The Sorption of Hydrophobic Organic Chemicals to Bacteria

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Sorption of Hydrophobic Organic Chemicals to Coastal Sediment Bacteria and Biofilms

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 non-growing) bacteria as well as to dead bacteria. For PCP (an ionizing chemical), bacterial growth decreased and death increased as the PCP concentration increased. In sorption experiments, the partition coefficient was affected by (a) active uptake of PCP by living bacteria but not by dead bacteria, (b) death of the living bacteria due to PCP toxicity, and (c) saturation of site specific sorption as the PCP concentration increased. HCB (a non-ionizing chemical) did not affect the growth or death of the bacteria at all HCB concentrations investigated. In sorption experiments, 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 non-ionizing chemical with equilibrium partition coefficient similar to that of HCB) were similar to those for HCB.

INTRODUCTION

Bacteria are a major component of the aquatic environment and serve not only for 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. It is therefore 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 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 non-growing) bacteria as well as to dead bacteria. In the sorption experiments, results are presented in terms of the partition coefficient, K_p, defined as

$$K_p = \frac{C_B}{C_w} \tag{1}$$

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 in some countries (e.g., Sweden, Germany, Finland) or has been totally discontinued in others (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, molluscicide, defoliant, herbicide, and wood preservative. Even in those countries where 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 dichlorobiphenyl are non-ionizing 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, biolocal, and chemical degradation. HCB has been used as a pesticide and industrial chemical since 1933 when it was introduced as a commercial fungicide for wheat and as an industrial raw material for synthetic rubber. Most of the toxic effects of HCB appear to be correlated with disruption of the endocrine system [3]. DPCB has been used as a heat-transfer and insulating fluid in cooling systems and electrical equipment, in sealants, rubber, paints, plastics, printing ink, and pesticides. Studies strongly suggest that PCBs are probable human carcinogens [4]. Production of all PCBs has been banned in several industrialized countries; the United States stopped producing PCBs in 1977.

EXPERIMENTAL METHODS

Bacteria

The bacteria used for these experiments were *Rhodococcus 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. They are prevalent in coastal marine and aquatic sediments. *R. rhodochrous* are generally 1 × 3 μm rod-shaped bacteria that have a tendency to form aggregates as they multiply but may also fragment into cocci. They are hydrocarbon degraders that have been shown to tolerate high concentrations of some HOCs [7].

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 per liter (CFU/L) using the plate count method [8].

Sorption

Sorption experiments were conducted for each of three HOCs (PCP, HCB, and DPCB) using a method adapted from HOC-sediment sorption experiments [7]. Experiments were performed with living as well as dead bacteria. Food-enriched (fed) bacteria were given acetone (5, 10, or 20 μL depending on the experiment) as a food source as well as all the necessary nutrients required for growth. Non-food-enriched (non-fed) bacteria were deprived of a food source although they were supplied with all the necessary inorganic nutrients required for survival.

Aggregation of bacteria

In experiments with PCP, it will be seen that sorption is quite rapid so that 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 doubling times for bacteria and K_p generally will not be at its equilibrium value during the experiments with living bacteria. As with sediments, sorption rates for bacteria depend on their state of aggregation. Because of this, 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. When bacterial growth was zero, aggregates disintegrated with time due to 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. In addition, aggregation for the fed (10 μL acetone) and non-fed bacteria was measured and photographed using a microscope and a Canon PowerShot S400 Digital ELPH camera at times of 0, 4 hour, 8 hour, 1 day, 2 day, 3 day, and 6 day. Aggregate sizes were quantified by calibrating the scale on the microscope lens to Polybead® Polystyrene Microspheres of known sizes (24.9 μm and 45.6 μm) at magnifications of 10x and 40x. In addition, settling speeds and densities of aggregates were determined based on methods developed by Burban et al. [9].

RESULTS AND DISCUSSION

For the purpose of remaining concise and to address a more general audience, some of the more detailed results have been omitted from discussion. Only a brief description of the major findings is reported here.

Pentachlorophenol

The toxic response of both non-fed and fed bacteria to a range of PCP concentrations was investigated. For low PCP concentrations, non-fed bacteria remained unaffected and the fed bacteria (20 μL of acetone) grew through 24 and 48 hours. For both non-FED and FED bacteria at PCP concentrations greater than 5×10^{-8} kg/L, the bacteria were greatly inhibited in their growth or died from 24 to 48 hours. At these high concentrations, the percentage of bacteria that died increased with increasing PCP concentration. The non-fed and fed bacteria were therefore similar in their response to PCP and began to die at approximately the same concentration.

PCP sorption experiments were conducted with dead, non-fed, and fed (10 μL acetone) bacteria; total PCP concentrations ranged from 4×10^{-9} to 7×10^{-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 minutes. After this, K_p was independent of time but did depend on the PCP concentration.

For each of the three bacterial treatments (dead, non-fed, and fed) and for each PCP concentration, the K_ps were averaged over time; these are shown as a function of C_w in Figure 1. Consider the results for dead bacteria first. For low PCP concentrations, K_p is approximately constant at 1300 L/kg. At high PCP concentrations, K_p decreases 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 [5] investigated the sorption of eight different HOCs to

sediments for a range of HOC concentrations. For each HOC, they found that K_p was constant at low HOC concentrations but decreased as the HOC concentration increased.

Karickhoff [6] also observed nonlinear sorption for HOCs in sediments. He attributed the nonlinearity to the sorbed pollutant being bound by highly site-specific mechanisms rather than 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; C_B then 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; it is therefore likely to be of significance where ionic mechanisms are involved, such as is the case with PCP. In the present experiments, because the bacteria in these experiments consist largely of whole, intact cells, site-specific sorption most likely occurs primarily to the hydrophobic portions of the cell membranes.

For non-fed bacteria, Figure 1 shows that, at low PCP concentrations, K_p is reasonably constant at approximately 3600 L/kg, a value significantly greater than that for dead bacteria (1300 L/kg). The reason for this is uptake of PCP by living bacteria. At a pH of 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 [10], this benzene ring can be actively taken up through the cell membrane of living bacteria.

At higher PCP concentrations, K_p for non-fed bacteria first decreases rapidly with increasing C_w, then more slowly, and approaches the K_p for dead bacteria. At these high PCP concentrations, toxic effects are present and, at doses of PCP greater than about 1×10^{-8} kg/L, the number of viable non-fed bacteria decreases with increasing concentrations

of PCP, i.e., the number of bacteria capable of PCP uptake decreases. Consequently, the decrease of the K_p for non-fed bacteria is most likely a combination of the effect of PCP toxicity and the effect of site-specific sorption.

Figure 1 also shows that, for fed bacteria at low PCP concentration, K_p is approximately 3800 L/kg. This is approximately 3 times greater than the K_p value for dead bacteria but equivalent to that for non-fed bacteria. Again, this increased value of K_p compared to that of the dead bacteria is a result of the bacteria's active uptake of PCP. For PCP concentrations greater than a C_w of 1×10^{-8} kg/L, K_p decreases with increasing C_w and approaches K_p for dead bacteria. As with the non-fed bacteria, the decrease of K_p is a combination of the effect of PCP toxicity which reduces the uptake of PCP by the fed bacteria and the effect of site-specific sorption which causes saturation.

At higher C_w, the K_ps of non-fed and fed bacteria approached 10 L/kg while the K_p for dead bacteria was somewhat higher at 200 L/kg. At these high concentrations, the non-fed and fed bacteria are mostly dead; nevertheless, they probably have a significantly greater percentage of whole, intact cells than do the dead bacteria (who have been dead for a longer period of time and therefore have more time to lyse). In this case, the non-fed and fed bacteria would be more involved in site-specific sorption to the cell membranes; this would decrease the K_p relative to the dead bacteria.

Aggregation of bacteria and its effect on sorption

The mean bacterial aggregate sizes of the non-fed and fed bacteria were measured using a particle sizer as a function of time. For non-fed bacteria, aggregates are approximately 75 μm in diameter at time zero but decrease rapidly to approximately 10 μm in diameter by day 3. At day 10 and day 30, the diameter decreased to about 2.5 μm,

approximately the size of individual bacteria (1 × 3 μm). This decrease in size of the aggregates with time is due to the decreased cohesivity of the non-fed bacteria as time increases and to the continuous turbulence caused by the motion of the shaker. For fed bacteria at zero time, the aggregate size is equal to that of the non-fed bacteria at approximately 75 μm in diameter; it then increases to approximately 100 μm after 1 day. At day 3, the aggregate size has decreased to approximately 40 μm; thereafter, it decreases gradually to approximately 15 μm at day 5 and then to approximately 3 μm at day 60.

The time-dependent aggregation of bacteria was also investigated using microscopy at different times up to 6 days. For non-fed bacteria at time zero, photographs indicated that the aggregates were approximately 25 to 75 μm in diameter, consisted of loosely spaced bacterial cells, and had low density. As time increased, they gradually disintegrated to individual bacterial cells. At 3 days, the aggregates were on the order of 10 μm or less and were loosely bound. For fed bacteria, photographs of aggregates are shown in Figure 2. At time zero, the aggregates were similar to those for non-fed bacteria. Through the first day, they increased in size. During this time, the aggregates were loosely bound and had low densities. The aggregate shown at day 1 is larger than the usual bacterial aggregate (typically 100 to 150 μm) at this time; however, this photograph was selected because it can be seen that, while the bacterial aggregation size increases, the density of the aggregates is still quite small. At days 2 and 3, the size of the aggregates is smaller (approximately 25 to 75 μm in diameter); however, they are visibly denser and more compact than those at earlier times. After 6 days of growth, the aggregates consist 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 Stokes law to determine the effective densities. From this, the effective density for the aggregates at day 1 was determined to be 1.0047 g/cm^3 while the effective density for the aggregates at day 6 was 1.29 g/cm^3.

In the analyses of the experiments on 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 to be given by [5, 9]

$$t = \frac{d^2}{24D} \tag{2}$$

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

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

where D_m is the molecular diffusion coefficient, f is a correction for toruosity, ϕ is the porosity of the particle or floc, and ρ_p is the density of the solid particle.

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 therefore the effective diffusion coefficient (Eqn. (3)) is relatively high and the time to equilibrium (Eqn. (2)) must therefore be relatively low. For the sorption of HCB to dead and non-growing live bacteria, experiments have demonstrated that the sorption time is on the order of a few hours, longer than PCP due to 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 less).

For live, fed bacteria, the bacteria are growing aggregates which increase rapidly in size immediately after the initiation of the sorption experiment and are approximately 110 μm in diameter after one day. Since 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 decreases but the density of the aggregate increases, i.e., the porosity decreases. The decrease in porosity decreases the diffusion coefficient (Eqn. (3)) and increases the time to equilibrium. This more than compensates for the decrease in t due to the decrease in 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. Only results for HCB will be described here.

For HCB, both non-fed and fed bacteria were exposed to a range of chemical concentrations and were independent of HCB concentration and time. The bacteria did not utilize HCB as a food source and HCB did not toxically affect the number of viable bacteria.

Short-term (3 day) experiments with dead and non-fed bacteria were conducted first. In both cases, the bacterial mass concentration and K_p remained constant with time. For dead bacteria, K_p was approximately 1.5×10^5 L/kg. The K_p for non-fed bacteria was approximately 1.0×10^5 L/kg, 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. Non-fed bacteria were introduced into the experiments near the end of the exponential growth phase when a majority of the bacteria are whole with cell membranes intact. Thus, sorption to non-fed bacteria is to whole, intact cells while 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 non-fed bacteria is less than that to dead bacteria (see [14, 15, 16] for a similar conclusion).

Bacterial mass and K_p for live bacteria were then measured as a function of time for longer time periods (up to 60 days) with the amount of acetone (0, 5, 10, and 20 μL) added to the solution as a parameter. The mass concentrations as a function of time are shown in Figure 3. For non-fed bacteria, the concentration remained constant with time. For the fed bacteria, consider the period from zero time to 10 days first. For all fed bacteria, the concentrations increased rapidly at first and then more slowly until they became constant. Maximum values were 1.5, 2.1, and 3.7×10^4 kg/L for the experiments with 5, 10, and 20 μL added acetone, respectively. This corresponds to an increase of approximately 0.8×10^8 kg/L for each 5 μL of added acetone. For short time, sufficient food (acetone) was available for all fed bacteria and they all grew essentially at the same rate. However, as they exhausted their food supply, the growth rate decreased and then became zero with the amount of growth proportional to the amount of food available. After 10 days, the bacterial mass concentration with 10 μL acetone remained constant with time. Although measurements of bacterial mass with 5 and 20 μL of acetone were not made after 10 days, it can be assumed that these would also remain constant with time.

The partition coefficients for non-fed and fed bacteria are shown in Figure 4. For non-fed bacteria, K_p remained constant with time. For fed bacteria, consider the first 10 days. In this time period, K_p decreased rapidly at first, then more slowly, and then remained approximately constant for the rest of the 10 day 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 is 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 1.0×10^5 L/kg) and the new bacteria (a K_p of zero). On this basis, the K_ps (multiplied by 10^{-5}) at 10 days 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 μL acetone. The observed values are approximately 0.38, 0.20, and 0.11, values very close to the theoretical values.

As shown above, for growing bacteria during the first day, aggregates are quite large and hence sorb relatively slowly. After this initial period, the aggregates decrease in size but become more dense; their sorption rate decreases even further. Therefore, as a first approximation for the first 10 days, the sorption rate is relatively low while the growth rate is relatively high, and little HCB is sorbed to the growing bacteria.

For bacteria with 10 μL acetone and after 10 days, Figure 4 shows that K_p slowly increases until it reaches its initial (and equilibrium) value of 1×10^5 L/kg. During this time period, (a) aggregates are dense but are decreasing in size, (b) the sorption rate is low but is probably increasing with time, while (c) bacterial growth is zero. All bacteria eventually sorb until they equilibrate with their surroundings.

SUMMARY AND CONCLUDING REMARKS

The toxicity and time-dependent sorption of three HOCs to living (both growing and non-growing) and dead *Rhodococcus rhodochrous* were investigated. The three HOCs were PCP (an ionizing chemical with a moderate K_p), HCB, and DPCB (two non-ionizing chemicals whose K_ps were similar to each other but much larger than the K_p 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 three times greater than the K_p for dead bacteria. The reason for this is that live bacteria can actively take up ionized PCP while dead bacteria can not. At higher PCP concentrations, the K_ps for both the dead and living bacteria decreased rapidly and then more slowly as the PCP concentration increased. This is attributed to (a) death of the living bacteria due to PCP toxicity and (b) saturation of site specific sorption for all bacteria.

HCB did not affect the growth or death of the bacteria at all HCB concentrations investigated. In time-dependent HCB sorption experiments with dead and non-growing bacteria, K_p remained constant with time; with growing bacteria, K_p decreased with time during the first four days, stayed almost constant for about the next 10 to 15 days, and then gradually increased to its initial value over a period of about 40 days. The maximum decrease in K_p depended on the amount of food provided. The initial decrease was primarily due to the rate of bacterial growth being high enough and the HCB

sorption rate 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 similar K_p 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, the transfer of chemicals through aggregates of cells, ionizing versus non-ionizing compounds, and nonlinear sorption) should also be significant in more complex organisms.

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PUBLICATIONS

Publications that have resulted from this research are still pending. One paper has been submitted for review to *Environmental Toxicology & Chemistry* and is in the process of revision by the authors. This paper is:

Lunsman TD and Lick W. 2004. The sorption of hydrophobic organic chemicals to bacteria. *Environmental Toxicology & Chemistry*.

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FIGURE LEGENDS

Figure 1. K_p as a Function of C_w for Dead, Non-Fed, and Fed Bacteria.

Figure 2. Aggregation of Fed (10 μL acetone) Bacteria as a Function of Time. Magnification = 40x.

Figure 3. Bacterial Mass Concentration as a Function of Time for Non-Fed and Fed Bacteria (5, 10, and 20 μL acetone).

Figure 4. K_p as a Function of Time for Non-Fed and Fed Bacteria (5, 10, and 20 μL of acetone).