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Effects of Polychlorinated Biphenyls on Growth and Respiration of Heterotrophic Marine Bacteria†

RICHARD P. BLAKEMORE††* AND ANNE E. CAREY
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

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A number of marine bacterial isolates from both near-shore and open-ocean environments were tested for growth inhibition with exposure to low concentrations (1 to 100 μg/liter) of Aroclor 1254, a commercial mixture of polychlorinated biphenyls (PCBs). Of over 17 bacterial cultures tested, growth of only two open-ocean isolates, one a pseudomonad and the other a tetrad-forming coccus, was consistently inhibited by Aroclor at concentrations as low as 10 μg/liter (10 ppb). Growth inhibition was dose dependent over a concentration range of 10 to 100 μg/liter. The effects upon division rates and final cell yields of each bacterial isolate were greatest when PCBs were added to cultures with low cell densities or with lower specific growth rates. The pseudomonad also had reduced carotenoid levels and an altered filamentous morphology with Aroclor present at a concentration of 10 μg/liter, or more. The effects noted were reversible for at least 18 h after initial exposure. Concentrations of Aroclor in excess of those needed to stop growth had no detectable effect upon the respiration rate of cells of either culture. This suggests that the reduced division rates observed were not due to inability of PCB-treated cells to transport or catabolize the carbohydrate or amino acid substrates tested.

Polychlorinated biphenyls (PCBs) are chemically and physically stable compounds which have been released to the environment solely as a result of human activity. They have become ubiquitously distributed throughout the world and its biota within the past 20 years (8, 9, 14, 15). Like many other halogenated aromatic hydrocarbons, PCBs are toxic to eucaryotes, in some instances at concentrations not much above present environmental levels (5, 6, 13, 14).

Others have shown that PCBs inhibited growth of selected pure cultures of bacteria when added to liquid growth medium (2). Sensitivity of bacteria at PCB concentrations lower than 1 mg/liter (1 ppm) has not commonly been reported, however, and information concerning the physiological basis of PCB toxicity in bacteria is meager.

This study grew out of a survey of marine microorganisms for effects of Aroclors. Of 17 bacterial isolates tested, two remained sensitive to low levels (10 to 100 μg/liter) during extended laboratory culture. Presented here are our findings of the effects of Aroclor 1254 on the growth and respiration of these two isolates in batch culture.

† Contribution no. 4052 of the Woods Hole Oceanographic Institution.

†† Present address: Department of Microbiology, University of New Hampshire, Durham, NH 03824.

MATERIALS AND METHODS

Organisms. The organisms used in this study were isolated by E. R. Gonye, C. C. Remsen, K. M. Ulmer, and one of us (A.E.C.). Isolate 41 was recovered from seawater in the North Atlantic Ocean at 23°01′ N, 45°02′ W on 16 October 1973 (*R.V. Atlantis II*, cruise 78). Organism F1-21 was isolated at 13°39′ N, 65°39′ W on 9 August 1974 (*Atlantis II*, cruise 84). Both were isolated by plating surface seawater samples on marine nutrient agar.

Cells of isolate 41 were gram-negative, rod-shaped, motile bacteria, each with a single polar flagellum. They produced pink to yellow-orange pigments (depending upon the nutrient concentration of the culture medium) with absorption spectra typical of carotenoids. The organism was a strict aerobe that oxidized sugars and amino acids and degraded proteins and complex carbohydrates. It was tentatively identified as a pseudomonad (3).

Cells of isolate F1-21 were gram-positive, non-motile, yellow cocci occurring in pairs or tetrads typical of cells of the genera *Micrococcus* or *Aerococcus* (3). This organism oxidized glucose at significant rates but not several single amino acids tested.

Culture conditions. Marine nutrient agar consisted of (grams per liter of distilled water): nutrient agar (Difco), 23; NaCl, 29; $MgCl_2$, 12.1; KCl, 1.5. For growth experiments, cells were cultured in either dilute nutrient broth-salts (DNS) or seawater supplemented with Casamino Acids and yeast extract (SWCAA). DNS consisted of (grams per liter of distilled water): nutrient broth (Difco), 0.8; NaCl, 29; $MgCl_2$, 12.1; KCl, 1.5. For some experiments, lower

concentrations of nutrient broth were used in DNS, but the salt concentrations remained the same. SWCAA consisted of (g/750 ml of filtered, aged Sargasso Sea water): Casmino Acids (Difco, technical), 0.5; distilled water, 250. The seawater medium was adjusted to pH 4 with HCl and autoclaved. After cooling to room temperature, the medium was adjusted to pH 7.4 to 7.6 with sterile NaOH. A sterile solution of 10% (wt/vol) yeast extract in distilled water was then added to give a final concentration of 0.05 to 0.1 g/liter.

Cultures were grown in Erlenmeyer flasks or in 250-ml Nephelo flasks (Kontes no. 881800-0414). Culture media were inoculated to 1% of their volume with a 12-h culture to an initial cell density of approximately 5×10^5 cells per ml. Cultures were incubated on a gyratory shaker (125 rpm) at 22°C under constant illumination of 1300 lx. To prevent volatilization of PCBs, flasks were sealed with rubber stoppers covered with aluminum foil or with screw caps with Teflon liners.

All glassware used in culturing bacteria, as well as respirometer reaction vessels, were washed in warm detergent, rinsed with distilled water, and then rinsed with reagent grade 95% ethanol and/or Nanograde hexane (Mallinkrodt Chemical Co., St. Louis, Mo.). Glassware was then baked in an oven at not less than 100°C for 6 to 24 h. After this treatment, there were no detectable traces of PCBs on glassware as determined by gas chromatographic analysis using established procedures (7). The method was sufficiently sensitive to detect 0.2 ng of Aroclor.

Aroclor 1254 (Monsanto Chemical Co., St. Louis, Mo., lot no. AA-1), a commercial mixture of polychlorinated biphenyl isomers that averages 54% chlorine by weight, was used throughout this study. Because Aroclor is only slightly soluble in water, stock solutions were prepared in Nanograde methanol (Mallinkrodt Chemical Co., St. Louis, Mo.) and diluted 10,000-fold by volume in the cultures. Aroclor solutions or comparable volumes of methanol were injected beneath the surface of inoculated medium.

Estimates of cell numbers. Direct cell counts were made with an improved Neubauer cell-counting chamber. Optical density measurements were taken at 540 nm, using a Bausch & Lomb Spectronic 20 colorimeter or a Beckman model B spectrophotometer with a holder to accommodate culture tubes and sidearms of Nephelo flasks. Optical density readings were occasionally converted to cell numbers from standard curves constructed from direct cell counts.

Attempts to determine viability of PCB-treated cells by plating fractions of serially diluted cultures gave variable and non-reproducible results. Dry weight measurements did not give values representative of cell weights because of the high concentration of salts in buffers used to suspend cells.

Preparation of cell suspensions. Exponentially growing cells from 0.5 to 2 liters of medium were harvested by centrifugation at $5,000 \times g$ for 20 min at 4°C in a Sorvall RC-2B centrifuge. The cell pellets were washed twice by suspending them in 10 times their volume of 0.05 M potassium phosphate buffer (pH 6.3 or 7.4) containing 29 g of NaCl or 40 g of Seven-Seas Marine Mix (Utility Chemical Co., Patterson, N.J.) per liter. The suspensions were centrifuged, and the pellet of washed cells was resuspended in buffer and, unless otherwise indicated, stored overnight at 4°C to deplete cells of endogenous oxidizable reserve materials. The protein content of washed-cell suspensions was determined by the method of Lowry et al. (12), using crystalline bovine serum albumin (K & K Laboratories, Plainview, N.Y.) as the standard.

Respiration measurements. Measurements of oxygen uptake by cell suspensions were made at the growth temperature (22°C) with a Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, Wis.) by conventional manometric techniques (16). Reaction volumes were 3.1 ml. Aroclor or methanol was added to the sidearm containing substrate. O_2 uptake was measured after a 15-min equilibration period.

RESULTS

The aerobic growth response of 17 isolates of heterotrophic marine bacteria to Aroclor 1254 at concentrations from 10 to 100 μg/liter was tested in liquid medium. When treated with up to 100 μg of Aroclor per liter, most open-ocean and near-shore isolates showed no detectable change in their division rate, final cell yield, motility, or morphology when compared with untreated or methanol-treated control cultures. Several isolates initially sensitive to Aroclor gradually became more resistant upon repeated transfer in standard laboratory medium.

Bacterial isolates 41 and F1-21 were chosen for additional study since they remained sensitive to low levels of Aroclor 1254 (10 μg/liter) for many months in laboratory culture. In addition, these two isolates differed morphologically and physiologically.

Organism 41 grew with a generation time of 1.2 h in DNS medium (Fig. 1A) and in SWCAA medium (Fig. 2A). Cell growth was completely inhibited by 10 to 100 μg of Aroclor per liter added immediately after inoculation (Fig. 1H). Cell division diminished (Fig. 1C; Fig. 3C) or stopped (Fig. 1G) within one doubling period of adding 100 μg of Aroclor per liter to exponentially growing cells. Methanol had no discernible effect upon division rates or final cell yields when added to cultures at the concentration at which it was used as a PCB carrier (Fig. 1, cf. A with B, D with E; Fig. 3, cf. A with B, D with E, G with H, J with K).

The extent of growth inhibition was dose dependent at 10 and 100 μg of Aroclor per liter (Fig. 1F and G). Aroclor inhibited growth to a greater percentage of the control values when added at low cell densities (Fig. 1, cf. F with H), or when added to exponentially growing cells with low specific growth rates (Fig. 1, cf. C with G) or to cultures with lower concentrations of nutrients in the medium (Fig. 3). At PCB con-

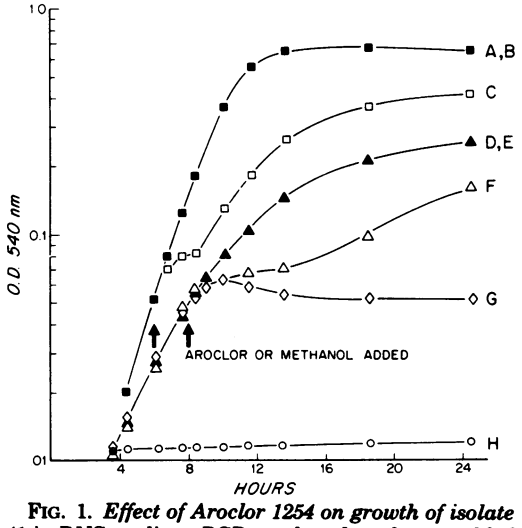

FIG. 1. *Effect of Aroclor 1254 on growth of isolate 41 in DNS medium. PCBs and methanol were added at the time indicated by arrows, except H. A through C are for DNS medium. D through H are for 1/10-strength DNS medium. C and G are for 100 μg of Aroclor per liter. F represents 10 μg of Aroclor per liter. B and E are for methanol (100 μl/liter) controls. A and D represent untreated controls. H indicates the response when Aroclor (10 or 100 μg/liter) was added at the time of inoculation.*

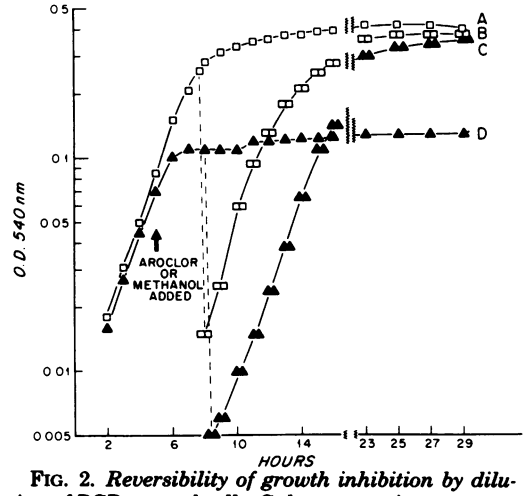

FIG. 2. *Reversibility of growth inhibition by dilution of PCB-treated cells. Cultures growing exponentially in 100 ml of SWCAA medium were treated (arrow) with 200 μg of Aroclor (D) or methanol (A) per liter. Approximately 3 h later, a 10-ml fraction of each culture was diluted into 100 ml of fresh medium (dashed lines) and incubated (B and C).*

centrations that did not completely arrest division (Fig. 1C and F; Fig. 3C), cells grew at reduced rates and to lower final yields than did untreated or methanol-treated controls. Effects of PCBs on growth rates were detectable within several hours after exposure to Aroclor. Treated cells exhibited lower levels of carotenoid pigments and more prevalent chaining than controls. Cells whose growth had been totally inhibited by 200 μg of Aroclor per liter, when transferred into PCB-free medium, achieved, without lag, normal growth rates (Fig. 2), and, at the end of exponential growth the cell yield, pigment content, and morphology were normal. Effects of PCBs were reversible within 3 and for at least 18 h after exposure. Cell lysis was not microscopically detectable in cultures exposed to Aroclor at the concentrations used in our studies.

Because of the lipophilic nature of PCBs, they are most likely to accumulate in cell membranes. Their toxic effect could be to inhibit membrane-associated functions such as transport and/or oxidation of energy sources required for growth. To test this possibility, we studied respiration of various substrates by isolates 41 and F1-21 in the presence and absence of Aroclor.

Washed cells of isolate 41 oxidized glucose at the same rate (3.5×10^{-8} nmol of O_2 consumed/cell per min) in the presence of greater than growth-inhibiting amounts of Aroclor (300 μg/liter) as did control cells. Methanol in concentrations as high as 0.5% (vol/vol) had no detectable effect on endogenous or substrate-facilitated respiration rates by cell suspensions.

We also measured respiration rates of cells whose growth had been inhibited by Aroclor and

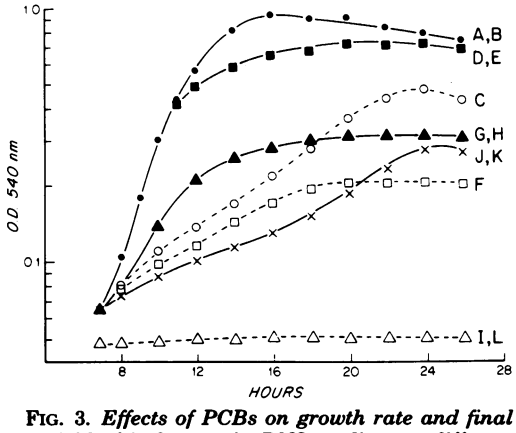

FIG. 3. *Effects of PCBs on growth rate and final cell yield of isolate 41 in DNS medium at different nutrient concentrations. PCBs or methanol were added at the time of inoculation. A through C are for DNS medium; D through F represent 1/2-strength DNS. G through I are for 1/10-strength DNS. J through L are for 1/20-strength DNS. A, D, G, and J indicate untreated controls. B, E, H, and K are for methanol (100 μl/liter) controls. C, F, I, and L are for Aroclor (100 μg/liter)-treated cultures.*

compared them with rates by untreated cells. Cell suspensions were prepared from cultures to which either Aroclor (100 μg/liter) or methanol was added when the density of the exponentially growing bacteria reached 4.0×10^7 cells per ml. Cells, in exponential growth, were harvested 3.5 h later when the density of the PCB-treated culture (6.4×10^7 cells per ml) was 40% of that of the control. Cell respiration rates, with glucose as substrate, were immediately determined. Cells pretreated with Aroclor (Fig. 4C) consumed O_2 at a rate (1.75×10^{-8} nmol/cell per min) similar to that (1.64×10^{-8} nmol/cell per min) of methanol-treated cells (Fig. 4B). Cells from cultures to which no PCBs had been added (Fig. 4A and B) had similar respiration rates for over 3 h from the time glucose was supplied whether Aroclor was present with the substrate (A) or not (B). Aroclor had no effect upon the endogenous respiration rate.

Isolate 41 was most sensitive to Aroclor 1254 (1 to 10 μg/liter) when growing in DNS medium. Nevertheless, cells pretreated with 100 μg of Aroclor per liter during growth in this medium had similar rates of O_2 consumption (2.7 ± 0.14 nmol/μg of cell protein per h) with glucose as substrate as did controls either with, or without, 300 μg of Aroclor per liter. Rates are per amount of cell protein due to difficulty in obtaining accurate cell counts in this medium.

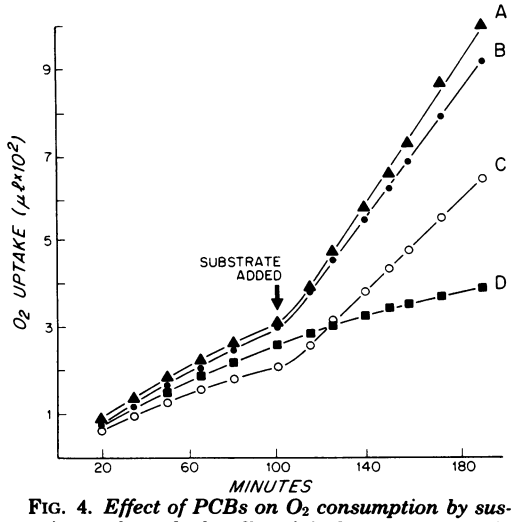

FIG. 4. *Effect of PCBs on O_2 consumption by suspensions of washed cells of isolate 41 grown in SWCAA medium. Each reaction vessel contained cells suspended in 3.0 ml of filtered seawater containing 150 μmol of potassium phosphate buffer (pH 6.8). A, B, and D were each obtained with 1.76×10^{10} cells from a culture to which methanol (100 μl/liter) was added 3.5 h before harvest. C shows the rate obtained with 1.2×10^{10} cells from a culture to which Aroclor (100 μg/liter) was added 3.5 h before harvest (See Fig. 1.). At the time indicated by the arrow, glucose (50 μmol) was supplied to cell suspensions used to obtain A, B, and C. Aroclor (1 mg/liter) was added with glucose to cells used to obtain A. All values are uncorrected for the endogenous respiration rate (D).*

We studied the effect of PCBs and methanol on the rate of amino acid oxidation by cell suspensions of strain 41 grown in SWCAA. Oxygen consumption rates (2×10^{10} cell per reaction flask) with 40 μmol of leucine as substrate were similar by cells treated with 1 mg of Aroclor per liter (9.96×10^{-9} nmol/cell per min), by methanol-treated cells (9.90×10^{-9} nmol/cell per min), and by untreated cells (9.00×10^{-9} nmol/cell per min). The values were corrected for the endogenous rate of respiration, which remained constant over the 1-h interval studied. Similar results were obtained by using glycine and arginine.

Growth of isolate F1-21 was consistently inhibited by 10 to 100 μg of Aroclor per liter added to cultures at the time of inoculation or during exponential growth (data not shown). Methanol had no discernible effect upon division rates or final cell yields of this organism when added to cultures at the same concentration at which it was used as an Aroclor solvent.

Although initially inhibited by 1 μg of Aroclor per liter, this organism's growth response became unpredictable at PCB levels less than 10 μg/liter after 18 months in laboratory culture. For this reason, F1-21 was not studied as thoroughly as isolate 41.

Respiration rates for washed cells of isolate F1-21 were determined in the presence and absence of Aroclor. Cells were cultured in SWCAA medium containing 0.05% (wt/vol) glucose. Glucose was oxidized by the cells treated with greater than growth-inhibiting amounts of PCBs (1 mg/liter) at a rate (5.0×10^{-9} nmol/cell per min) similar to that of untreated cells (5.4×10^{-9} nmol/cell per min) and methanol-treated cells (5.5×10^{-9} nmol/cell per min).

DISCUSSION

Measurements of PCB concentrations in bacteria from natural environments or in laboratory experiments have not been reported. However, concentration factors of 3.4×10^5 for PCBs with suspended matter in seawater (8) and 1.4×10^4 for dichlorodiphenyltrichloroethane with bacteria in water (10) have been measured. PCB concentrations as high as 31 ppm (mg/kg, wet weight) have been reported in marine phytoplankton (17). PCBs partition rapidly and with a high affinity to surfaces (8), including those to which bacteria may be attached, or to bacterial cells themselves in aqueous environments. Thus, concentrations of chlorinated hydrocarbons in

bacterial cells in natural waters are probably within the range of PCB levels used in our experiments.

Concentration factors for other chlorinated hydrocarbons with bacteria in water increase in inverse proportion to the amount of cellular material present (10). Increased PCB sensitivity of our isolates at low cell densities can be explained by similar dosage considerations. PCB sensitivity may also depend upon the physiological state of the cell. Thus, cells growing at lower rates were more sensitive than faster-growing populations of the same species when treated with the same amount of Aroclor at similar population densities. Cells growing at faster rates were also growing with larger amounts of organic material present. This suggests the possibility that organic constituents of the medium might have bound or complexed with PCBs and thereby reduced the effective dose per cell.

Our observations are consistent with the possibility that, in natural environments, heterotrophic bacteria growing at low cell densities with low growth rates in water low in organic content might be more sensitive to Aroclor 1254 than when tested under laboratory conditions. Fisher and Wurster (6) have shown that phytoplankton were more sensitive to PCBs when growing under suboptimal conditions (temperature) than under optimal conditions for growth.

The effects of Aroclor 1254 on the sensitive bacteria studied was immediate (e.g., detectable as a modified growth rate within one doubling time of treatment) and reversible for periods of at least 18 h after initial exposure. The reversibility of effects of PCBs on growth rates and cell yields indicates that these compounds are bacteriostatic for cells of isolate 41 at the concentrations used in our work. The reversibility of these effects also indicates that PCBs that may have bound to cells of this organism were not irreversibly bound. Escape from growth inhibition was probably due to establishment of a new equilibrium among cells, flask walls, vapor phase, and medium when treated cells were placed in fresh medium.

Since cell lysis was not detected microscopically in PCB-treated cultures and, as mentioned, the PCB-induced inhibitory effects were reversible, we presumed that PCBs did not cause degradation of preformed structural components such as the cell wall or membrane.

Results of our respiration studies were consistent with the proposed bacteriostatic role of PCBs. Washed-cell suspensions exposed simultaneously to growth-inhibiting concentrations of Aroclor and oxidizable substrates showed no effect of PCBs on respiration rates. Cells whose growth had been inhibited by PCBs had similar respiratory activity for the carbohydrate and amino acid substrates tested as did untreated cells. Failure to observe measurable changes in cell respiration was not due to a possible effect resulting from harvesting and washing cells since additional PCBs added together with the substrates had no effect on respiration rates per cell in cells from cultures grown with or without PCBs in the medium. Inasmuch as we supplied test substrates exogenously, transport of these compounds through the cell membrane was also apparently unaffected by toxic concentrations of Aroclor.

While our work was in progress, a report appeared (11) indicating that concentrations of Aroclor (300 to 800 μg/liter) sufficient to inhibit growth of the green alga *Scenedesmus* failed to inhibit its respiration. Similar findings were also recently reported for *Euglena* (5). The failure of PCBs to affect this aspect of metabolism may be quite general.

The apparent uncoupling of transport and oxidation of selected substrates from growth in the bacteria studied suggested the possibility that PCBs might interfere with the biosynthetic rather than catabolic capacity of sensitive bacteria. Studies of the effect of Aroclor 1254 on macromolecular synthesis in strain 41 are reported in the accompanying manuscript (1).

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