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Importance of Passive Diffusion in the Uptake of Polychlorinated Biphenyls by Phagotrophic Protozoa†

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Unicellular protozoan grazers represent a size class of organisms where a transition in the mechanism of chlorobiphenyl (CB) introduction, from diffusion through surface membranes to ingestion of contaminated prey, could occur. This study compares the relative importance of these two processes in the overall uptake of polychlorinated biphenyls by protists. Uptake rates and steady-state concentrations were compared in laboratory cultures of grazing and nongrazing protozoa. These experiments were conducted with a 10-μm marine scuticociliate (*Uronema* sp.), bacterial prey (*Halomonas halodurans*), and a suite of 21 CB congeners spanning a range of aqueous solubilities. The dominant pathway of CB uptake by both grazing and nongrazing protozoa was diffusion. Organic-carbon-normalized CB concentrations (in the protozoan cell) were equivalent in grazing and nongrazing protozoa for all congeners studied. Rate constants for uptake into and loss from the protozoan cell were independently determined by using [3,3′,4,4′-^{14}C]tetrachlorobiphenyl (IUPAC no. 77), 0.38 ± 0.03 min^{-1} and (1.1 ± 0.1) × 10^{-5} (g of organic carbon)$^{-1}$ min^{-1}, respectively. Magnitudes of the uptake and loss processes were calculated and compared by using a numerical model. The model result was consistent with data from the bioaccumulation experiment and supported the hypothesis that diffusive uptake is faster than ingestive uptake in phagotrophic unicellular protozoa.

Polychlorinated biphenyls (PCBs) are persistent organic pollutants ubiquitous in the global environment which have been shown to have adverse effects on the health of many aquatic organisms (5, 16, 17, 20, 24). Limited biotransformations result in efficient transfer of many chlorinated biphenyls between trophic levels and accumulation in lipid-rich tissues (9, 19). Diffusion through cellular membranes is the only chlorobiphenyl (CB) uptake pathway available in nonphagocytotic unicellular organisms such as phytoplankton. For large organisms such as fish and marine mammals, PCBs accumulate through ingestion (6, 9, 12). Uptake via gill and dermal exposure has been suggested to play a minor role in the overall uptake of PCBs by fish and other large marine organisms (9, 19, 22).

Previous studies have explored the kinetics of CB equilibration with phytoplankton (25, 28), but to date, no work has been done concerning the accumulation of PCBs in organisms that can ingest contaminated prey. Heterotrophic protozoa offer a unique opportunity to study the relative rates and contributions of diffusive and ingestive uptake because they are capable of ingesting small particulate material yet are the same size as nonphagocytotic organisms already studied.

Within a bulk solution, CB speciation is determined by the nature and concentration of organic carbon in both dissolved and particulate pools (23). Studies have shown that PCBs associated with organic material (dissolved and/or particulate) are relatively unavailable for biological uptake in comparison to PCBs truly dissolved in the aqueous phase (for reviews, see references 10 and 18). Truly dissolved PCBs can enter a cell by diffusing through outer membranes. Ingestion of prey, on the other hand, involves the encapsulation of a parcel of water containing both free and complexed PCBs via invagination of the cellular membrane. Prey CB concentration is an important parameter in determining the CB uptake via ingestion and is affected by both prey size (i.e., surface-area-to-volume ratio) and cellular composition.

The steady-state CB concentration within the protozoan cell is the equilibrium value predicted by the CB congener K_{ow} (the *n*-octanol-water partition coefficient) and the relative size and composition of all organic carbon pools in the system. The uptake pathway does not affect the equilibrium concentration in the protozoan cell, simply the time needed to achieve this value (8, 9). Swackhamer and Skoglund (28) predicted that full equilibration of aquatic organisms (15 to 20 μm) with aqueous CB concentrations occurs on day time scales. Generation times of many aquatic organisms are shorter than days, and thus, the rate of uptake is an important factor in determining the accumulation of PCBs within the food web. Organisms such as phagotrophic protozoa can potentially access an additional pool of PCBs and achieve equilibrium faster than nonphagocytotic cells.

Initial calculation. An initial calculation for 3,3′,4,4′-tetrachlorobiphenyl (IUPAC no. 77), however, showed that the diffusion uptake pathway should be faster than ingestion of contaminated prey. The calculation assumed that the rate-limiting step of diffusive uptake was transfer across the lipid membrane. The other slow step in this process, diffusion across the unstirred water boundary layer, was not considered to be due to the difficulty of estimating the width of this layer. Constant movement of surface cilia associated with filter feeding and swimming will lower the thickness of the unstirred water boundary layer.

The rate of uptake via diffusion is defined as the flux through the phospholipid membrane multiplied by protozoan surface area (normalized to a single protozoan cell):

$$\left(\frac{d[CB]_{prot}}{dt}\right)_{diff} = \text{flux} \times SA_{prot} = \frac{D_m K_{lw} SA_{prot}}{\Delta z}[CB]_d \quad (1)$$

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TABLE 1. Parameters used in estimates of uptake rates via diffusion and ingestion

Uptake method	Parameter[a]	Units	Value	Reference or source
Diffusion	D_m	m^2/s	10^{-13} or 10^{-19} (10^{-16} used)	13
	K_{lw}	Nondimensional	$10^{6.33}$	27
	SA_{prot}	m^2	1.2×10^{-9}	From $SA = 4\pi r^2$ and $r = 10$ μm
	Δz	m	5×10^{-9}	1
Ingestion	CR	ml/s	9.4×10^{-10}	7
	BC	cells/ml	10^7	Experimental condition
	BV	m^3/cell	5.2×10^{-19}	From $V = 4/3\ \pi r^3$ and $r = 0.5$ μm
	F_{lip}	Nondimensional	0.15	28

[a] Parameter abbreviations are the same as used in the formulas shown in the text.

where $[CB]_{prot}$ and $[CB]_d$ are the CB concentrations in the protozoan (mole of CB per cubic meter) and dissolved phase (mole of CB per cubic meter), respectively; SA_{prot} is the surface area of the protist (square meter); D_m is the diffusion coefficient of a CB congener through the lipid membrane (square meter per second); K_{lw} is the lipid-water partition coefficient; and Δz is the thickness of the lipid membrane (meters). Values are contained in Table 1.

Uptake via ingestion is equal to prey ingestion rate multiplied by prey CB concentration:

$$\left(\frac{d[CB]_{prot}}{dt}\right)_{ing} = CR \times BC \times BV \times [CB]_{bact} \quad (2)$$

where $[CB]_{prot}$ and $[CB]_{bact}$ are CB concentrations in the protozoa and the bacteria (mole of CB per cubic meter), respectively; CR is the protozoan clearance rate (milliliters per second); BC is the bacterial concentration (cells per milliliter); and BV is the bacterial cell volume (cubic meter per cell). $[CB]_{bact}$ is the product of the bacterial lipid fraction (F_{lip}), the lipid-water partition coefficient (K_{lw}), and the dissolved CB concentration ($[CB]_d$). The ratio of diffusive uptake (*Diff*) to ingested uptake (*Ing*) shows the relative speeds of CB influx via two methods:

$$\frac{Diff}{Ing} = \frac{D_m \times K_{lw} \times SA_{prot} \times [CB]_d}{\Delta z \times CR \times BC \times BV \times F_{lip} \times K_{lw} \times [CB]_d} = 3.3 \times 10^4 \quad (3)$$

This ratio between uptake rates predicts that diffusion through the membrane delivers PCBs faster than ingestion of contaminated prey by a factor of 10^4. This occurs even when a midrange value for diffusive uptake and an upper limit for ingested uptake (100% assimilation at maximum clearance rate) are used. Ingestion of CB-dissolved organic carbon (DOC) complexes is not included in the above calculation because it represents a minor contribution to the overall uptake of PCBs via ingestion (13).

In this paper, we present the results of a study designed to experimentally verify the predicted significance of diffusion and ingestion as CB uptake pathways in protozoans. We compared CB uptake in prey-limited and prey-replete cultures of protozoa. Protozoa rapidly equilibrated with dissolved CB concentrations in the surrounding aqueous medium. Equivalence of organic-carbon-normalized CB concentrations in protozoa in the two treatments indicated that diffusion was the dominant uptake pathway. Protozoan uptake and loss rate constants were measured by using a radiolabeled congener and were compared with an estimated bacterial loss constant by using a four-box numerical model. Model results were consistent with observations from the bioaccumulation experiment.

MATERIALS AND METHODS

Growth of organisms. Vineyard Sound seawater (VSW), used in all growth media, was collected by using a Masterflex pump during an incoming tide at Woods Hole, Mass. Seawater was stored in polycarbonate carboys in the dark at room temperature. Before media preparation, seawater was filtered through a 1.2-μm-pore-size in-line Versapor filter (Gelman) and a 0.2-μm-pore-size in-line nylon filter (Whatman). The water filtered through the 0.2-μm pores was autoclaved for at least 30 min and was stored at room temperature. All glassware used for culturing was washed with Citranox detergent (Fisher Scientific), was soaked in 10% ethanol-HCl in Milli-Q water overnight, and was rinsed with Milli-Q water and sterilized.

The organisms used in these experiments were (i) a 10- to 15-μm scuticociliate (*Uronema* sp., clone BBCil) and (ii) a 0.5-μm marine bacterium (*Halomonas halodurans*), both from the collection of D. Caron, University of Southern California. Both organisms were chosen because of their relative hardiness during experimental manipulations. The bacterium species is ubiquitous in the marine environment and has been assumed to be representative of marine heterotrophic bacteria (3).

A variation on the protocol of Lim et al. (14) was used to grow high-density ciliate cultures (10^5 to 10^6 cells/ml). Bacteria (*H. halodurans*) were grown on 0.04% yeast extract in sterile, filtered VSW (0.2-μm pore size) and were rinsed three times to remove excess yeast extract (13). An aliquot of bacterial concentrate was diluted with sterile VSW to a bacterial concentration of 10^8 to 10^9 cells/ml (total volume, 1 liter). Initial *Uronema* concentrations were 500 to 1,000 cells/ml. Cultures were shaken on a table rotary shaker at 30 to 40 rpm to ensure an oxygenated medium.

A two-step centrifugation protocol was developed to selectively remove bacterial prey from protozoan cultures. Aliquots (40 ml) of protozoan culture were centrifuged in polycarbonate tubes at 6,800 rpm ($5,169 \times g$) for 17 min. The top two-thirds of the supernatant of each centrifuge tube was removed as quickly as possible by vacuum aspiration. Fresh VSW was added to each tube to return the volume to the original level (40 ml in this case). The contents of tubes were then mixed to resuspend the bottom pellet and were centrifuged again at 6,000 rpm ($4,024 \times g$) for 12 min to sediment bacterial aggregates. The tubes were then left undisturbed to allow the protozoans to swim away from the bacterial pellet. After 15 to 20 min, the supernatant was removed with a pipette while avoiding any dislodged bacterial aggregates. This supernatant was considered the protozoan concentrate. Recovery of protozoans from this separation protocol varied depending on the condition of protists prior to centrifugation and the composition of the culture medium. Protozoan cell recoveries varied from 60 to 10% (range of all trials performed). Bacterial cell concentrations were reduced by 50 to 90% (range of all trials performed; high rates of protozoan recoveries were not necessarily coincidental with high rates of bacterial recovery). This reduction lowered bacterial cell concentrations in the protozoan concentrate to $\leq 10^5$ cells/ml.

Experimental protocol. The PCBs used in this experiment were purchased (catalog no. C-CCSEC-R, lot no. 124-269; AccuStandard, New Haven, Conn.) as a mixture of 21 congeners (approximately 100 μg of each congener per ml of acetone), spanning a range of hydrophobicities (Table 2). All experimental cultures contained approximately 0.4 ng (total) of each CB congener per ml. For each congener, the aqueous concentration in each flask at the beginning of the experiment was significantly lower than the aqueous solubility (<10%), with the exception of three congeners: IUPAC no. 195 (11%), IUPAC no. 206 (44%), and IUPAC no. 209 (52%).

Four 2.5-liter Fernbach flasks were used in this experiment—two designated as grazing flasks and two designated as nongrazing flasks. The nongrazing flasks contained 13 μl of CB spike (approximately 200 ng of congener/ml) and 450 ml of sterile VSW. The grazing flasks contained 50 ml of bacterial slurry (equilibrated with 13 μl of CB spike) and 400 ml of sterile VSW. After the VSW solutions equilibrated for 1 h on a table rotary shaker, protozoan concentrate (550 ml) was added to all four flasks (total volume, 1 liter). Addition of protists (initial concentration, 1,000 cells/ml) was considered the start of the experiment. Bacterial concentrations in the grazing and nongrazing flasks were 10^7 and 10^4

TABLE 2. CB congeners used in experiments

Congener (IUPAC no.)	Dichlorobiphenyl structure	Log K_{ow}	Total CB concn in each flask (ng/ml)
8	2,4′-	5.07	0.303
18	2,2′,5-	5.24	0.306
28	2,4,4′-	5.67	0.303
44	2,2′,3,3′-	5.75	0.304
52	2,2′,5,5′-	5.84	0.304
66	2,3′,4,4′-	6.2	0.303
77	3,3′,4,4′-	6.36	0.307
101	2,2′,4,5,5′-	6.38	0.303
105	2,3,3′,4,4′-	6.65	0.303
118	2,3′,4,4′,5-	6.74	0.306
126	3,3′,4,4′,5-	6.89	0.304
128	2,2′,3,3′,4,4′-	6.74	0.304
138	2,2′,3,4,4′,5′-	6.83	0.305
153	2,2′,4,4′,5,5′-	6.92	0.306
170	2,2′,3,3′,4,4′,5-	7.27	0.303
180	2,2′,3,4,4′,5,5′-	7.36	0.306
187	2,2′,3,4′,5,5′,6-	7.17	0.303
195	2,2′,3,3′,4,4′,5,6-	7.56	0.303
199	2,2′,3,3′,4,5,6,6′-	7.2	0.307
206	2,2′,3,3′,4,4′,5,5′,6-	8.09	0.303
209	Decachlorobiphenyl	8.18	0.306

cells/ml, respectively, at 2 h and increased during the experiment in all flasks (Table 3).

Samples were taken every 6 min (on average) for the first hour and then once every 2 h until 6 h. At each time point, 40-ml culture aliquots were removed and filtered through 5.0-μm-pore-size silver (Ag) filters (Osmonics, Livermore, Calif.) by using positive-pressure reverse-flow filtration through stainless steel tubing and an in-line 5.0-μm-pore-size Ag filter housed in a 47-mm stainless steel in-line filter holder (Gelman). The filtration system was cleaned with Milli-Q water and acetone between samples. Size fractionation through 5.0-μm-pore-size silver filters was used to separate bacteria from protozoa. Silver membranes were chosen for this purpose because of their low retention of dissolved PCBs and their capacity to cleanly separate the two organisms.

Filters were covered with 1:1 hexane-acetone in 40-ml combusted-glass screw-cap vials and were stored in a refrigerator. Filtrates were stored in 40-ml combusted-glass vials. At later time points (2 h and onward), 9-ml aliquots were preserved with 1% glutaraldehyde for population enumeration. In addition, 40-ml aliquots were stored in combusted-glass screw-cap vials for total PCB analyses. At the last time point (6 h), additional aliquots were filtered through 0.2-μm-pore-size Ag filters: 40 ml from each nongrazing, or diffusion, flask and 40 ml of a 1:2 dilution from each grazing, or ingestion, flask. All size fractionations (5.0 and 0.2 μm) were repeated at the 6 h time point for organic carbon analyses. Filters for organic carbon analyses were folded into quarters, wrapped in combusted Al foil, and stored at −4°C in a freezer until analysis.

CB analyses. Congeners IUPAC no. 14 (3,5-dichlorobiphenyl) and IUPAC no. 198 (2,2′,3,3′,4,5,5′,6-octachlorobiphenyl) were used as surrogate recovery standards in all samples. Congener IUPAC no. 103 (2,2′,4,5′,6-pentachlorobiphenyl) was used as the gas chromatography (GC) external quantitation standard. Individual congeners were purchased from AccuStandard (lot no. 024-212 [14], 081-186 [103], and 085-005 [198]), all at a concentration of 35 μg/ml in isooctane). Prior to use, anhydrous Na_2SO_4 (Fisher Scientific) was combusted for at least 4 h at 450°C and was stored in a desiccator. All solvents (hexane and acetone) were Ultra Resi-Analyzed grade (J. T. Baker, Phillipsburg, N.J.).

At least 12 h prior to analysis, 150 μl of each surrogate recovery standard was added to each sample. Filters were extracted three times with fresh 1:1 hexane-acetone by sonic probe extraction (VibraCell, Sonics and Materials, Inc., Danbury, Conn.) (conditions, pulse for 15 min at 60% duty cycle with output 5.0). All extracts were combined in a round-bottom flask. Aqueous samples were acidified with hexane-extracted 1 N HCl (4 to 5 drops) to pH 2 to 3 and were extracted five times with hexane. All extracts (and surface emulsions, if any) were combined in a round-bottom flask and were dried with anhydrous Na_2SO_4. (Note that the presence of emulsions in aqueous extractions was correlated with high bacterial concentrations and resulted in a concomitant loss of PCBs.) Each extract was solvent exchanged into hexane and was reduced in volume to 1 to 2 ml via rotary evaporation.

Extracts were then cleaned with concentrated H_2SO_4 after the method of Bergen et al. (4). Each extract and half its volume of concentrated H_2SO_4 were vortexed for 1 min in a combusted 15-ml glass tube and then allowed to sit at least 45 min. The hexane phase was removed, and the acid phase was reextracted twice more with hexane. All hexane phases were combined in a 4-ml combusted-glass vial and reduced to approximately 150 μl with ultra-high-purity N_2 after the addition of 150 μl of GC quantitation standard.

Final extracts were transferred to a combusted GC vial with a 200-μl insert and were analyzed on a gas chromatograph (Hewlett-Packard [HP] 5890, series II) with an electron capture detector (HP model no. G1223A) and a 60-ml DB-5 capillary column (0.25-μm inside diameter; J. T. Baker) installed. Analysis conditions consisted of the following temperature program: 60°C for 2 min, ramp at 6°C/min to 170°C, ramp at 1°C/min to 240°C, hold for 10 min, ramp at 3°C/min to 298°C, and hold for 5 min, with He as a carrier gas flowing at 1.2 ml/min. Standards were run every six samples to monitor column conditions. Chromatograms were integrated with HP ChemStation software by using a five-point external standard curve. Surrogate recovery standard recoveries averaged 91.6% ± 20.2% for no. 14 and 90.7% ± 17.6% for no. 198 (range, 52.2 to 155.1% for no. 14 and 52.3 to 149.3% for no. 198; $n = 99$). GC detection limits were 1 to 5 pg for the congeners studied.

Ancillary analyses. Ag filters were dried overnight in a 60°C oven and were then cut into quarters, weighed, folded, and wrapped in a Sn boat (Microanalysis, Manchester, Mass.). Three of the four quarters were combusted and analyzed on a Fisons Instruments EA 1108 elemental analyzer. The average of the three quarters is presented in Table 3.

DOC and total organic carbon (TOC) samples were acidified with 50% (vol/vol) H_3PO_4 (200 μL per 40-mL sample) and were measured by high-temperature combustion (21). DOC concentrations were significantly higher than measured TOC concentrations, therefore contamination was suspected. TOC concentrations were used instead of DOC concentrations, so it should be noted that these values represent an upper limit of actual DOC concentrations.

Bacterial and protozoan populations were enumerated within 2 weeks of glutaradehyde preservation by using acridine orange (15).

Radioactive experiments. Short (15-min) radioactive experiments were conducted with ^{14}C-labeled 3,3′,4,4′-tetrachlorobiphenyl ([^{14}C]TCB) (IUPAC no. 77) (specific activity, 52.1 μCi/μmol [courtesy of J. Stegeman, Woods Hole Oceanographic Institution, Mass]) to better determine the protozoan uptake rate constant. This experiment was performed by using protozoan cultures with low concentrations of bacteria and was not repeated with high concentrations of bacteria. Filtrates (<5.0 μm) of the same protozoan culture were used to test retention of dissolved PCBs by Ag filters. In each experiment, an aliquot (600 ml) of either culture or filtrate was inoculated with [^{14}C]TCB (in an acetone carrier) to a final concentration of 0.25 ng/ml (approximately 100 dpm/ml). While the concentration of radiolabel was low relative to typical radiofractionation studies, higher activities would have required higher CB mass concentrations, and the

TABLE 3. Population and organic carbon data for all experiments[a]

Flask and replicate no. (t in h)	Bacterial cells ± 1σ (cells/ml)	Protozoan cells ± 1σ (cells/ml)	Organic carbon in 5.0-μm fraction (fg/ml)	Organic carbon per protozoan cell ± 1σ (fg)
Diffusion replicate 1 (2)	7.63×10^4 (1.08×10^4)	1.94×10^3 (6.15×10^2)		
Diffusion replicate 2 (2)	4.88×10^5 (2.78×10^4)	5.18×10^3 (8.22×10^2)		
Ingestion replicate 1 (2)	1.25×10^7 (1.13×10^5)	3.05×10^3 (1.03×10^3)		
Ingestion replicate 2 (2)	1.45×10^7 (2.00×10^6)	2.78×10^3 (6.57×10^2)		
Diffusion replicate 1 (6)	1.21×10^6 (1.31×10^4)	1.39×10^3 (3.16×10^2)	4.94×10^9 (2.97×10^8)	3.56×10^6 (8.38×10^5)
Diffusion replicate 2 (6)	7.80×10^6 (1.08×10^5)	3.52×10^3 (5.55×10^2)	7.80×10^9 (1.98×10^8)	2.22×10^6 (3.55×10^5)
Ingestion replicate 1 (6)	2.53×10^7 (2.95×10^6)	4.44×10^3 (1.03×10^3)	6.35×10^9 (2.51×10^7)	1.43×10^6 (3.33×10^5)
Ingestion replicate 2 (6)	2.12×10^7 (3.19×10^5)	2.64×10^3 (7.08×10^2)	1.15×10^{10} (1.84×10^8)	4.35×10^6 (1.17×10^6)

[a] Population and organic carbon data for all experiments. Bacterial and protozoan cell concentrations are the average of 16 random fields corrected for volume of aliquot filtered. Errors (in parentheses) are ±1 standard deviation. Organic carbon data are averages of three filter sections with errors of ±1 standard deviation. Amount of organic carbon per cell was calculated by dividing organic carbon concentration by protozoan cell concentration. Errors were propagated from errors on protozoan population counts and organic carbon analyses.

results would have not been comparable to the earlier study. No loss of sensitivity in measurements was observed, since 5 to 10 ml of solution and filters were analyzed on the scintillation counter (counts ranged from 700 to 1,400 dpm per sample). Blanks averaged 55 ± 7 dpm and were subtracted from all samples.

Addition of the congener was considered zero time. Three replicate samples of culture and two replicates of culture filtrate were tested. In each experiment, 50-ml aliquots of solution were removed as quickly as possible and were vacuum filtered through 5.0-μm-pore-size Ag filters. Filters were placed in scintillation vials with 5 mL of ScintiVerseII scintillation cocktail (Fisher Scientific) and were counted to ±2% on a Beckman Scintillation Counter. Filter radioactive counts were normalized to total aliquots removed in the middle of the experiment. Prior to [^{14}C]TCB inoculation, solution aliquots were also removed for bacterial and protozoan cell enumeration as well as analysis of total and dissolved organic carbon (<0.2 μm).

Numerical model. A four-box model was written to compare protozoan uptake and loss rate constants with an estimated bacterial loss rate constant. In addition, we compared the model results to the data from the bioaccumulation experiments. Initial values for the bacterial, DOC, and aqueous phases were assumed to be equal to the equilibrium values predicted by K_{oc}. The protozoan size class contained no PCBs at zero time. The model was run with 0.1-min time steps for 60 min. The following equations described the fluxes between pools:

$$B_n^{diff} = B_{n-1}^{diff} - k_{dep} \times B_{n-1}^{diff} \times \Delta t \quad (4)$$

$$B_n^{ing} = B_{n-1}^{ing} - k_{dep} \times B_{n-1}^{ing} \times \Delta t - IR \times B_{n-1}^{ing} \times \Delta t \quad (5)$$

$$W_n = W_{n-1} + Y \quad (6)$$

$$Y = k_{dep} \times B_{n-1} \times \Delta t - k_{for} \times Aq_{n-1} \times \Delta t + k_{rev} \times [P] \times [OC]_P \times P_{n-1} \times \Delta t \quad (7)$$

$$Aq_0 = (1 + K_{oc} \times [DOC]) \times W_0 \quad (8)$$

$$Aq_n = Aq_{n-1} + \left(\frac{1}{1 + [DOC] \times K_{OC}}\right) \times Y \quad (9)$$

$$D_n = W_n - Aq_n = D_{n-1} + \left(\frac{[DOC] \times K_{OC}}{1 + [DOC] \times K_{OC}}\right) \times Y \quad (10)$$

$$P_n^{diff} = P_{n-1}^{diff} + k_{for} \times Aq_{n-1} \times \Delta t - k_{rev} \times P_{n-1}^{diff} \times [P] \times [OC]_P \times \Delta t \quad (11)$$

$$P_n^{ing} = P_{n-1}^{ing} + k_{for} \times Aq_{n-1} \times \Delta t - k_{rev} \times P_{n-1}^{ing} \times [P] \times [OC]_P \times \Delta t + IR \times B_{n-1} \times \Delta t \quad (12)$$

B_n, W_n, Aq_n, D_n, and P_n refer to the mass of CB congener in the bacterial, water (aqueous and DOC combined), aqueous, DOC, and protozoan pools, respectively. Superscripts refer to the case study (*diff* for diffusion and *ing* for ingestion), whereas subscripts refer to the time step number n. The other parameters are as follows: k_{dep}, bacterial depuration rate constant (per minute); IR, ingestion rate (cells per minute); k_{for}, protozoan uptake rate constant (per minute); k_{rev}, protozoan loss rate constant (per gram of organic carbon per minute); [P], protozoan concentration (cells per milliliter); and $[OC]_P$, organic carbon per protozoan (grams of organic carbon per cell). Equations 15 and 22 are used for grazing protozoa only (ingestion case study).

RESULTS

Bioaccumulation experiments. In the prey-limited, or diffusion, flask, bacterial cell numbers remained below the protozoan grazing threshold (10^6 cells/ml) until the end of the experiment. In the prey-replete, or ingestion, flask, however, the prey concentration remained above the grazing threshold during the entire experiment. The protozoan population in each flask did not change significantly over the time course of the experiment (Table 3). Qualitatively, however, the health of the protozoa in the two flasks was different. It was observed microscopically that cells in the diffusion flask were very thin and contained few (<5) food vacuoles, whereas protozoa in the ingestion flask were robust with many (15 to 20) food vacuoles.

Particulate organic carbon concentrations in the >0.2- and >5.0-μm size classes were similar in prey-limited flasks because protozoa represented the major particulate pool. Conversely, the total particulate organic carbon (>0.2 μm) in the prey-repleted cultures was approximately twice that in the protozoa (>5.0 μm) due to contributions of bacterial biomass in the 0.2- to 5.0-μm size class. In both flasks, bacterial aggregates constituted a small fraction of the >5.0-μm size class (0.24 ± 0.31% in the diffusion flasks and 7.1 ± 6.4% in the ingestion flasks). The amount of organic carbon per protozoan was calculated in each of the experimental bottles by dividing the organic carbon concentration in the >5.0-μm fraction by protozoan number (Table 3).

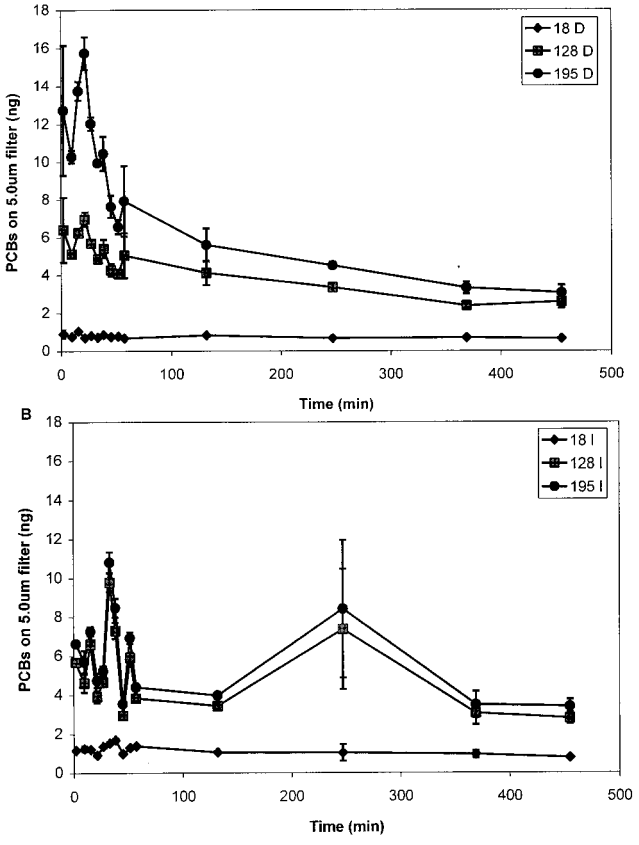

FIG. 1. Mass of three selected congeners (18, 128, and 195) retained on a 5.0-μm-pore-size filter versus time. (A) Diffusion flask replicate 1; (B) ingestion flask replicate 1. Open symbols are used for samples in which one of the recovery standards in the filtrate was too low and only one recovery standard could be used to estimate CB content.

Quantities of each congener occurring in the protozoan size class in both flasks increased rapidly and achieved maximal values within 20 min of CB inoculation (Fig. 1). To circumvent volatilization and emulsion complications, congener concentrations were normalized to the total extracted at each time point. The maximum percentage of each congener within the protozoan size class (>5.0 μm) was achieved quickly. Relative quantities of each congener in the protozoan size class followed the trend expected from the hydrophobicity (K_{ow} values) of the congeners, i.e., high K_{ow} (high-molecular-weight) congeners should have higher concentrations in the organic phase than low-K_{ow} congeners. In addition, there was no time lag associated with the diffusive uptake pathway. Significant losses of PCBs were observed during the experiment. The dynamics and magnitude of this loss were consistent with volatilization (13).

Organic-carbon-normalized CB concentrations. CB concentrations in the protozoan size class were normalized to organic carbon content by dividing PCBs caught on the 5.0-μm-pore-size filter (at 6 h) by the >5.0-μm particulate organic carbon (POC) concentration. Organic-carbon-normalized CB concentrations were calculated for each congener in each experimen-

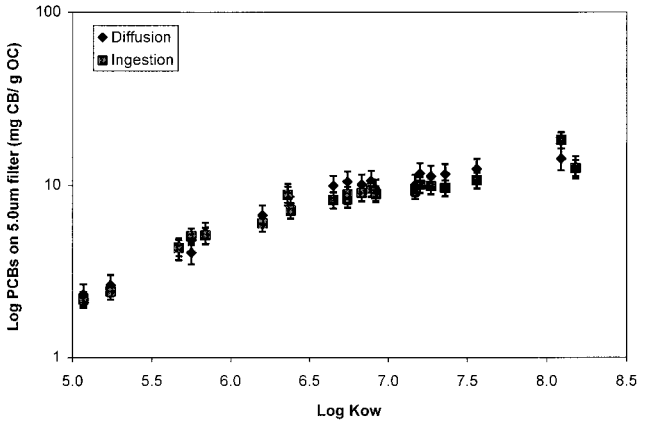

FIG. 2. Organic-carbon (OC)-normalized CB concentrations in the protozoan size class for each congener in the experimental flasks. The log of OC-normalized CB concentrations for the diffusion and ingestion flasks were plotted against the log K_{ow} of the appropriate congener. OC-normalized concentrations were calculated by the following formula: (number of PCBs caught on a 5.0-μm-pore-size filter)/(POC > 5.0 μm). Errors were propagated from errors on both CB and POC measurements.

tal flask and were plotted against log K_{ow} (Fig. 2). Data from the two treatments (diffusion and ingestion) were compared by using a Student's t test (95% confidence level) and two-way analysis of variance ($\alpha = 0.05$; $F = 3.58$; $F_{crit} = 4.35$). For both tests, the organic-carbon-normalized concentrations for each congener were statistically indistinguishable in the two flasks, suggesting that PCBs had accumulated in the protozoan size class of each experimental flask according to organic carbon content.

Rate constants for protozoan uptake and bacterial loss of PCBs. The Ag filters adsorbed a small fraction of the [^{14}C]TCB (on average, 15.5 ± 2.0 dpm/ml filtered; $n = 34$, roughly 15% of the total [^{14}C]TCB added). Background filter-associated [^{14}C]TCBs were subtracted from the 5.0-μm-pore-size filters in the protozoan culture experiment to determine the amount associated with the protozoa (Fig. 3). The data from the short-term radioactive diffusion experiment was assumed to exhibit

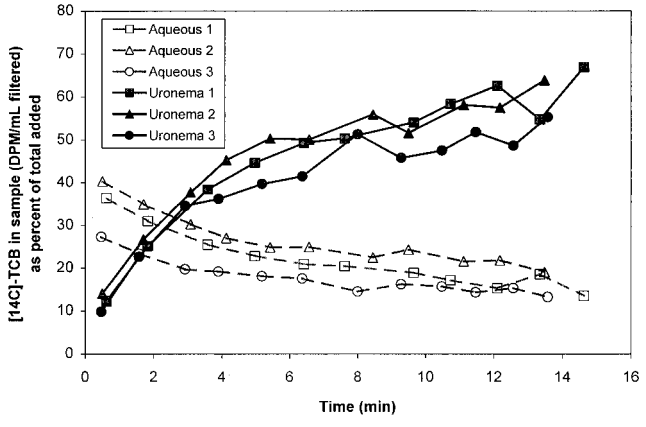

FIG. 3. Radioactive bioaccumulation experiment. Fifty milliliters of a protozoan culture was filtered through a 5.0-μm-pore-size filter for each time point (solid lines). Three replicate experiments are shown. Aqueous CB concentrations are also shown as a function of time for three replicates (dashed lines) and are corrected for CBs associated with DOC. Organic carbon concentrations used for each replicate were as follows: experiment no. 1, 5.62 mg per liter; experiment no. 2, 4.63 mg per liter; and experiment no. 3, 9.16 mg per liter.

pseudo-first-order uptake of [^{14}C]TCB by the protozoa (>5.0-μm size class).

The following system of equations was solved analytically:

$$\frac{d[CB]_{Aq}}{dt} = \frac{k_{rev}}{[P] \times [OC]_p}[CB]_{prot} - k_{for}[CB]_{Aq} - [DOC] \times K_{OC} \times \frac{d[CB]_{Aq}}{dt} \quad (13)$$

$$[CB]_{DOC} = K_{OC} \times [DOC] \times [CB]_{Aq} \quad (14)$$

$$[CB]_{Tot} = [CB]_{Aq} + [CB]_{DOC} + [CB]_{prot} \quad (15)$$

where $[CB]_{Aq}$, $[CB]_{DOC}$, and $[CB]_{prot}$ are the CB concentrations in the aqueous, DOC, and protozoan pools, respectively (disintegrations per minute per milliliter); k_{for} and k_{rev} are the uptake and loss rate constants (per minute); $[DOC]$ is the concentration of DOC (grams of organic carbon per milliliter); $[P]$ is the protozoan concentration (cells per milliliter); $[OC]_P$ is the organic carbon per protozoan cell (grams of organic carbon per cell) as determined in Table 3; and K_{OC} is the organic-carbon-to-water partition coefficient (disintegrations per minute of CB per gram of organic carbon per disintegrations per minute of CB per gram of water) from Schwarzenbach et al. (23). The analytical solution to this system of equations is as follows:

$$[CB]_{Aq,t} = [CB]_{Aq,0}\, e^{-Xt} + \frac{k_{rev} \times [CB]_{Tot}}{k_{rev} \times (1 - K_{OC} \times [DOC]) + k_{for} \times [P] \times [OC]_P}(1 - e^{-Xt})$$

$$\text{where: } X = \frac{k_{rev}}{[P] \times [OC]_P} + \frac{k_{for}}{1 + K_{OC} \times [DOC]} \quad (16)$$

The fit of this analytical solution to the radioactive data was performed by using the Levenberg-Marquardt method and generated values for the two rate constants: $k_{for} = 0.38 \pm 0.03$ min^{-1} and $k_{rev} = 1.1 \pm 0.1 \times 10^{-5}$ (g of organic carbon)$^{-1}$ min^{-1} (Fig. 4).

The time scales of protozoan uptake and of bacterial loss of PCBs were then compared to ensure that the protozoan diffusive uptake could be supplied adequately by loss from the bacterial pool. The bacterial loss rate constant was estimated by using a variation on the calculation for diffusive uptake presented in the introduction (13). From this calculation, we concluded that the protozoan uptake rate constant and bacterial loss rate constant were of similar magnitude, and thus, the bacterial loss rate constant, k_{dep}, was estimated to be approximately 0.38 min^{-1}.

Comparison of protozoan uptake rate and bacterial depuration rate. The numerical model was run with the protozoan rate constants derived from the regression analysis of the radioactive uptake experiment. The total activity of [^{14}C]TCB in the model system was 100 dpm (in a 1-ml system). As written, the model did not include uptake into the bacteria. If alternate values were used for a bacterial uptake rate constant ($10 \times k_{dep}$, $2 \times k_{dep}$, and $0.1 \times k_{dep}$), the relative amounts of PCBs in bacterial and protozoan pools changed, but the time required for equilibration did not (<15 min). The addition of ingestive uptake of PCBs did not change the model results for mass of PCBs within the protozoan pool or for time to equilibration (Table 4).

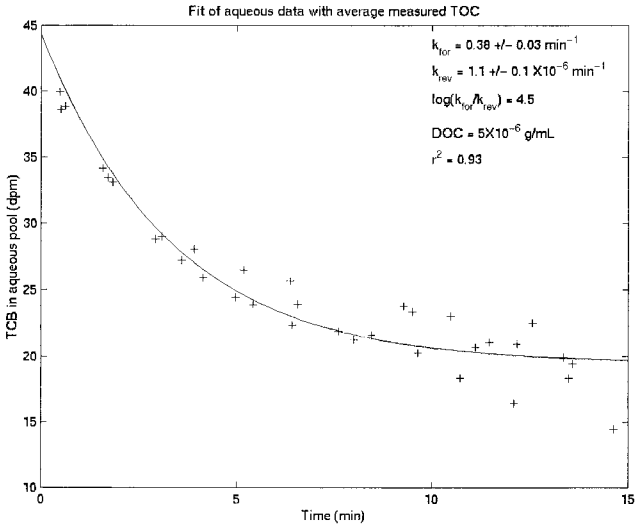

FIG. 4. Nonlinear regression fits for data from radioactive experiment. Data points are [^{14}C]TCB aqueous concentrations as calculated from DOC concentrations and [^{14}C]TCB (>5.0 μm). Average concentration of DOC was 5×10^{-6} g/ml. The aqueous pool refers to the concentration of truly dissolved CB. The analytical solution derived in the text was fit to the radioactive data by using the Levenberg-Marquardt method of nonlinear regression.

DISCUSSION

This data set demonstrates that the ingestion pathway does not contribute additional PCBs to the protozoan cell above those assimilated through diffusion. If ingestion was contributing PCBs over those accumulated via diffusion, a significant increase in organic-carbon-normalized concentration in the ingestion flasks would be observed. The observations in this study are consistent with the hypothesis that CB uptake is driven by diffusion and the steady-state cellular CB concentration is determined by the hydrophobicity of the CB congener.

The change in slope of log organic-carbon-normalized concentration versus log K_{ow} (log $K_{ow} > 6.5$ [Fig. 2]) has been observed by other investigators (26). They assumed that this plateau in particulate CB concentrations indicated the presence of a short-term surface adsorption constant that was independent of congener hydrophobicity (25). They hypothesized subsequent slow secondary uptake into internal cellular pools. Yet, the similarity of organic-carbon-normalized concentrations suggests that PCBs have been assimilated into all organic-carbon-containing cellular compartments (Fig. 2). Due to increased hydrophobicity, a greater percentage of higher-chlorinated congeners is associated with DOC. Low aqueous concentrations and high CB-DOC complex concentrations can potentially lead to decreased availability for diffusive uptake. DOC concentrations of approximately 7 mg per liter would be sufficient to explain lower concentration of organic-carbon-normalized CB in this system (13). This value is within the range of DOC concentrations observed in cultures of this organism (2 to 15 mg per liter).

TABLE 4. Results from numerical model runs[a]

k_{dep} (min^{-1})	Ingestion	Time to equilibrium (min)	Final concn (% at 60 min)
0.38	N	7.3	31
0.38	Y	7.3	31
0.038	N	7.0	31
0.038	Y	7.0	31
3.8	N	6.4	31
3.8	Y	6.4	31

[a] Initial conditions: concentration of protozoa = 10^4 cells/ml, concentration of bacteria = 10^7 cells/ml, concentration of DOC = 4×10^{-6} g/ml, ingestion rate = 0.56 bacteria/min, CB in bacterial cells = 8.06%, CB in aqueous phase = 45.87%, CB in DOC pool = 46.07%, k_{for} = 0.38 min^{-1}, $k_{rev} = 1.1 \times 10^{-5}$ (g of organic carbon)$^{-1}$ min^{-1}.

The equivalence of organic-carbon-normalized CB concentrations in both diffusion and ingestion flasks (Fig. 2) is compelling evidence in this study that diffusion is the primary method of CB uptake for the ciliate studied here. This conclusion was further bolstered by subsequent radioactive experiments in which three replicate trials of CB no. 77 uptake into the ciliate exhibited pseudo-first-order kinetics with respect to aqueous CB concentration. In our study, equilibrium among different organic carbon pools was achieved quickly, and diffusion dominated CB uptake. Results of the numerical model supported our hypothesis in that equilibration occurred quickly (<10 min) and ingestion played a minor role in overall CB uptake.

These results could be extended to other prey species such as cyanobacteria or phytoplankton. Differences in cell composition among these various species would affect prey CB concentrations. However, the ratio of diffusive to ingestion uptake rates is so large that small variations in prey CB concentrations should not affect the general conclusions of this study. Changes in prey species will also influence protozoan clearance rates. Unless the increase in clearance rate is a factor of 10 or higher, diffusion will still out-compete ingestion and the conclusions of this study will remain unchanged.

The conclusion of this study has implications for the prediction of uptake pathways in other organisms. We have shown that diffusion dominates uptake in protozoa less than 15 μm in size. Trophic transfer studies have shown that ingestion dominates CB uptake in macroscopic organisms (22). Therefore, there must be a transition in the size spectrum of organisms between diffusion- and ingestion-dominated CB uptake. We can estimate this transitional size by comparing the ratios of diffusion and ingestion rates between species within a phylum. We assumed that cellular membrane characteristics did not change within this group of organisms, and thus, all parameters in equation 3 were held constant except those relating to cell size and ingestion and/or clearance rates.

The transitional size where uptake via diffusion and ingestion are equivalent was estimated in two ways: first, by varying feeding rates in a series of ciliate species and second, by varying clearance rates and optimal prey concentrations for the same series of species. We used feeding rates for a number of ciliate species ranging from 4 to 400 μm in diameter from Fenchel (11) and substituted them into the relationship between maximum ingestion rate and cell size from Fig. 2 of Fenchel (11): $IR_{max} = 2.78 \times 10^{-4} \times \text{Vol}^{0.85}$, where IR is ingestion rate (in cubic meters per second) and Vol is cell volume (in cubic meters). After maximum ingestion rates for $CR \times BC \times BV$ are substituted in equation 3, the ratio between diffusion and ingestion reduces to a function of cell radius ($0.00834r^{-0.55}$). From this relationship, the cell radius at which diffusion and ingestion are equal is approximately 166 μm, corresponding to a cell diameter of 332 μm.

To obtain an independent estimate, maximum clearance rates (CR) and optimal prey concentrations ($BC \times BV$) for each species studied by Fenchel (11) can be employed in a manner similar to that shown above. In this estimate, diffusion and ingestion were equivalent at approximately 50-μm cell radius or 100-μm cell diameter. This second estimate was consistent with field data of Axelman et al. (2), who observed that

particles greater than 20 μm had lower CB concentrations than predicted from equilibrium calculations.

Both calculations presented above probably overestimate the importance of diffusion, because certain factors were not considered. After PCBs are incorporated into the cellular membrane, they are transported to other cellular compartments by diffusion and/or internal mixing. In equation 1, the cellular mixing rate was assumed to be practically instantaneous so that the rate-limiting step for cellular CB uptake was transport through the phospholipid membrane. As cell size increases, mixing within the cell will play a larger role in the overall accumulation of PCBs by lengthening the time for diffusive equilibration.

Full equilibration with internal cellular compartments will be further inhibited by cellular growth and addition of biomass, most noticeably in larger cells. This phenomenon was not observed in our cultures, but it is possible that the large surface-area-to-volume ratio of the ciliate obscured this effect. However, biomass dilution was observed in algal cultures by Swackhamer and Skoglund (28). Since algae (20 to 30 μm) were not capable of ingesting CB-laden particles, they were dependent on diffusion as an uptake mechanism and thus were potentially affected by the surface-area-to-volume dependence of diffusive equilibration. While the calculations above predicted diffusive equilibrium for organisms within this size range, it is possible that these organisms were large enough to be affected by internal equilibration barriers.

Lastly, there was no attempt in the above calculation to address the effect of cellular surface composition or increased surface area due to the presence of frustules (e.g., diatoms) or reticulopodia (e.g., foraminifera and radiolaria). These morphological features are composed of materials that are lipid poor and thus should have much lower affinity for PCBs than phospholipid bilayers. However, increases in surface area should increase the diffusive flux to the cell surface. The overall effect of these counterbalancing parameters will be species dependent. Being mindful of the limitations of these calculations, the best estimate at this time for the transitional size where diffusion is approximately equal to ingestion is a 50- to 170-μm cell radius (or a 100 to 340-μm cell diameter).

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