Biomagnification of \( p,p' \)-DDT and Methoxychlor by Bacteria

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Aerobacter aerogenes and Bacillus subtilis accumulated \( p,p' \)-DDT and methoxychlor directly from water. Uptake of both \(^1^4\)C-labeled organochlorine insecticides was rapid; 80 to 90% of the 24-h residues were reached within 30 min. Total cellular residues varied linearly with concentrations of DDT and methoxychlor in water ranging from 0.5 to 5.0 \( \mu \)g/liter. The residue magnification factors from water were between 1,400- to 4,300-fold, but were independent of insecticide concentrations in water. When the insecticide-exposed microbial cells were washed with pesticide-free water, DDT residues were 45% in \( A. \) aerogenes and 30% in \( B. \) subtilis, whereas the methoxychlor level decreased nearly 75% in both organisms. Subsequent washing did not further reduce the insecticide residue. Autoclave-killed bacteria also rapidly adsorbed DDT and methoxychlor from water and, in some instances, residues were higher than in the living cells. Molecular polarity and lipid solubility appear to influence the retention of the organochlorine insecticides by bacterial cells.

High residues of certain organochlorine contaminants have been found frequently in fish and birds (7, 16). Some investigators have hypothesized that the food-chain "magnification" of these chemicals directly act as an important causative factor in producing such residue levels in higher vertebrates (8, 12, 13, 19). The food-chain magnification hypothesis is predicated on the assumption that chemical pollutants enter the food chain and are accumulated and magnified as they pass through succeeding trophic levels, resulting in high chemical residues in top level consumers. Conceivably, this could mean that a chemical just within the limits of detection in water, essentially innocuous, may rapidly reach potentially lethal or detrimental concentrations as the result of biomagnification.

Microorganisms are undoubtedly an important nutrient source for a broad spectrum of aquatic micro- and macroscopic filter-feeding organisms (9). If such microorganisms can accumulate pollutants of the aquatic environment and concentrate them thousands of times above ambient water levels, that is, act as biomagnifiers, the microorganisms-filter-feeder link in the food chain of fish and higher vertebrates could be an important avenue for massive movement of these pollutants from one trophic level to another. In this manuscript we define "biomagnification" as the active or passive accumulation by a biological system of an aquatic chemical contaminant above ambient levels. Although reports of pesticide accumulation by microorganisms have been published (4, 11, 17), no evidence has been presented that quantifies the biomagnification process necessary in predicting the environmental impact of an aquatic chemical. Our goal is to develop a series of simple food chains, with fish as the top consumers, that may act as a model to predict the potential biomagnification of an aquatic pollutant via a food chain. This study with microorganisms represents a first step in developing such a model system, one that can quantitatively measure the movement of chemicals from or within a specific trophic level(s).

Specifically, this study reports the potential biomagnification of two organochlorine insecticides, \( p,p' \)-DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane] and methoxychlor [1,1,1-trichloro-2,2-bis(p-methoxyphenyl)ethane]. Both organochlorine insecticides represent a class of compounds that are used widely; they are persistent, lipid chlorinated (14), and each occurs as an aquatic pollutant which is known
or suspected to undergo biomagnification in various organisms (10). Methoxychlor was of particular interest in this study because it is a potential substitute for DDT; though chemically similar, it is less toxic to fish, birds, and mammals than DDT (15).

MATERIALS AND METHODS

Organisms and cultural conditions. The strains of *Aerobacter aerogenes* and *Bacillus subtilis* used in this investigation were obtained from collections maintained at the Fish-Pesticide Research Laboratory, Columbia, Missouri. Although the bacteria were routinely isolated from freshwater ponds in central Missouri, they should not be interpreted as the dominant microflora in a freshwater ecosystem. We used these bacteria as gram-negative and gram-positive type organisms. However, the relatively clear taxonomic position of the organisms, their availability, and cosmopolitan nature were all considerations in their selection for the microbial segment of the model food chain. During the current experiment, they were routinely grown from the freeze-dried state in 250 ml-batches of a commercial formulation of brain-heart infusion broth (BBL, Baltimore, Maryland) in a shake culture at 25 C.

Labeled insecticides and assay. Both *4C ring labeled p,p'-DDT* (19.1 mCi/mmol, 0.05 mCi) and methoxychlor (7.64 mCi/mmol, 0.1 mCi) were purchased from Nuclear-Chicago Corp. Both compounds were dissolved in acetone. Standard exposure concentration to the bacteria was 1 part per billion (1 \( \mu \)g/liter *4C*-labeled DDT or methoxychlor, unless otherwise indicated. Gas-liquid chromatography and thin-layer chromatography-autoradiography analyses indicated both *4C-p,p'-DDT* and *4C-methoxychlor* were 99+\% pure.

Radioisometric methods were used to determine total residues (both adsorption and absorption by bacterial cells of parent compound and degradation products, if present) of *4C-labeled p,p'-DDT* and methoxychlor. The bacteria were exposed to a *4C-labeled insecticide* in 50 ml of sterile distilled water at pH 7.0 ± 0.5 for various times and centrifuged, and the pellets were resuspended in 15 ml of Triton-X-Fluorarolloy mixture (10). The suspensions were quantitatively transferred to glass scintillation vials and counted in a Beckman 200-L liquid scintillation counter with a variable discriminator set for *4C* \( \beta \)-particle energy spectrum. Counting efficiency was 90%. All samples were taken in triplicate, and counts represent mean value ± standard error. Acetone was employed as a carrier for the two insecticides. The concentration of acetone used never exceeded 100 \( \mu \)l/50 ml of water (vol/vol). The fluor: bacterial suspensions were permitted to stabilize overnight prior to scintillation counting.

We were unable to detect any loss of the pesticides from centrifugation at the 1 \( \mu \)g/liter concentration. However, the pesticides did readily adhere to the glass containers. We found that the adsorption of the pesticide by the containers was correlated with time and agitation of the sample. These residue values were considered in determining the final pesticide concentrations in bacteria; therefore, only the insecticide extracted from water was considered available to the bacteria.

Experiments designed to determine uptake kinetics and retention of DDT and methoxychlor by bacteria were performed by using both live and autoclave-killed cells. Experimental procedures were similar for both organisms and compounds. Sterile 0.05 M phosphate buffer and reconstituted water at pH 7.0 ± 0.5 were also used to suspend bacteria for determination of the insecticide accumulation. Reconstituted water, employed as a standard in freshwater toxicity texts, was prepared by adding the following salts to 1 liter of deionized water: 30 mg of MgSO\(_4\), 30 mg of CaSO\(_4\), 48 mg of NaHCO\(_3\), and 2 mg of KCl. Neither distilled, buffered, or reconstituted water influenced the bacterial uptake of *4C*-DDT or methoxychlor.

Ten-milliliter samples were removed from the batch bacterial culture in either exponential or early stationary growth phase, centrifuged at 10,000 \* g, washed, recentrifuged and introduced into a 1 \( \mu \)g/liter *4C-labeled insecticide solution. The cells were vigorously agitated with a Vortex mixer and incubated at 22 C. Samples were removed from the insecticide:water mixture at 30 min and 1, 4, and 24 h and centrifuged. The cell pellet was mixed with the emulsifier:fluor and counted in a liquid scintillation counter. The supernatant fluid was discarded. Uptake kinetics studies in variable concentrations of insecticides by bacteria were done similarly, except that bacteria were exposed for only 4 h to 0.1, 0.5, 1.0, 2.5, and 5.0 \( \mu \)g/liter of DDT or methoxychlor.

After uptake equilibrium for the insecticide became known, we removed bacteria containing these concentrations to determine residue retention in cells resuspended in pesticide-free media. The bacterial cells were centrifuged, resuspended in distilled water, and vigorously agitated on a reciprocal shaker. Samples were removed at 0, 1, 2, 4, 24, and 48 h, and the residues were analyzed by radiometric assay.

Residues are expressed as pesticide weight per dry biomass. Samples from each bacterial batch culture were removed for biomass determination. All pesticide residues, whether from water or bacteria, are based on a weight-to-weight ratio. The term "magnification factor" is defined to mean the total insecticide residue (ng/mg, dry weight of biomass) divided by the insecticide water concentration (ng/g of H\(_2\)O). These cells were washed and dried at 90 C to constant weight. All samples were taken in triplicate and expressed as the mean value ± standard error. To enable our pesticide residue data to be compared and correlated with other fish-food chain studies, which, in many cases, are calculated on a wet-weight basis, we also determined the water content of these bacteria. The values obtained are listed in Table 3.

Water samples were extracted twice in a separatory funnel with petroleum ether-diethyl ether (95:5, vol/vol) combined, dried over anhydrous sodium sulfate, and carefully evaporated to 1 ml. The emulsifier: fluor was added to the extract, and the sample was counted as previously described. Extraction efficiency from water for both compounds was 99%.

Qualitative analyses of the insecticide residues
were made with thin-layer chromatography and autoradiography. After exposure of the bacteria to the insecticide, the sample was extracted three times with petroleum ether: diethyl ether (95:5, vol/vol) and dried over anhydrous sodium sulfate. The sample extracts were spotted on Eastman precoated silica gel sheets with fluorescent indicator. The DDT samples were developed in hexane: diethyl ether: acetic acid (200:2:2, vol/vol/vol); methoxychlor samples were developed in hexane: chloroform: methanol (3:2:1, vol/vol/vol).

Authentic standards of the insecticide and known degradation products were cochromatographed with the experimental extract and located under ultraviolet light. An autoradiogram was made by exposing the chromatograms to Kodak no-screen X-ray film for 7 days. Identification of 14C-labeled degradation products was made by comparing the autoradiogram with the authentic standards on the chromatogram.

RESULTS AND DISCUSSION

Both A. aerogenes and B. subtilis accumulated and magnified p,p'-DDT and methoxychlor directly from water (Table 1). We found no evidence to suggest that either was degraded during the various incubation periods. Uptake of both 14C-labeled DDT and methoxychlor was rapid; 80 to 90% of the 24-h residue was accumulated within the first 30 min of exposure. A total residue magnification factor for the two insecticides in bacterial cultures with approximately 0.1 mg/ml of biomass ranged from about 1,400 to 4,300 times greater than the insecticide concentration in water during the 24-h exposure. B. subtilis accumulated nearly 2.5 times as much methoxychlor as did A. aerogenes. However, no significant difference in the biomagnification of DDT was observed between the two organisms.

When total insecticide residues were plotted against concentrations of 0.5 to 5.0 µg/liter, the relationships were linear (Fig. 1). Therefore, the biomagnification factor of A. aerogenes and B. subtilis is independent of DDT and methoxychlor concentrations in water within these limits. However, this correlation disappears when the biomass changes. For example, when the biomass of A. aerogenes was decreased by 50% from 200 to 100 µg/ml, the magnification of

| Table 1. Biomagnification of p,p'-DDT and methoxychlor by Aerobacter aerogenes and Bacillus subtilis |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Insecticide | Bacteria | Exposition (h) | Insecticide residue (ng/mg) | Biomagnification factor | Recovery of insecticide by bacteria (%) |
| 14C-methoxychlor | A. aerogenes | 0.5 | 1.14 ± 0.02 | 1139 ± 22 | 4.5 ± 0.11 |
| | | 1 | 1.38 ± 0.03 | 1258 ± 29 | 5.5 ± 0.12 |
| | | 2 | 1.43 ± 0.04 | 1432 ± 45 | 5.7 ± 0.17 |
| | | 4 | 1.40 ± 0.19 | 1324 ± 18 | 5.6 ± 0.07 |
| | | 24 | 1.56 ± 0.03 | 1422 ± 32 | 6.2 ± 0.14 |
| | B. subtilis | 0.5 | 3.25 ± 0.06 | 3069 ± 61 | 12.9 ± 0.26 |
| | | 1 | 3.03 ± 0.03 | 2854 ± 29 | 12.0 ± 0.11 |
| | | 2 | 3.18 ± 0.08 | 3005 ± 80 | 12.5 ± 0.33 |
| | | 4 | 3.33 ± 0.08 | 3146 ± 77 | 13.2 ± 0.32 |
| | | 24 | 3.56 ± 0.17 | 3366 ± 161 | 14.1 ± 0.65 |
| 14C-DDT | A. aerogenes | 0.5 | 3.91 ± 0.21 | 3258 ± 180 | 13.0 ± 0.74 |
| | | 1 | 3.44 ± 0.13 | 2809 ± 110 | 11.5 ± 0.45 |
| | | 2 | 3.50 ± 0.06 | 2923 ± 53 | 11.8 ± 0.14 |
| | | 4 | 4.07 ± 0.27 | 3396 ± 223 | 13.6 ± 0.88 |
| | | 24 | 4.48 ± 0.41 | 3736 ± 340 | 14.9 ± 1.34 |
| | B. subtilis | 0.5 | 2.27 ± 0.52 | 3392 ± 780 | 13.4 ± 3.1 |
| | | 1 | 2.03 ± 0.28 | 3029 ± 426 | 12.1 ± 1.1 |
| | | 2 | 1.78 ± 0.22 | 2661 ± 333 | 10.5 ± 1.3 |
| | | 4 | 2.33 ± 0.31 | 3482 ± 458 | 13.8 ± 1.8 |
| | | 24 | 2.88 ± 0.24 | 4303 ± 363 | 17.0 ± 1.4 |

* Dry weight.

* DDT concentration in water was 0.676 and 1.20 µg/liter for A. aerogenes and B. subtilis, respectively; the methoxychlor concentration in water was 1.01 µg/liter for both microorganisms. Data represent the mean value of at least triplicate samples (± S.E.). Magnification factor = total insecticide residue in bacteria (ng/mg) per insecticide water concentration (ng/g).

* Significant difference in magnification found between organisms (P < 0.0005, Fisher’s “t”).

* No significant difference in magnification existed between organisms (P < 0.05, Fisher’s “t”).
methoxychlor increased 68% (Table 2). A similar inverse relationship was noted with DDT; the magnification of the insecticide is about doubled from 2,666 to 5,235 times the exposure concentration when the biomass of A. aerogenes was reduced by one-half (Table 2). In essence, the biomagnification factor(s) is not absolute, but is correlated to both the DDT or methoxychlor concentrations in water and the biomass of bacteria.

We interpret these data (Table 2) to suggest that many more "receptor sites" for the insecticides are available in (or on) the bacterial cell; that is, the finite equilibrium point has not been reached. One might speculate on the significance of this observation in a eutrophic aquatic ecosystem. The relatively high algal and microbial biomasses (and consequently a large number of receptor sites associated with such ecosystems) would, within limits, distribute pesticide residues more broadly than in oligotrophic waters. In effect, this action would dilute the pesticide residues available on a biomass basis for uptake by planktonic and primary consumers. In addition, such microflora would eventually sink to the bottom carrying the residues, introducing relatively large amounts of the chemicals to the benthos community via a filter-feeding food chain. The ultimate consequence of such a sequence may explain for instance the pesticide distribution in Lake Poinsett, South Dakota. Hannon et al. (6) observed about a 10-fold-greater concentration of pesticides by aquatic insects over fish.

Accumulation of the two insecticides appears to be a passive process in bacteria. Autoclave-killed cells also rapidly adsorbed 14C-methoxychlor and 14C-DDT (Fig. 2). In all cases, the magnification rate was greater than that for the living cells. For instance, after autoclaving the cells of A. aerogenes, the total DDT and methoxychlor residue for both compounds increased over 100 and 200%, respectively (Fig. 2 and 3).

Washing of insecticide-exposed bacteria in pesticide-free water caused desorption of both DDT and methoxychlor. Most of this desorption occurred during the initial wash of the bacteria, and subsequent washings did not further reduce the insecticide residues substantially. The initial wash of methoxychlor-exposed bacteria, both A. aerogenes and B. subtilis, reduced total residues by nearly 75%. However, DDT was more resistant to desorption, and only 45% was removed from A. aerogenes, and 30% was removed from B. subtilis. Less than 5% of the

| Bacteria      | Insecticide concentration (µg/liter) | Microbial* biomass (µg/ml) | Mean magnification* factor ± Sx |
|---------------|-------------------------------------|---------------------------|---------------------------------|
| A. aerogenes  | DDT, 0.64                           | 25                        | 10639 ± 920                     |
|               |                                     | 50                        | 5235 ± 606                      |
|               |                                     | 75                        | 4030 ± 453                      |
|               |                                     | 100                       | 2666 ± 235                      |
|               |                                     | 200                       | 1784 ± 15                       |
| B. subtilis   | DDT, 0.64                           | 43                        | 13880 ± 1650                    |
|               |                                     | 87                        | 5902 ± 374                      |
|               |                                     | 130                       | 4533 ± 295                      |
|               |                                     | 174                       | 3245 ± 169                      |
|               |                                     | 348                       | 1805 ± 41                       |
| A. aerogenes  | Methoxychlor 1.06                   | 50                        | 2758 ± 112                      |
|               |                                     | 100                       | 1704 ± 82                       |
|               |                                     | 150                       | 1324 ± 19                       |
|               |                                     | 200                       | 1014 ± 18                       |
|               |                                     | 400                       | 461 ± 4                         |
| B. subtilis   | Methoxychlor 1.06                   | 26                        | 8138 ± 100                      |
|               |                                     | 52                        | 5004 ± 55                       |
|               |                                     | 78                        | 4021 ± 10                       |
|               |                                     | 104                       | 3088 ± 83                       |
|               |                                     | 208                       | 2114 ± 24                       |

* Dry weight.

* Magnification factor = total insecticide residue in bacteria per insecticide water concentration. Exposure duration 4 h for all samples. N = 3 samples.
remaining DDT or methoxychlor residue was lost during the subsequent 48 h of washing.

Some physical and chemical characteristics of the insecticides and bacteria were compared to suggest factors that may influence microbial biomagnification of DDT and methoxychlor (Table 3). Lipid solubility, the number and position of the chlorine atoms, and molecular polarity seem to influence the higher retention of DDT than methoxychlor by bacterial cells. We postulate that the biomagnification of DDT and methoxychlor by bacteria occurs as the result of both absorption and desorption. The mechanism proposed by Hamelink et al. (5), suggesting that biological magnification of pesticides by fish is dependent on sorption and solubility differences, may partially explain DDT and methoxychlor uptake and retention by microorganisms; however, there are inconsistencies. Ether-extractable lipid contents of *A. aerogenes* and *B. subtilis* were similar, approximately 5 to 6% per dry weight. With this similarity, one would expect little difference in biomagnification between the two organisms due to lipid content. Predictably, we observed no significant difference in DDT magnification. However, with methoxychlor, *B. subtilis* magnified twice as much of the compound as *A. aerogenes*, even with the similarity in lipid content.

**Table 3. Physical and chemical factors that may influence biomagnification of DDT and methoxychlor by bacteria**

| Physical and chemical factors | Pesticides | Bacteria |
|------------------------------|------------|---------|
|                              | DDT    | Methoxychlor | *A. aerogenes* | *B. subtilis* |
| Molecular weight             | 354.0  | 345.2       |                 |              |
| *p*-value*                   | 0.38   | 0.069       |                 |              |
| *T*<sub>R</sub> relative<sup>b</sup> to *T*<sub>R</sub> Aldrin | 3.63   | 5.99        |                 |              |
| Lipid content<sup>c</sup>    |         |             |                 |              |
| Surface area/mg<sup>d</sup>  |         |             |                 |              |
| Water content<sup>e</sup>    |         |             |                 |              |

<sup>a</sup>*p*-value = fraction of the total solute that distributes itself in the nonpolar phase of an equivolume solvent pair (1, 2).
<sup>b</sup>Gas-liquid chromatography retention times (*T*<sub>R</sub>; reference 3).
<sup>c</sup>Diethyl ether extraction, based on dry weight.
<sup>d</sup>Surface area = 2πr<sub>1</sub> + 4πr<sub>2</sub>. Biomass dry weight.
<sup>e</sup>Mean value ± standard error. N = 3 samples.

![Fig. 2. Magnification of DDT by live and autoclave-killed bacteria. Magnification factor = total cellular residue per pesticide water concentration. DDT and methoxychlor concentration = 1.0 μg/liter. Biomass/10 ml = 1.0 to 1.2 mg. Points represent mean value of triplicate samples.](http://aem.asm.org/)

![Fig. 3. Same as Fig. 2, except magnification is of methoxychlor by live and autoclave-killed bacteria.](http://aem.asm.org/)
If the uptake phenomenon were dependent on sorption, surface area should play an integral role. We found that A. aerogenes, which has a surface area approximately 10 times greater than B. subtilis (Table 3), actually accumulated no more residue than B. subtilis. Although lipid content apparently did not directly influence DDT or methoxychlor uptake by bacteria, the retention of these pesticides followed the lipid-partition hypothesis for magnification. For instance, the partition coefficient value (p-value) for DDT and methoxychlor in a hexane-acetonitrile solvent system is 0.38 and 0.069, respectively (Table 3), and indicates that methoxychlor is more polar or less lipid soluble than DDT. This may explain why two to three times as much DDT was retained than methoxychlor by both organisms after they were washed with pesticide-free water.

Perhaps the most puzzling data relate to the uptake of insecticides by autoclave-killed cells. One might logically suspect that autoclaving would result in massive lysis of the cells, thereby increasing the adsorptive sites for insecticide accumulation. However, we found no such evidence from microscopic examination of the autoclave-killed cells. Our hypothesis is that changes in the cellular membrane occur as the result of autoclaving the bacteria, and this action apparently increased the DDT and methoxychlor uptake sites. Actually, the statement that insecticide accumulation is a passive phenomenon in bacteria is at best tenuous. We do not know whether live bacteria actively or passively incorporate the pesticide through the multiple physical and chemical barriers that separate the cell from the external environment. We might postulate that the integrity of the cell membrane has been disrupted by autoclaving and, as indicated from the increased pesticide residue, more receptor sites are available for insecticide retention.

Seemingly, the potential role of microorganisms in aquatic pollution extends beyond that of the traditional decomposer. Microorganisms may act as physical agents in movement of chemical pollutants in an aquatic ecosystem in addition to influencing the chemical nature of the pollutant through degradation (18). In a model food chain, bacteria may be a valuable, simple adjuvant in predicting potential biomagnification of trace aquatic contaminants prior to field investigations.

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