multipurpose Scintillation Counter (Fullerton, CA, USA). These measurements were then used to calculate the concentrations of HOCs associated with the bacteria and of HOCs dissolved in the water as a function of time until a steady-state equilibrium was attained. All experiments were performed at a constant temperature (25°C) and salinity (~20 ppt) and at an initial pH 6.8. Mass balances were calculated for each and every experiment; every experiment with a mass balance less than 85% was discarded.

Experiments were performed with living as well as dead bacteria. Food-enriched (fed) bacteria were given acetone (5, 10, or 20  $\mu\text{l}$ , depending on the experiment, per 25 ml of solution) as a food source as well as all the necessary nutrients required for growth. Control experiments were conducted to ascertain the effect of acetone on sorption. It was determined that neither the acetone nor the medium acted as a cosolvent and that neither affected sorption. Bacteria that were not enriched with food (unfed) were deprived of a food source, although they were supplied with all the necessary inorganic nutrients required for survival. In experiments, both fed and unfed bacteria were exposed to the carbon that is a component of the HOC compound. All experiments with living bacteria were estimated to begin with the bacteria between the end of the exponential phase and the beginning of the stationary phase. Dead bacterial cells were created by heat-treating living cells via autoclaving at 121°C for 25 min. These cells remained whole and intact; however, some cell membrane disruption was later observed via microscopy at 1, 4, 8, and 24 h after autoclaving.

### Aggregation of bacteria

In experiments with PCP, sorption is quite rapid, so  $K_p$  is at its equilibrium value in all experiments after the first few minutes. For HCB, this is no longer true: Times for sorption may be quite slow relative to the doubling times for bacteria, and  $K_p$  generally will not be at its equilibrium value during

the experiments with living bacteria. As with sediments [7], sorption rates for bacteria depend on their state of aggregation. Therefore, the aggregation of bacteria was investigated and quantified.

All bacterial experiments were put on a rotary shaker and exposed to approximately 240 rpm. During bacterial growth, aggregation occurred. However, when bacterial growth was zero, aggregates disintegrated with time because of the continuous imposed shear of the shaker. Average sizes of bacterial aggregates were determined as a function of time by use of a Malvern Mastersizer Particle Sizer 2000X (Worcestershire, UK). In addition, sizes of aggregates for the fed (10  $\mu\text{l}$  of acetone) and unfed bacteria were measured and photographed using a microscope and a Canon PowerShot S400 Digital ELPH (Lake Success, NY, USA) camera at 0, 4, and 8 h as well as at 1, 2, 3, and 6 d. Aggregate sizes were quantified by calibrating the scale on the microscope lens to Polysciences Polybead® Polystyrene Microspheres (Warrington, PA, USA) of known sizes (24.9 and 45.6  $\mu\text{m}$ ) at magnifications of  $\times 10$  and  $\times 40$ .

Settling speeds and densities of aggregates were determined based on methods developed by Burban et al. [19]. Measurements of settling speeds were made in a carefully insulated, square tube approximately 1 m in length and 10 cm in width by measuring the time required for an aggregate to settle a specific distance. A total of 20 to 30 measurements were made in this way. From these measurements, an effective aggregate density can then be calculated from the Stokes law, which is

$$w_s = \frac{gd^2\Delta\rho}{18\mu} \quad (4)$$

where  $\Delta\rho = \rho_a - \rho_w$ ,  $\rho_a$  is the effective density of the aggregate,  $\rho_w$  is the density of water,  $\mu$  is the molecular viscosity of water,  $g$  is the acceleration caused by gravity,  $w_s$  is the settling speed, and  $d$  is the diameter of the aggregate.

## RESULTS AND DISCUSSION

### Pentachlorophenol

The toxic response of both unfed and fed bacteria to a range of PCP concentrations was investigated. For unfed bacteria, the initial concentration of viable bacteria was  $9 \times 10^7$  CFU/L ( $\pm 30\%$ ) and the initial bacterial mass was  $0.5 \times 10^{-4}$  kg/L ( $\pm 5\%$ ). Bacterial viability at 24 and 48 h as a function of total PCP concentration is shown in Figure 1. At low PCP concentrations ( $1 \times 10^{-9}$  to  $5 \times 10^{-8}$  kg/L), the bacteria did not grow significantly through 24 and 48 h. At PCP concentrations greater than approximately  $1 \times 10^{-7}$  kg/L, the bacteria died; the percentage of bacteria that died increased with increasing PCP concentration.

For fed bacteria (20  $\mu\text{l}$  of acetone), the initial concentration of viable bacteria was  $2 \times 10^8$  CFU/L, and the initial bacterial mass was  $0.5 \times 10^{-4}$  kg/L. Figure 2 shows the bacterial viability at 24 and 48 h for a range of total PCP concentrations. For low PCP concentrations ( $1 \times 10^{-9}$  to  $5 \times 10^{-8}$  kg/L), the bacteria grew through 24 and 48 h. For higher PCP concentrations ( $5 \times 10^{-8}$  to  $5 \times 10^{-7}$  kg/L), the bacteria showed inhibited growth; at doses greater than  $5 \times 10^{-7}$  kg/L, the bacteria were greatly inhibited in their growth or died. At these high concentrations, the percentage of bacteria that died increased with increasing PCP concentration. The unfed and fed bacteria therefore were similar in their response to PCP and began to die at approximately the same concentration.

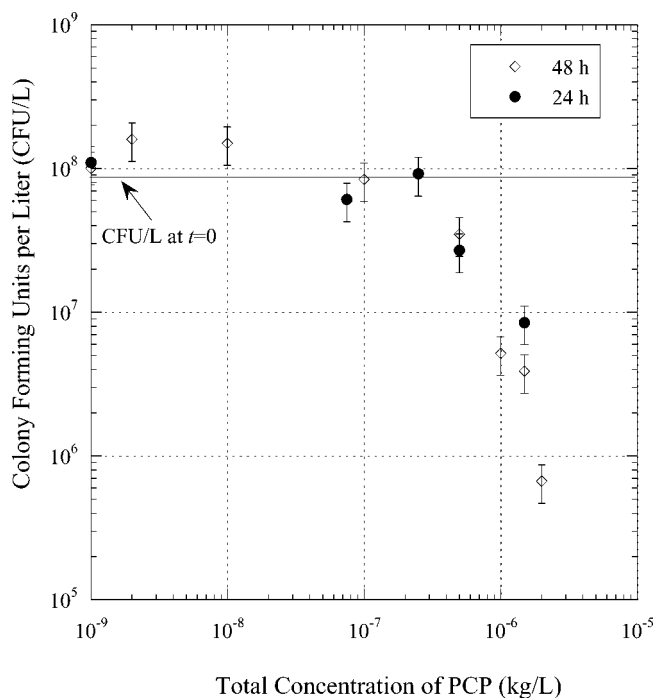


Fig. 1. Viability of unfed bacteria as a function of pentachlorophenol (PCP) concentration at  $t = 0, 24,$  and  $48$  h. CFU = colony-forming unit.

The PCP sorption experiments were conducted with dead, unfed, and fed ( $10 \mu\text{l}$  of acetone) bacteria; total PCP concentrations ranged from  $4 \times 10^{-9}$  to  $7 \times 10^{-6}$  kg/L. For each PCP concentration,  $K_p$  was determined as a function of time until well after the chemical reached steady-state equilibrium. In all cases, the experimental data indicated a rapid initial partitioning of PCP that reached a steady state before the minimal experimental measurement time of 2 min. After this,  $K_p$  was

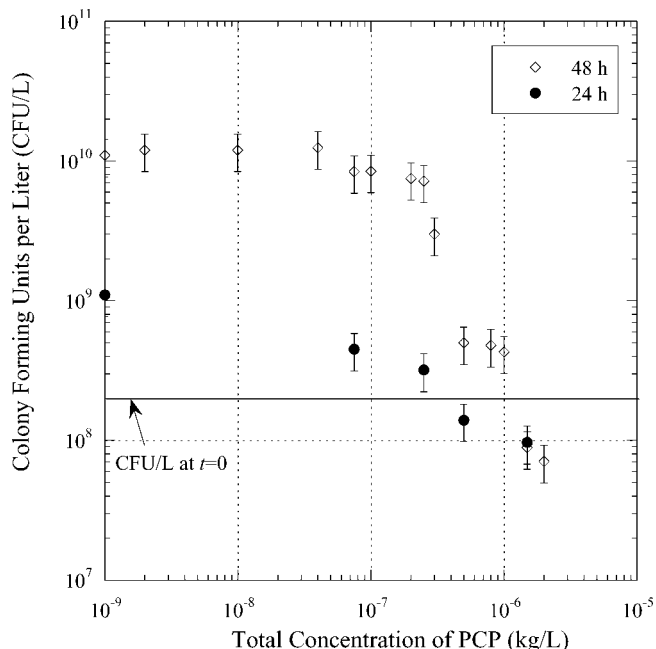


Fig. 2. Viability of fed ( $20 \mu\text{l}$  of acetone) bacteria as a function of pentachlorophenol (PCP) concentration at  $t = 0, 24,$  and  $48$  h. CFU = colony-forming unit.

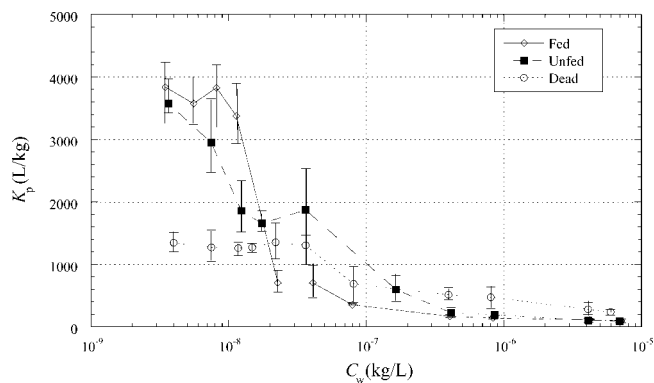


Fig. 3. Partition coefficient ( $K_p$ ) as a function of the mass of chemical dissolved in the water divided by the volume of water ( $C_w$ ) for dead, unfed, and fed bacteria.

independent of time but dependent on the PCP concentration. The  $K_p$  was measured at specific time intervals (2, 5, 10, and 30 min; 1, 4, and 8 h; and 1, 2, and 3 d). The  $K_p$  fluctuated because of experimental variations. However, no temporal trend was observed, and  $K_p$  was essentially independent of time.

For each of the three bacterial treatments (dead, unfed, and fed) and each PCP concentration, the  $K_p$  values were averaged; these are shown as a function of  $C_w$  in Figure 3. To analyze these results more carefully, it is convenient to introduce the quantities  $C_{oc}$  (kg/kg), which is the mass of an HOC sorbed to the bacteria divided by the mass of organic carbon in the bacteria (kg/kg), and  $K_{oc}$  (L/kg), which is a partition coefficient normalized to the organic carbon content of the bacteria and is defined as

$$K_{oc} = \frac{C_{oc}}{C_w} \quad (5)$$

In Figure 3, consider the results for dead bacteria first. For low PCP concentrations (i.e., for  $C_w$  from  $4 \times 10^{-9}$  to  $\sim 3.6 \times 10^{-8}$  kg/L),  $K_p$  was approximately constant at 1,300 L/kg. Because the organic carbon content of the bacteria is 50%, this gives a  $K_{oc}$  of 2,600 L/kg; this is the same value as the  $K_{oc}$  for sediments reported by Jepsen and Lick [4]. At high PCP concentrations (i.e., for  $C_w$  from  $3.6 \times 10^{-8}$  to  $\sim 6 \times 10^{-6}$  kg/L),  $K_p$  decreased with increasing PCP concentration. This decrease of  $K_p$  with increasing  $C_w$  is typical of the nonlinear sorption of organic chemicals to sediments observed by several investigators. For example, Jepsen and Lick [4] investigated the sorption of eight different HOCs to sediments for a range of HOC concentrations. For each HOC, those authors found that  $K_p$  was constant at low HOC concentrations but decreased as the HOC concentration increased. For sediments and all HOCs, this decrease began to occur when  $C_{oc}$  was approximately  $10^{-3}$  kg/kg.

Figure 4 shows  $C_B$  as a function of  $C_w$  for the three cases. For dead bacteria and low  $C_B$ ,  $C_B$  increases with  $C_w$  at a constant rate such that  $C_B = K_p C_w$  and  $K_p$  is constant. When  $C_B$  is approximately  $6 \times 10^{-5}$  kg/kg (i.e., when  $C_{oc}$  is  $\sim 1.2 \times 10^{-4}$  kg/kg), the rate of increase of  $C_B$  decreases as  $C_w$  increases (i.e.,  $K_p$  decreases as  $C_w$  increases). This value of  $1.2 \times 10^{-4}$  kg/kg for  $C_{oc}$  is somewhat smaller (by a factor of eight) than the  $1 \times 10^{-3}$  kg/kg observed by Jepsen and Lick [4] for sediments.

Karickhoff [6] also observed nonlinear sorption for HOCs in sediments. He attributed the nonlinearity to the sorbed pol-

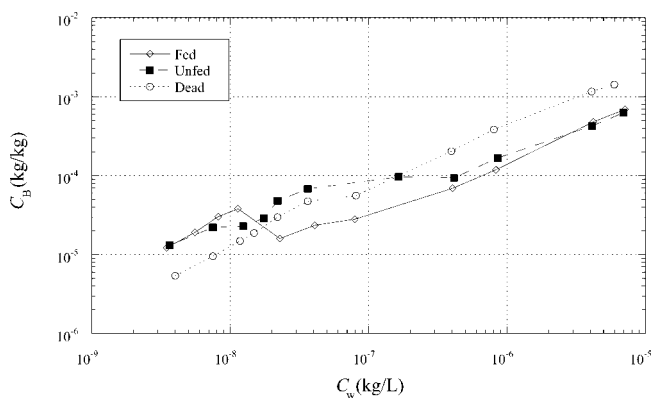


Fig. 4. Mass of chemical sorbed to the bacteria divided by the mass of bacteria ( $C_B$ ) as a function of the mass of chemical dissolved in the water divided by the volume of water ( $C_w$ ) for dead, unfed, and fed bacteria.

lutant being bound by highly site-specific mechanisms rather than to sorption to the total mass of the sorbent. In site-specific sorption, the amount of sites available for sorption is more limited and saturation occurs more rapidly. Therefore,  $C_B$  increases at a slower rate and  $K_p$  decreases. Site-specific sorption frequently involves weak-acid sites and can vary with the system's pH and pollutant concentration; therefore, it is likely to be of significance where ionic mechanisms are involved, such as with PCP. Because the bacteria in the present study consisted largely of whole, intact cells, site-specific sorption most likely occurred primarily to the hydrophobic portions of the cell membranes.

For unfed bacteria, Figure 3 shows that at low PCP concentrations,  $K_p$  was approximately 3,600 L/kg, a value significantly greater than that for dead bacteria (1,300 L/kg). The reason for this is uptake of PCP by living bacteria. At pH 6.8, a large proportion of PCP (>99%) is in the ionized form. In this form, the  $H^+$  is cleaved from the phenol group, leaving an  $O^-$  in place of the phenol group on the benzene ring. According to Crosby [20], this benzene ring can be actively taken up through the cell membrane of living bacteria.

At higher PCP concentrations,  $K_p$  for unfed bacteria first decreased rapidly with increasing  $C_w$ , then more slowly, and approached the  $K_p$  for dead bacteria. At these high PCP concentrations, toxic effects were present (Fig. 1), and at doses of PCP greater than approximately  $1 \times 10^{-8}$  kg/L, the number of viable unfed bacteria decreased with increasing concentrations of PCP (i.e., the number of bacteria capable of PCP uptake decreased). Consequently, the decrease of the  $K_p$  for unfed bacteria most likely was a combination of the effect of PCP toxicity and the effect of site-specific sorption.

Figure 3 also shows that for fed bacteria at low PCP concentration,  $K_p$  was approximately 3,800 L/kg, which is approximately threefold greater than the  $K_p$  for dead bacteria but equivalent to that for unfed bacteria. Again, this increased value of  $K_p$  compared to that of the dead bacteria resulted from the active uptake of PCP by the bacteria. For PCP concentrations greater than  $C_w$  of  $1 \times 10^{-8}$  kg/L,  $K_p$  decreased with increasing  $C_w$  and approached the  $K_p$  value for dead bacteria. As with the unfed bacteria, the decrease of  $K_p$  was a combination of the effect of PCP toxicity, which reduced the uptake of PCP by the fed bacteria, and the effect of site-specific sorption, which caused saturation.

At higher  $C_w$ , the  $K_p$  values of unfed and fed bacteria ap-

proached 10 L/kg, whereas the  $K_p$  value of dead bacteria was somewhat higher at 200 L/kg. At these high concentrations, the unfed and fed bacteria were mostly dead; nevertheless, they probably had a significantly greater percentage of whole, intact cells than the dead bacteria, which had been dead for a longer period of time and, therefore, had more time to lyse. In this case, the unfed and fed bacteria would be more involved in site-specific sorption to the cell membranes; this would decrease the  $K_p$  value relative to that of the dead bacteria.

#### Aggregation of bacteria and its effect on sorption

The mean bacterial aggregate sizes of the unfed and fed bacteria were measured. These are shown as a function of time in Figure 5. For unfed bacteria, aggregates were approximately 75  $\mu\text{m}$  in diameter at time zero but decreased rapidly to approximately 10  $\mu\text{m}$  in diameter by day 3. At day 10 and day 30, the diameter was approximately 2.5  $\mu\text{m}$ , approximately the size of individual bacteria ( $1 \times 3 \mu\text{m}$ ). This decrease in the size of aggregates with time resulted from the decreased cohesivity of the unfed bacteria as time increased and to the continuous turbulence caused by the motion of the shaker. For fed bacteria at time zero, the aggregate size was equal to that of the unfed bacteria at approximately 75  $\mu\text{m}$  in diameter; it then increased to approximately 100  $\mu\text{m}$  at day 1. At day 3, the aggregate size had decreased to approximately 40  $\mu\text{m}$ ; thereafter, it decreased gradually to approximately 15  $\mu\text{m}$  at day 5 and then approximately 3  $\mu\text{m}$  at day 60.

The time-dependent aggregation of bacteria also was investigated using microscopy at different times up to 6 d. For unfed bacteria at time zero, photographs indicated that the aggregates were approximately 25 to 75  $\mu\text{m}$  in diameter, consisted of loosely spaced bacterial cells, and had low density. As time increased, the aggregates gradually disintegrated to individual bacterial cells. At day 3, the aggregates were on the order of 10  $\mu\text{m}$  or less and were loosely bound. For fed bacteria, photographs of aggregates are shown in Figure 6. At time zero, the aggregates were similar to those for unfed bacteria. Through the first day, they increased in size. During this time, the aggregates were loosely bound and had low densities. The aggregate at day 1 shown in Figure 6 is larger than the usual bacterial aggregate (typically 100–150  $\mu\text{m}$ ) at this time; however, this photograph was selected because it can be seen that whereas the bacterial aggregation size increased, the density of the aggregates was still quite small. At days 2 and 3, the size of the aggregates was smaller (~25–75  $\mu\text{m}$  in diameter); however, they were visibly denser and more compact than those at earlier times. After 6 d of growth, the aggregates consisted primarily of small, tightly packed bacterial cells.

The effective densities of the fed bacterial aggregates were further investigated and quantified by measuring the settling speeds of the bacterial aggregates and then using the Stokes law (Eqn. 4) to determine the effective densities. Figure 7 shows the settling speed as a function of aggregate diameter at day 1, when bacterial growth was fast, and at day 6, when bacterial growth was negligible. For the aggregates at day 1, the average diameter was 110  $\mu\text{m}$  (standard deviation [SD], 44  $\mu\text{m}$ ), and the average settling speed was  $3.1 \times 10^{-3}$  cm/s (SD,  $1.4 \times 10^{-3}$  cm/s). For the aggregates at day 6, the diameter was 22  $\mu\text{m}$  (SD, 11  $\mu\text{m}$ ), and the settling speed was  $7.7 \times 10^{-3}$  cm/s (SD,  $1.9 \times 10^{-3}$  cm/s). From this, the effective density for the aggregates at day 1 was determined to be 1.0047 g/cm<sup>3</sup>, whereas the effective density for the aggregates at day 6 was 1.29 g/cm<sup>3</sup>.

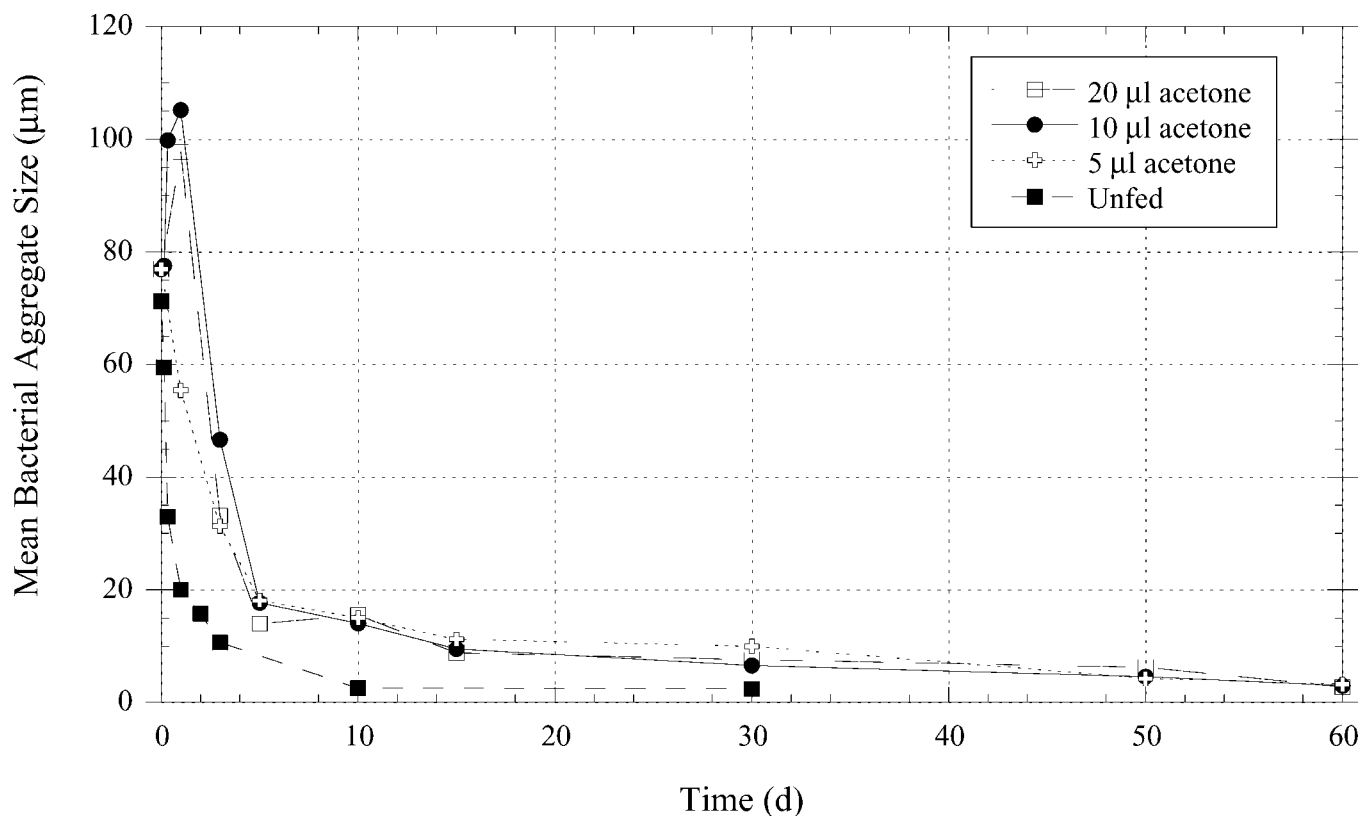


Fig. 5. Mean bacterial aggregate size for fed (5, 10, and 20 µl of acetone) and unfed bacteria as a function of time.

Experiments have demonstrated that the sorption of PCP to bacteria was relatively fast, on the order of a few minutes. This is consistent with the fact that the  $K_p$  for PCP is relatively low and, thus, that the effective diffusion coefficient (Eqn. 3) is relatively high and the time to equilibrium (Eqn. 2) therefore must be relatively low. In contrast, for the sorption of HCB to dead and nongrowing, live bacteria, experiments have demonstrated that the sorption time is on the order of a few hours, which is longer than the PCP sorption time because of the much higher  $K_p$  for HCB. For these bacteria, the sorption is to individual cells or small aggregates of cells (on the order of 10 µm or smaller).

For live, fed bacteria, the bacteria were growing aggregates that increased rapidly in size immediately after initiation of the sorption experiment and were approximately 110 µm in diameter after 1 d. Because the time to equilibrium is proportional to  $d^2$  (Eqn. 2), the sorption time during this period must be several orders of magnitude greater than it was at time zero. After the first day, the aggregate diameter decreased, but the density of the aggregate increased (i.e., the porosity decreased). The decrease in porosity decreased the diffusion coefficient (Eqn. 3) and increased the time to equilibrium. This more than compensated for the decrease in  $t$  caused by the decrease in  $d$ .

This can be demonstrated as follows. The density of the bacterial aggregate is

$$\rho_a = \phi \rho_w + (1 - \phi) \rho_b \quad (6)$$

where  $\rho_b$  is the density of the bacterial cell. It follows that

$$\Delta \rho = \rho_a - \rho_w = (1 - \phi)(\rho_b - 1) \quad (7)$$

where it is assumed that  $\rho_w = 1 \text{ g/cm}^3$ . From this equation as well as Equations 2, 3, and 4, it can be shown that

$$\frac{t_6}{t_1} = \frac{w_{s6} \phi_1}{w_{s1} \phi_6} \quad (8)$$

where  $t_6$  and  $t_1$  are the sorption times for the bacterial aggregates at days 6 and 1, respectively;  $w_{s6}$  and  $w_{s1}$  are the settling speeds at days 6 and 1, respectively; and  $\phi_1$  and  $\phi_6$  are the porosities of the particle or floc on days 1 and 6, respectively.

At day 6, the bacteria were closely packed, so the porosity must have been on the order of 0.1 to 0.3. For a porosity of 0.1, the bacterial cell density (from Eqn. 7) would be 1.32 g/cm<sup>3</sup>, which is in reasonable agreement with the measurements by Black [21] of 1.23 g/cm<sup>3</sup>. At day 1, an effective density of 1.0047 g/cm<sup>3</sup> indicates that the porosity is approximately 0.99. From this and the measured settling speeds, Equation 8 indicates that  $t_6/t_1$  is on the order of 7 to 25; this demonstrates that  $t_6$  is larger than  $t_1$ , and also several orders of magnitude larger than the sorption time for individual cells (~2 h). During rapid growth, the sorption time for the aggregates therefore must be on the order of 140 to 2,500 h, or 6 to 104 d.

#### Hexachlorobenzene

In both toxicity and sorption experiments, HCB and DPCB behaved in an almost identical manner. They are similar chemicals and had almost the same  $K_p$  value. Therefore, only the results for HCB will be described here.

For HCB, both unfed and fed bacteria were exposed to a range of chemical concentrations at an initial concentration of viable bacteria of  $1.7 \times 10^8$  CFU/L and an initial bacterial mass of  $0.55 \times 10^{-4}$  kg/L. The viability concentration and mass of unfed bacteria were measured at 0, 24, and 48 h. Both were independent of HCB concentration and time. The viability concentration and mass of fed bacteria (20 µl of acetone) also were measured at 0, 24, and 48 h. Both quantities in-

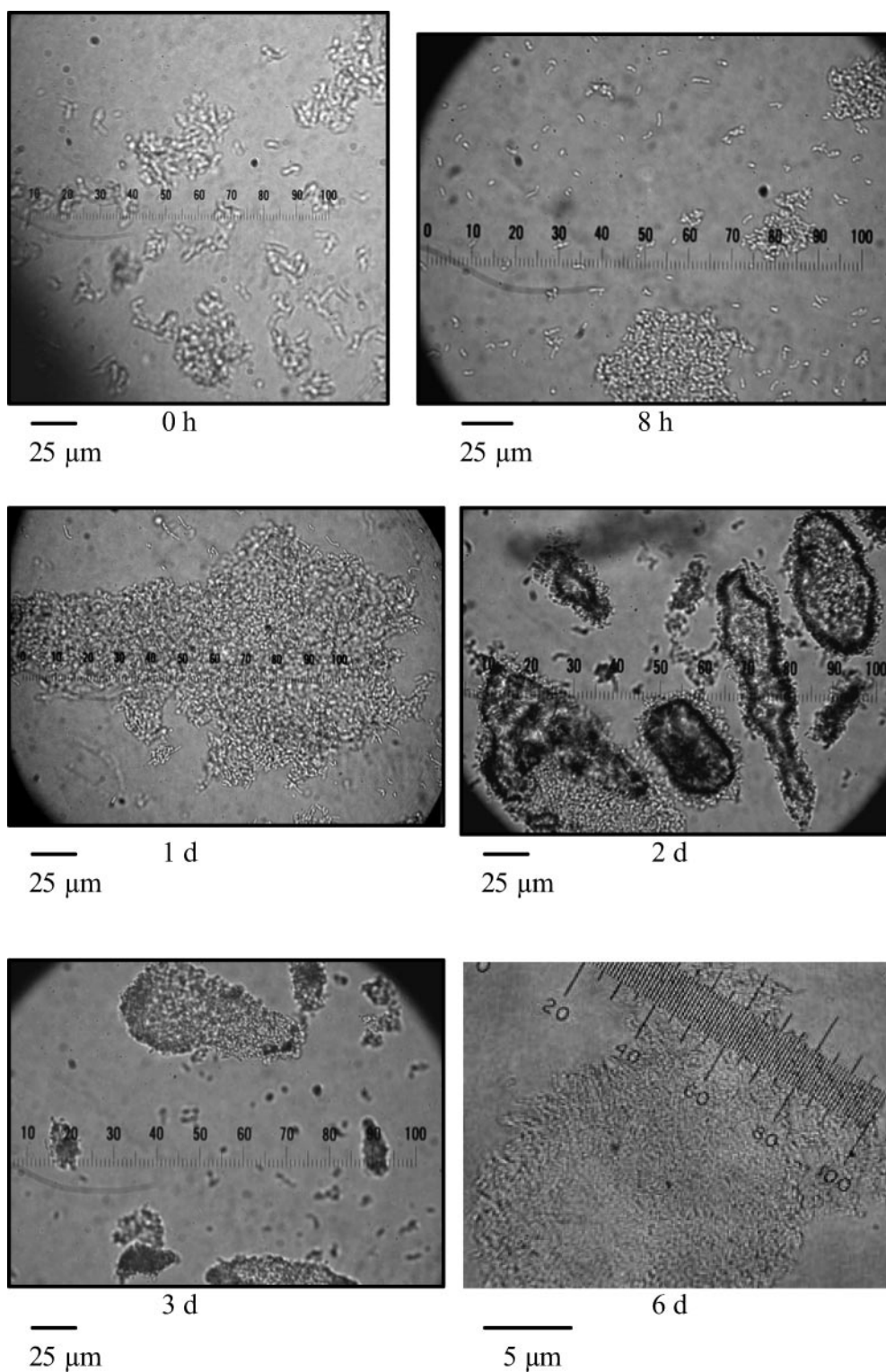


Fig. 6. Aggregation of fed ( $10 \mu\text{l}$  of acetone) bacteria as a function of time. Magnification  $\times 40$ .

creased with time, but they were not a function of HCB concentration. Thus, for both unfed and fed bacteria, the bacteria did not use HCB as a source of food, and HCB did not affect the number of viable bacteria.

Short-term (3-d) experiments with dead and unfed bacteria were conducted first. In both cases, the bacterial mass and  $K_p$  remained constant with time. For dead bacteria,  $K_p$  was approximately  $1.5 \times 10^5 \text{ L/kg}$ . Because dead bacteria are ap-

proximately 50% organic carbon,  $K_{oc}$  is approximately  $3 \times 10^5 \text{ L/kg}$ ; this is in reasonable agreement with the  $K_{oc}$  of sediments ( $4 \times 10^5 \text{ L/kg}$ ) measured by Jepsen and Lick [4]. The  $K_p$  for unfed bacteria was approximately  $1.0 \times 10^5 \text{ L/kg}$ , which is slightly less than that of the dead bacteria. Dead bacteria were produced by killing live bacteria using the autoclave; this can cause cell membrane disruption. Unfed bacteria were introduced into the experiments near the end of the exponential

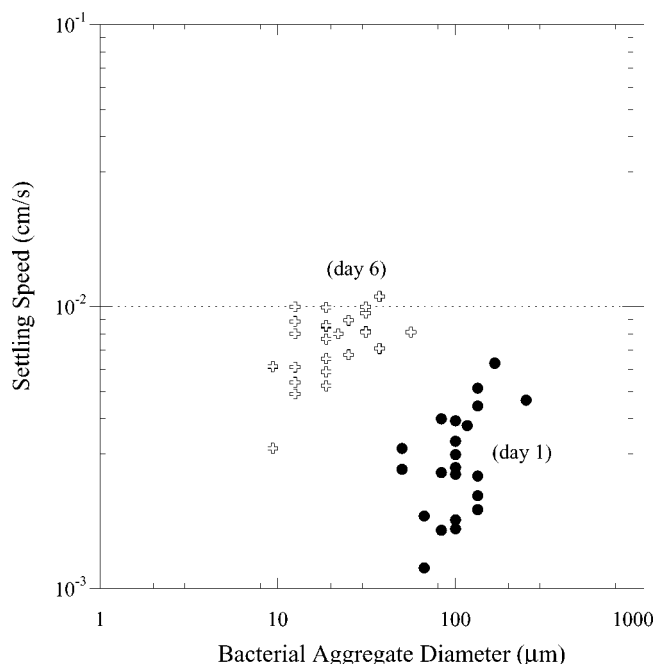


Fig. 7. Settling speed for fed bacteria as a function of bacterial aggregate diameter. Aggregates were measured after 1 and 6 d of growth.

growth phase, when a majority of the bacteria are whole and have intact cell membranes. Thus, sorption to unfed bacteria is to whole, intact cells, whereas sorption to dead bacteria is to cellular fragments as well as to whole cells. Intact cells (with intact membranes) probably restrict sorption. Because of this, sorption to unfed bacteria is less than that to dead bacteria (see [12–14] for a similar conclusion).

Bacterial mass and  $K_p$  for live bacteria were then measured as a function of time for longer periods (up to 60 d), with the amount of acetone added (0, 5, 10, and 20  $\mu\text{l}$ ) to the solution as a parameter. The mass concentrations as a function of time are shown in Figure 8. For the unfed bacteria, the concentration remained constant with time. For the fed bacteria, first consider the period from time zero to 10 d. For all fed bacteria in this time period, the concentrations increased rapidly at first and then more slowly, until they became constant. Maximum values were  $1.5$ ,  $2.1$ , and  $3.7 \times 10^{-4}$  kg/L for the experiments with 5, 10, and 20  $\mu\text{l}$ , respectively, of added acetone. This corresponds to an increase of approximately  $0.8 \times 10^{-4}$  kg/L for each 5  $\mu\text{l}$  of added acetone. For a short time, sufficient food (acetone) was available for all fed bacteria, and they all grew at essentially the same rate. However, as they exhausted their food supply, the growth rate decreased and then became zero, with the amount of growth being proportional to the amount of food available. After 10 d, the bacterial mass concentration with 10  $\mu\text{l}$  of acetone remained constant with time. Although measurements of bacterial mass with 5 and 20  $\mu\text{l}$  of acetone were not made after 10 d, it can be assumed that these would remain constant with time as well.

The partition coefficients for unfed and fed bacteria are shown in Figure 9. For unfed bacteria,  $K_p$  remained constant with time. For fed bacteria, consider the first 10 d. In this time period,  $K_p$  first decreased rapidly, then decreased more slowly, and then remained approximately constant for the rest of the 10-d period. As a first approximation, this behavior can be explained as follows: Assume that the bacterial growth rate is sufficiently high and the sorption rate sufficiently low that a negligible amount of HCB is sorbed to the growing bacteria. The measured  $K_p$  would then be a mass-weighted average of that for the bacteria initially present (a  $K_p$  of  $1.0 \times 10^5$  L/kg)

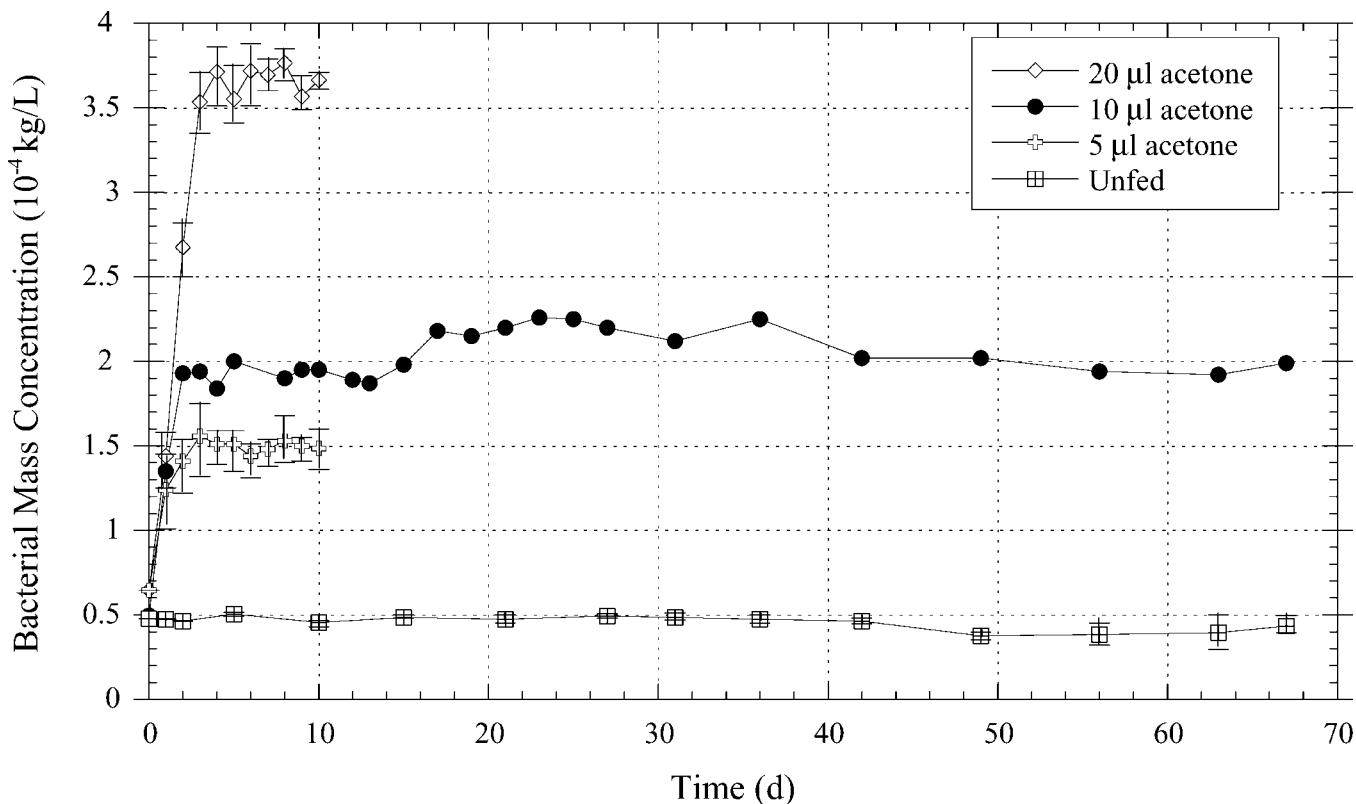


Fig. 8. Bacterial mass as a function of time for unfed and fed (5, 10, and 20  $\mu\text{l}$  of acetone) bacteria.

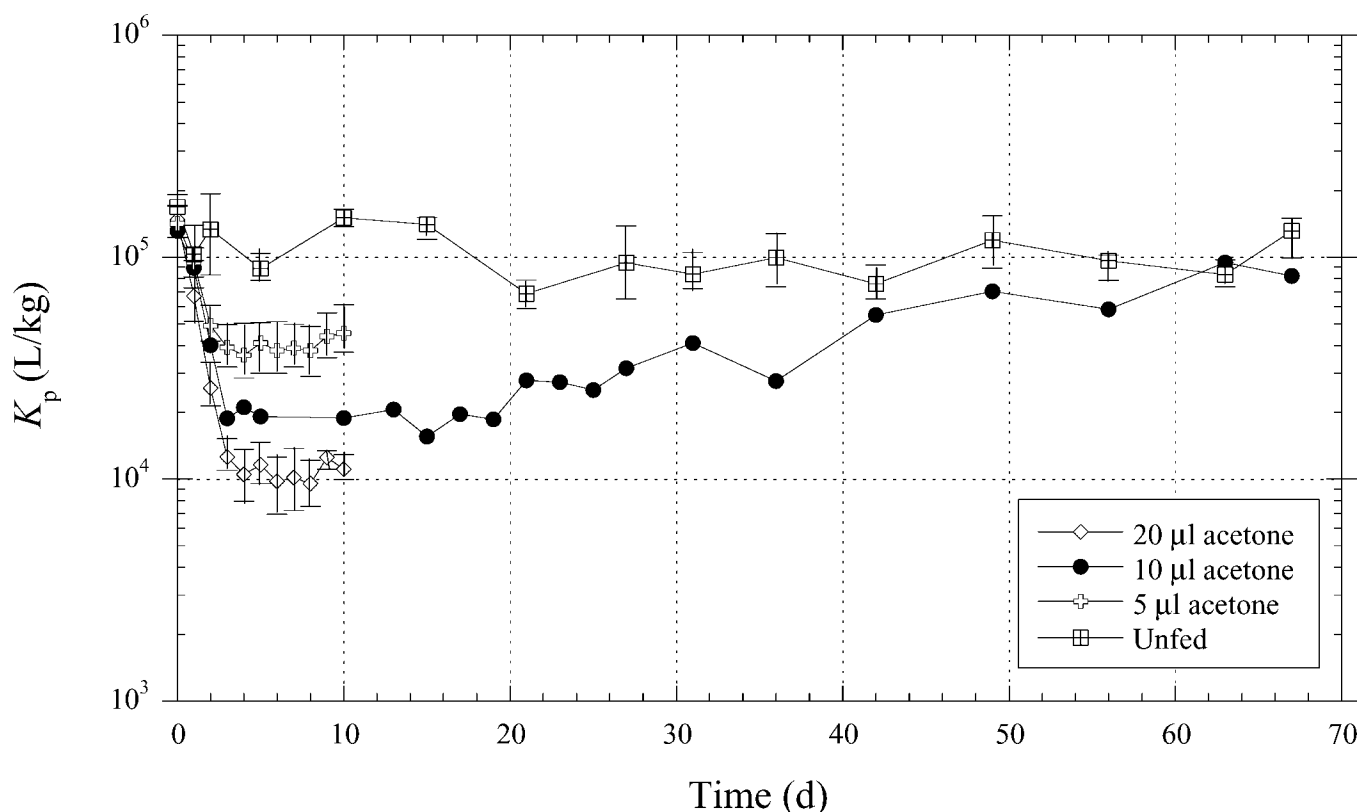


Fig. 9. Partition coefficient ( $K_p$ ) as a function of time for unfed and fed (5, 10, and 20  $\mu\text{l}$  of acetone) bacteria.

and the new bacteria (a  $K_p$  of zero). On this basis, the  $K_p$  values (multiplied by  $10^{-5}$ ) at 10 d should be 0.33 (0.5/1.5), 0.24 (0.5/2.1), and 0.135 (0.5/3.7) for the bacteria with 5, 10, and 20  $\mu\text{l}$  of acetone. The observed values are approximately 0.38, 0.20, and 0.11, which are all within 20% of the theoretical values.

As shown above, for growing bacteria during the first day, aggregates were quite large and, hence, sorbed relatively slowly. After this initial period, the aggregates decreased in size but became more dense, and their sorption rate decreased even further. Therefore, as a first approximation for the first 10 d, the sorption rate was relatively low, the growth rate was relatively high, and little HCB was sorbed to the growing bacteria.

For bacteria with 10  $\mu\text{l}$  of acetone and after 10 d, Figure 9 shows that  $K_p$  slowly increased until it reached its initial (and equilibrium) value of  $1 \times 10^5$  L/kg. During this time period, aggregates were dense but decreasing in size. The sorption rate was low but probably increasing with time, whereas bacterial growth was zero. All bacteria eventually sorbed until they equilibrated with their surroundings.

### CONCLUSION

The toxicity and time-dependent sorption of three HOCs to living (both growing and nongrowing) and dead *R. rhodochrous* were investigated. The three HOCs were PCP (an ionizing chemical with a moderate  $K_p$ ) as well as HCB and DPCB (two nonionizing chemicals with  $K_p$  values that are similar to each other but much larger than that for PCP).

For PCP at low concentrations, neither bacterial growth nor death was affected by the PCP. At higher concentrations, some of the bacteria died, and the percentage of bacteria that died increased with increasing PCP concentrations. In sorption studies at low PCP concentrations and at chemical equilibrium,

$K_p$  for living bacteria was almost threefold greater than the  $K_p$  for dead bacteria. The reason for this is that live bacteria can actively take up ionized PCP, whereas dead bacteria cannot. At higher PCP concentrations, the  $K_p$  values for both the dead and the living bacteria decreased rapidly and then more slowly as the PCP concentration increased. This is attributed to the death of the living bacteria because of PCP toxicity and saturation of site-specific sorption for all bacteria.

Hexachlorobenzene did not affect the growth or death of the bacteria at all the HCB concentrations investigated. In time-dependent HCB sorption experiments with dead and nongrowing bacteria,  $K_p$  remained constant with time; with growing bacteria,  $K_p$  decreased with time during the first 4 d, stayed almost constant for approximately the next 10 to 15 d, and then gradually increased to its initial value over a period of approximately 40 d. The maximum decrease in  $K_p$  depended on the amount of food provided. The initial decrease primarily resulted from the rate of bacterial growth being high enough, and the rate of HCB sorption being low enough, that chemical equilibrium between the bacteria and the surroundings could not be maintained. After the bacteria stopped growing, sorption was slow but continued until chemical equilibrium was reached. Sorption rates were heavily dependent on the sizes and densities of the bacterial aggregates; these changed with time. Results for DPCB, which had a  $K_p$  similar to that of HCB, were similar to those for HCB.

Bacteria are relatively simple organisms. Nevertheless, the same processes that affect sorption to bacteria (growth rates relative to sorption rates, aggregation, transfer of chemicals through aggregates of cells, ionizing vs nonionizing compounds, and nonlinear sorption) also should be significant in more complex organisms.

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