APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1994, p. 434–446
0099-2240/94/$04.00+0

Vol. 60, No. 2

Multicellular Organization in a Degradative Biofilm Community

G. M. WOLFAARDT,[1,2] J. R. LAWRENCE,[1]* R. D. ROBARTS,[1] S. J. CALDWELL,[3] AND D. E. CALDWELL[2]

National Hydrology Research Institute, Environment Canada, Saskatoon, Saskatchewan, Canada S7N 3H5,[1] and Department of Applied Microbiology[2] and Department of Veterinary Anatomy,[3] University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

Received 31 August 1993/Accepted 20 October 1993

Diclofop methyl, a commercial herbicide, was used as the sole carbon source to cultivate diclofop-degrading biofilms in continuous-flow slide culture. The biofilms were analyzed by using scanning confocal laser microscopy and image analysis. Spatial relationships among members of the community were distinctive to diclofop-grown biofilms. These relationships did not develop when the biofilms were grown on more labile substrates but were conserved when the biofilms were cultivated with other chlorinated ring compounds. The structures included conical bacterial consortia rising to 30 μm above the surrounding biofilm, grape-like clusters of cocci embedded in a matrix of perpendicularly oriented bacilli, and other highly specific patterns of intra- and intergeneric cellular coaggregation and growth. These unique consortial relationships indicated that syntrophic interactions may be necessary for optimal degradation of diclofop methyl and other chlorinated ring compounds.

Many microbial processes occurring in the environment are not possible with single-species populations but require consortial activities (11). Such activities typically are interactions between two or more populations in a given community, which enable organisms to maximize their metabolic capabilities and to maintain community integrity and stability. Much of our understanding of these interactions stems from work done on the oral microflora. In a review on coaggregation among oral bacteria, Kolenbrander (19) noted that nearly all human oral bacteria participate in intergeneric coaggregation. Sjollema et al. (33) used the term "cooperative effects" among oral bacteria to designate a microbial process by which adhering cells can modify their surrounding environment into a more favorable one for further attachment. Further, a unanimous conclusion from surveys of more than 700 strains of oral bacteria was that partner recognition during bacterial coaggregation is very specific (19).

Anaerobic digesters represent another area in which metabolic cooperation between bacteria has been extensively studied. Anaerobic digestion of waste, such as industrial and municipal effluent, is efficient only when microbial aggregates in the form of biofilms, sludge granules, and flocs are present. Thiele et al. (36) referred to syntrophic relationships, facilitated by the formation of mixed microbial aggregates (or consortia), in which the participating organisms share a common spatial microniche. Interspecies hydrogen transfer is an example of such a syntrophic relationship in anaerobic digesters (25, 36). Microorganisms also interact with their physical environment. Grotenhuis et al. (13) demonstrated that the morphological types of microorganisms found in sludge granules are dependent on the wastewater quality and the process parameters used, while it was suggested by Atlas (2) that changes in the environment lead to changes in community composition.

Although the importance of microbial consortia in degrading environmental contaminants was recognized by many workers (22, 31) and the use of microorganisms for treatment of toxic chemicals is now commonplace (32), very little is known about the interrelationships between the organisms involved. In addition, most of the work has involved studies of pure (mostly planktonic) cultures, making it difficult to assess the role of complementary metabolic activities among degradative populations.

Dworkin (8) suggested that a variety of strategies, based on cell-cell interactions, have evolved among microorganisms as alternatives to their unicellular nature. One possible strategy may be a spatial arrangement of microorganisms within biofilms that facilitates the metabolism of compounds which are resistant to degradation by microbial populations from a single cell line. There is thus a need for nondestructive experimental techniques which enable examination of biofilms in their natural state to provide a better understanding of these interactions. Application of scanning confocal laser microscopy (SCLM) techniques largely fulfills this requirement. The use of SCLM to study the formation and structure of biofilms, as well as advantages of this technique, has been described elsewhere (4, 23). The objective of the present study was to assess whether adaptive strategies, in terms of structure, cellular composition, and spatial arrangements, could be observed in degradative biofilms when they were grown with chlorinated organic compounds as sole carbon and energy source.

MATERIALS AND METHODS

Culture conditions and inoculum. Continuous-flow culture chambers (flow cells) were constructed of Plexiglas (Fig. 1). The flow chambers were 1 mm deep, 3 mm wide, and 42 mm long. A no. 1 microscope coverslip, mounted on the Plexiglas with silicon adhesive (General Electric RTV), allowed microscopic examination of biofilm development in the flow chambers. Previous studies have demonstrated laminar flow in these chambers (20). A 6% hypochloride solution was used to surface sterilize the flow cells. A peristaltic pump (Watson Marlow 201Z) was used to maintain flow at 0.2 mm s^{-1}. All incubations were conducted at room temperature (23 ± 2°C).

* Corresponding author. Mailing address: National Hydrology Research Institute, 11 Innovation Blvd., Saskatoon, Saskatchewan, Canada S7N 3H5. Phone: (306) 975-5789. Fax: (306) 975-5143.

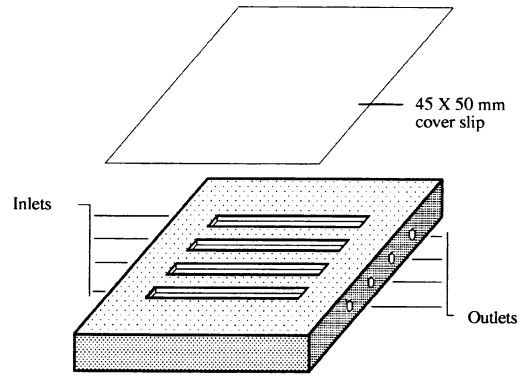


FIG. 1. Schematic diagram of the flow cell construction used to culture biofilms for microscopic examinations. The use of flow cells containing up to 10 growth chambers simplified handling and experimental replication when comparisons between biofilms grown on different carbon sources were made.

A degradative consortium, isolated from soil and maintained on the herbicide diclofop methyl, the methyl ester of 2-[4-(2,4-dichlorophenoxy)phenoxy]methyl propanoic acid (Riedel-de Haën; 99% purity), as the sole carbon source in a 250-ml continuous-culture system (D_t = 0.5), was used to inoculate flow cells. The inoculum (a single 0.5-ml pulse) was added directly upstream from the growth chamber while the pump was turned off. Flow was resumed 1 h after addition of inoculum.

Growth medium. Flow cells were irrigated with a minimal salts solution (6) amended with 14 μg of diclofop methyl per ml (7.8 μg of total carbon ml^{-1}) as the sole carbon and energy source for growth and biofilm development. The composition of the minimal salts solution (per liter) was as follows: for component A, 2 g of NaCl, 1 g of NH_4Cl, 0.12 g of $MgSO_4 \cdot 7H_2O$, and 1 ml of a trace element solution containing (per liter) 4 g of EDTA, 1.5 g of $CaCl_2$, 1 g of $FeSO_4 \cdot 7H_2O$, 0.35 g of $MnSO_4 \cdot 2H_2O$, and 0.5 g of $NaMoO_4 \cdot 2H_2O$; for component B, 4.24 g of Na_2HPO_4 and 2.7 g of KH_2PO_4. Components A and B were autoclaved separately and then mixed, and the diclofop was added after the mixture had cooled to room temperature. Diclofop methyl undergoes a rapid hydrolysis when mixed with water to form diclofop acid; therefore, for simplicity the term diclofop is used to indicate both methyl and acid forms. Biofilms were also grown on tryptic soy broth (TSB; Difco Co.) and diclofop-TSB mixtures. TSB concentrations applied were 18 μg ml^{-1} (7 μg of total carbon ml^{-1}) and 300 μg ml^{-1} (116 μg of total carbon ml^{-1}), respectively.

Biofilm responses to a change in carbon source were also investigated. In this case, development of separate biofilms, with diclofop or TSB as the sole carbon source, was monitored for 21 days and then the irrigation solutions were switched and the biofilms were examined to assess structural reorganization within biofilm communities when introduced to a new carbon source.

Biofilm formation on other chlorinated ring compounds. The ability of the diclofop-degrading consortia to grow and produce biofilms with other chlorinated ring compounds as the sole carbon and energy source was investigated by irrigating flow cells with the minimal salts solution plus 2,4-dichlorophenol, 1,3-dichlorobenzene, and 4-(2,4-dichlorophenoxy)phenol, a two-ring diclofop intermediate, at concentrations of 18, 16, and 14 μg ml^{-1}, respectively. These concentrations provided a carbon content equal to that supplied in the solution of 14 μg of diclofop ml^{-1}.

Laser microscopy. A scanning confocal laser (MRC-600; Bio-Rad Microscience, Mississauga, Ontario, Canada), mounted on a Nikon Microphot-SA microscope, was used to nondestructively obtain images of biofilms cultured in the flow cells. The microscope was equipped with a 60×, 1.4 numerical aperture oil immersion lens (Nikon Corp., Chiyoda-ku, Tokyo). The SCLM was operated as described previously (23). Optical thin sections in the *xy* plane, as well as *xz* sagittal images, were collected. Negative staining of microbial cells (at pH 6.6, the ambient pH in the flow cells) was achieved by fluorescence exclusion with fluorescein (5). Fluorescein absorbance is pH dependent and is quenched at low pH values. Therefore, to obtain positive staining of cells with fluorescein, the pH in the growth chamber was lowered to 4, and fluorescein that had penetrated the near-neutral internal pH in the cells fluoresced more brightly than in the surrounding medium. Biofilm material was also positively stained with Nile Red (Eastman Kodak Co., Rochester, N.Y.) a benzophenoxazinone dye specific for hydrophobic compounds (21). A staining solution at a final concentration of 5 μg of Nile red ml^{-1} in 50% aqueous glycerol was prepared from a 1-mg ml^{-1} stock solution of Nile red dissolved in acetone.

Image analysis. Biofilm thickness, cell density, and mean cell size in biofilms were compared for biofilms grown on diclofop and TSB. Biofilm thickness and percent cell area (cell density) in biofilms were assessed by image processing of *xz* sagittal and *xy* thin sections, respectively, of biofilms with a Northgate 80486 host computer and software provided by Bio-Rad. Mean cell size, measured as average cell area at a given horizontal plane, was determined by transferring images collected with the SCLM to an IBAS 2000 image analysis computer (Kontron, Eching, Germany). Cell boundaries were defined manually to differentiate noncellular from cellular material in biofilms. A detailed description of these methods and the application of image analysis in microbial ecology were presented by Caldwell et al. (4).

Electron microscopy. Diclofop-grown biofilms were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h, washed in three changes of 0.1 M phosphate buffer (pH 7.2), and postfixed with 1% osmium tetroxide in Ryter-Kellenberger buffer overnight. The biofilm material was then en bloc stained with 0.5% uranyl acetate in Ryter-Kellenberger buffer for 2 h and dehydrated in a graded ethanol series before infiltration and embedment in Epon 812 (7). Thin sections of the biofilms were viewed under a Philips 410LS electron microscope.

Total planktonic cell counts. The total number of bacterial cells in the effluent of flow cells was determined by a direct microscopic count. Cells were stained with 4′,6-diamidino-2-phenylindole and counted with an epifluorescence microscope according to the procedure described by Porter and Feig (29).

Characterization of flow cell communities. The BIOLOG microbial identification system (Biolog Inc., Hayward, Calif.) was used to compare flow cell and continuous-culture populations on the basis of community-level carbon source utilization. The wells of BIOLOG GN Microplates were filled (in triplicate) with 10^{-1} dilutions of effluents from flow cells and the continuous culture (which was used to inoculate flow cells). Plates were incubated at room temperature (23 ± 2°C) for 28 h, and the color reactions were recorded manually (utilization of a carbon source was indicated colorimetrically by reduction of tetrazolium violet contained in the wells). A description of the BIOLOG system and its use for the characterization of

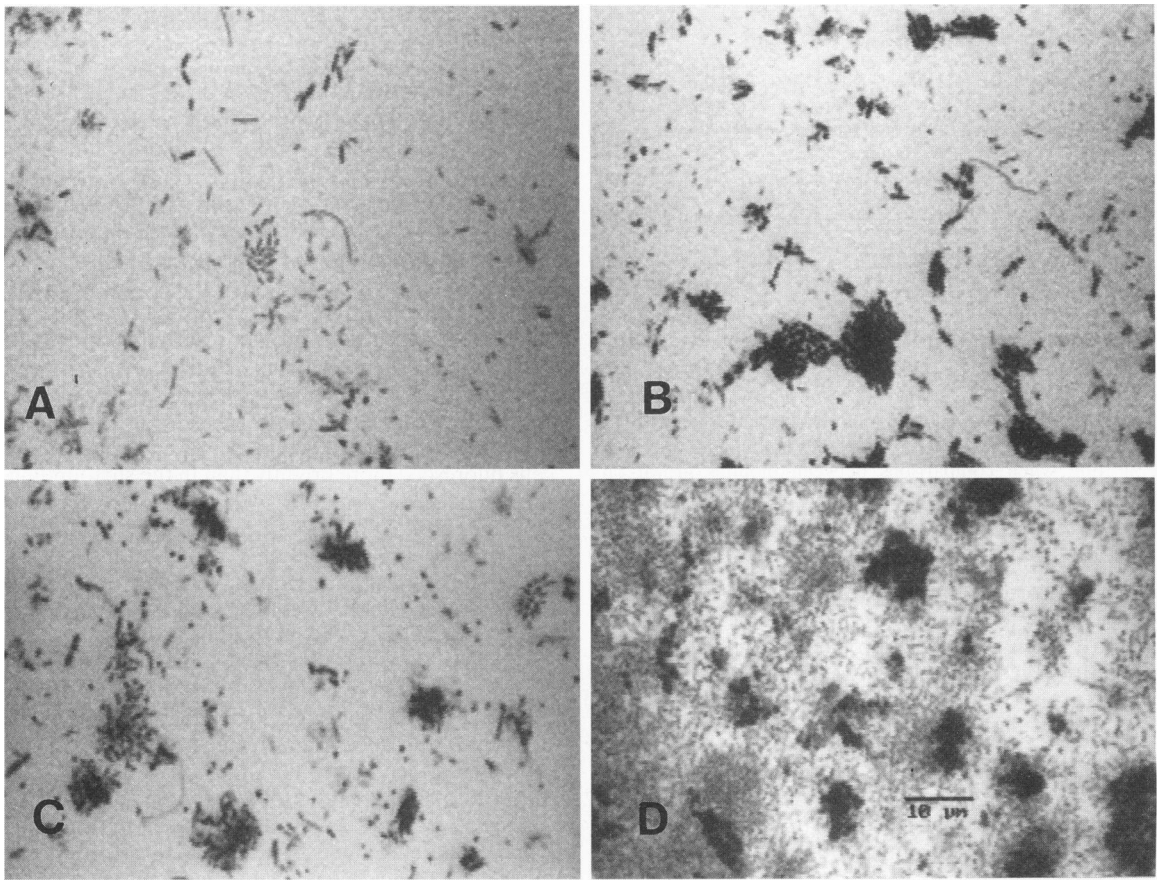


FIG. 2. Series of SCLM through-view images (0 to 5 µm) showing biofilm development in flow cells after 1 (A), 3 (B), 5 (C), and 8 (D) days, with diclofop (14 µg ml^{-1}) as the sole carbon and energy source.

heterotrophic microbial communities has been presented elsewhere (10, 17).

RESULTS

Horizontal organization of biofilms. Optical sectioning with the SCLM showed that biofilms grown on diclofop differed substantially from those grown on TSB. Biofilm formation followed a typical pattern when diclofop was provided as the sole carbon source; limited surface growth occurred during the early stages (first 7 days) (Fig. 2 and 3), with most cells being concentrated in clusters. Further biofilm development, in both the horizontal and vertical directions, was dominated by growth originating from these clusters. Once most of the surface was covered with biofilm material (±25% being cells, and the rest being exopolymers), the rate of vertical biofilm development increased with the simultaneous formation of additional clusters, containing up to 50 cells in a single horizontal plane (Fig. 2D). These clusters were randomly distributed through the biofilms (Fig. 4) and were on average 10.7 µm (standard deviation, 2.4 µm) apart in mature biofilms. Biofilm organization remained stable after 14 to 21 days; the films were then viewed as mature biofilms.

Cells from the same inoculum but grown on 18 or 300 µg of TSB ml^{-1} (with a total carbon content nearly equal to and 20

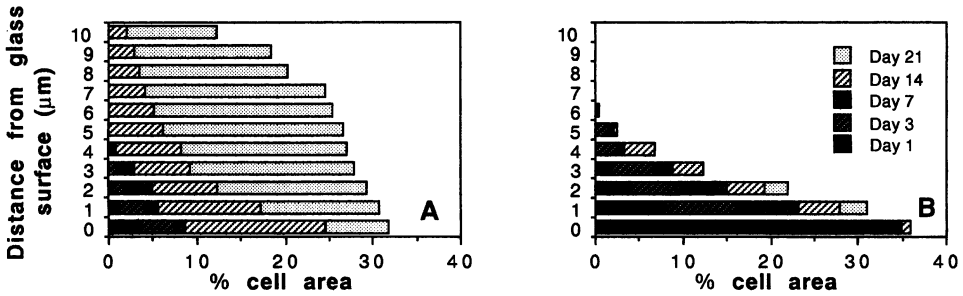


FIG. 3. Illustrations showing biofilm development (increase in area covered by cells at different depths with time) when the degradative consortium was grown on diclofop (A) and TSB (B) of equal carbon content.

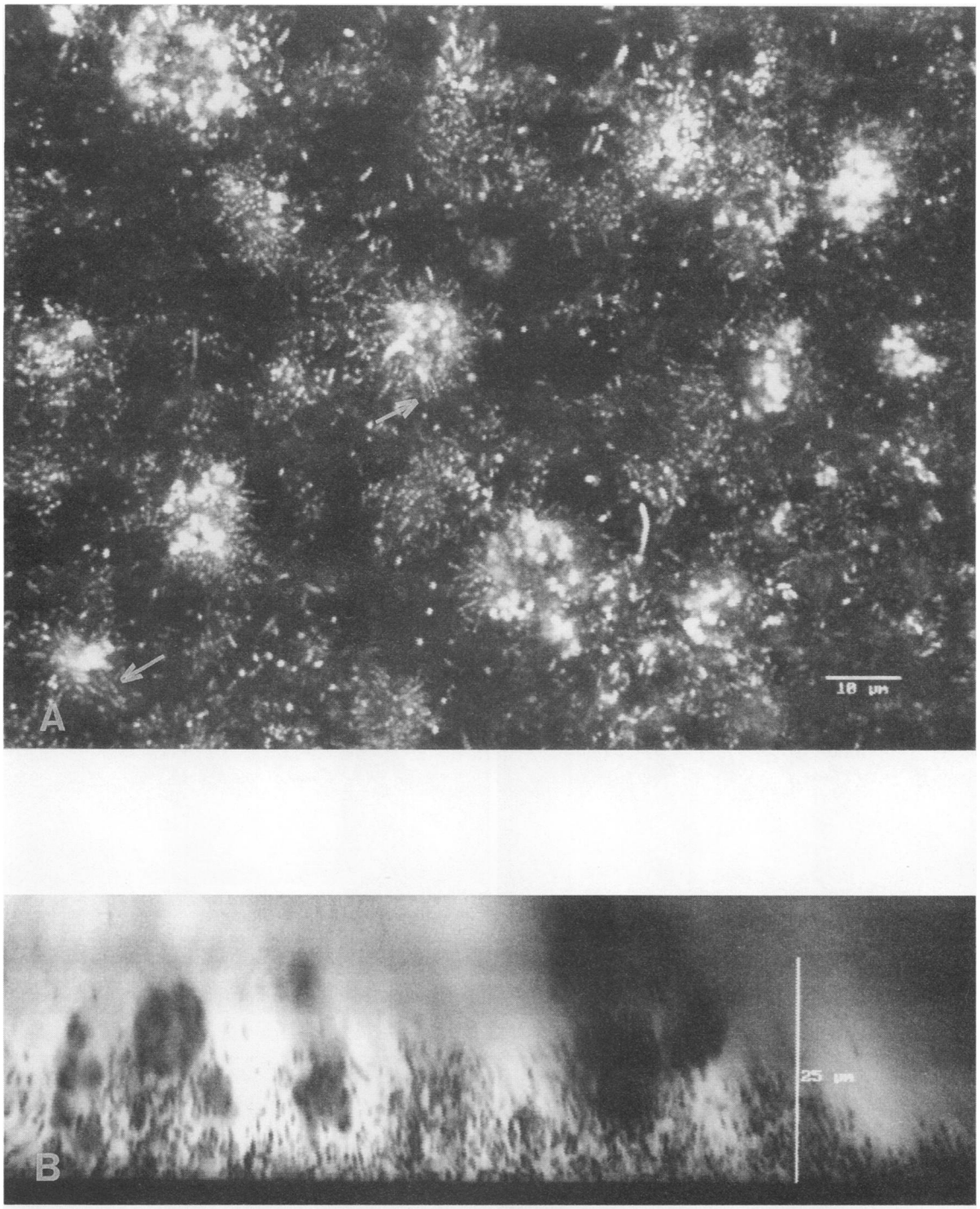
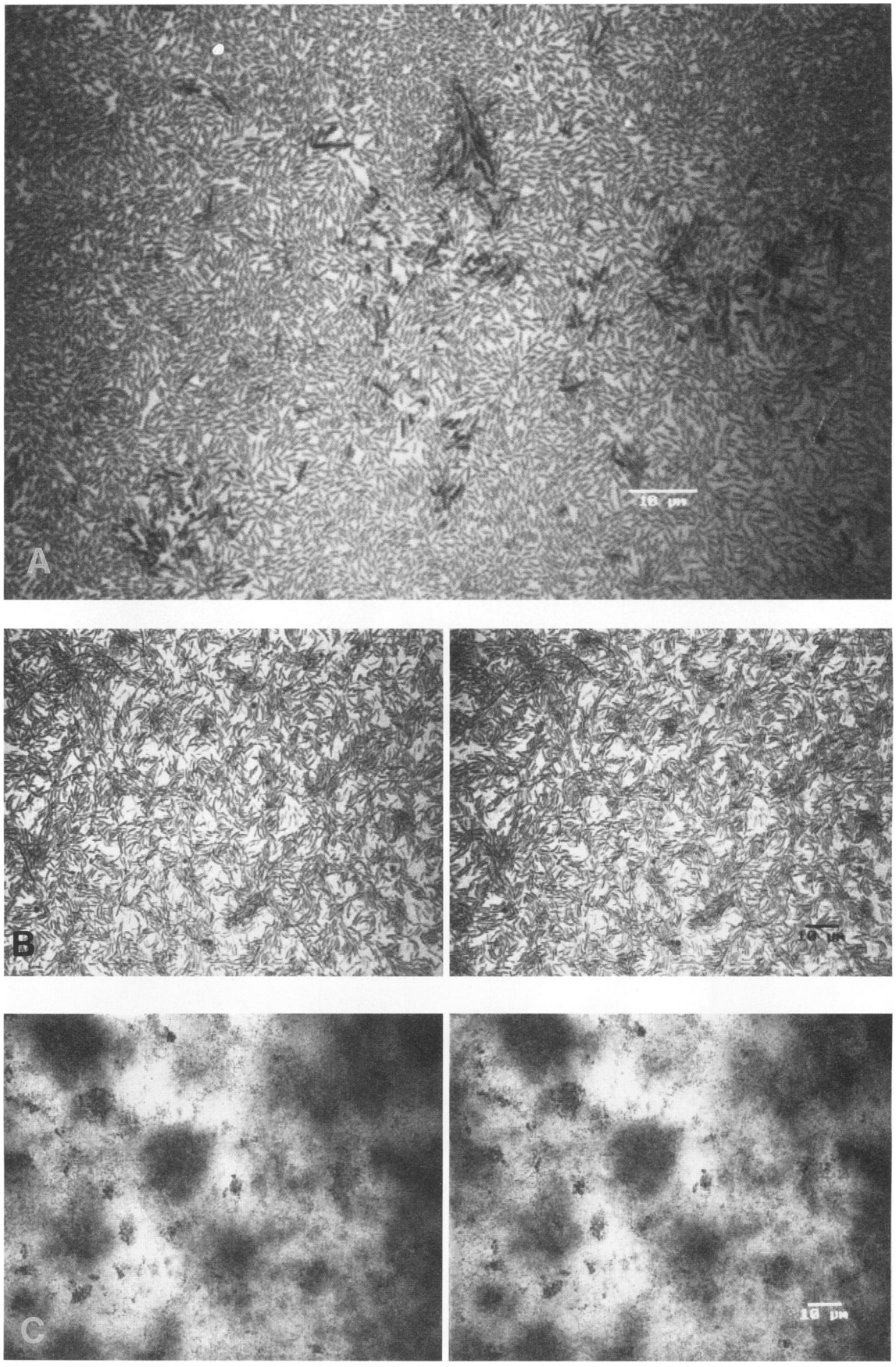


FIG. 4. Micrographs showing typical formation of clusters containing large coccoid cells in mature diclofop-grown biofilms. (A) A positively stained SCLM thin section, 8 µm from the base of the biofilm. Note the orientation of cells toward the clusters (arrows). (B) Sagittal *xz* image of a negatively stained biofilm showing the vertical distribution of clusters.

times the carbon content of 14 µg of diclofop ml^{-1}, respectively) were uniformly distributed in biofilms (Fig. 5A and B) and did not show the same degree of aggregation or differentiation as cells in the diclofop biofilms (Fig. 5C). TSB-grown biofilms developed confluent cell layers in a much shorter time (less than 1 day for both TSB concentrations) than did diclofop-grown biofilms and reached a stable architecture in less than 7 days. Figure 3B shows that for TSB-grown biofilms, there was little increase in the area covered by cells after 7 days. In contrast, the most significant increase occurred between days 14 and 21 in the diclofop-grown biofilms (Fig. 3A). The most notable difference was observed at the glass surface, where less than 10% of the area was covered by cells in the diclofop-grown biofilms after 7 days (Fig. 3A) whereas 35% of


A
10 µm
B
10 µm
C
10 µm

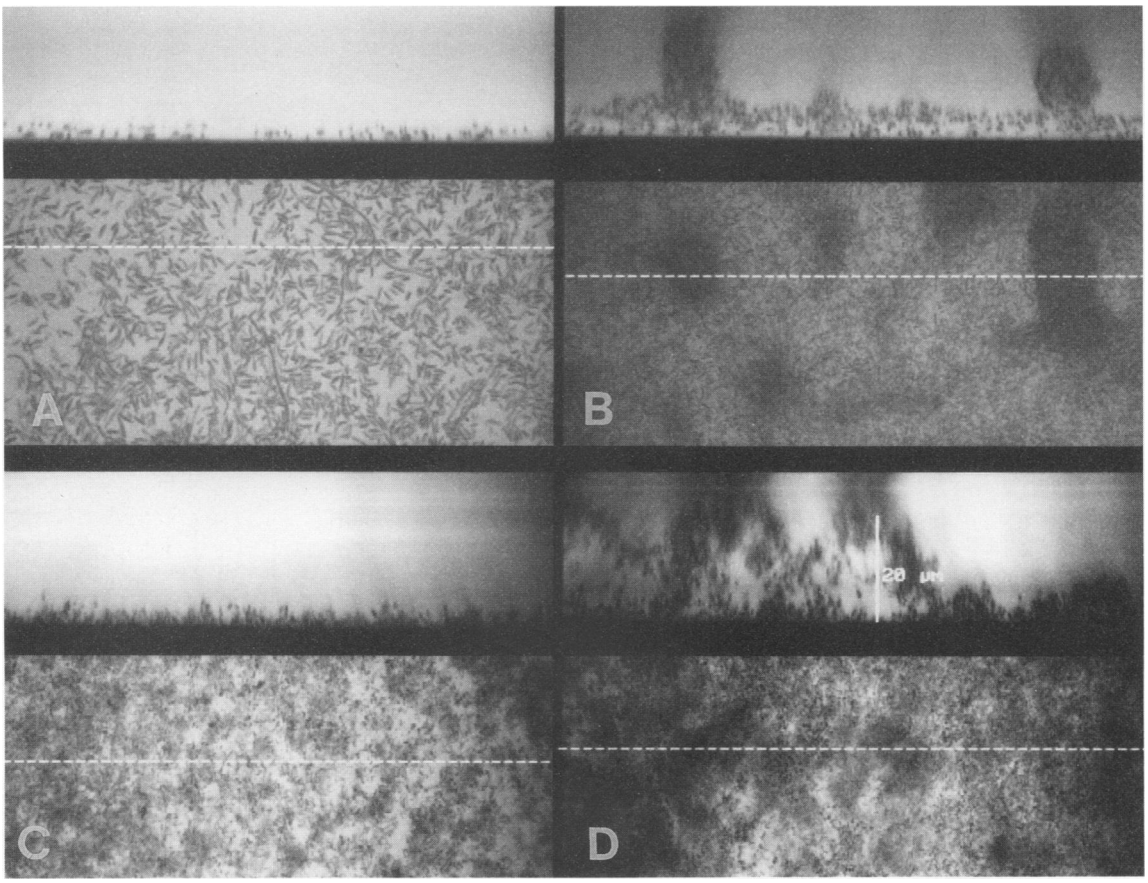


FIG. 6. SCLM images of negatively stained biofilms illustrating the response of biofilms grown on TSB when switched to diclofop. Each image shows an *xy* through-view image with the corresponding *xz* image, collected at the dotted line, directly above it. (A) Biofilm grown for 21 days on 300 μg of TSB ml^{-1}; (B) biofilm grown for 21 days on 300 μg of TSB ml^{-1} and then on diclofop (image obtained 2 days after switch); (C) biofilm grown for 21 days on 18 μg of TSB ml^{-1}; (D) biofilm grown for 21 days on 18 μg of TSB ml^{-1} and then on diclofop (image obtained 2 days after switch).

the area was covered by cells of the TSB-grown biofilm after 1 day (Fig. 3B).

Figures 6A and C show that cells in 21-day-old biofilms grown on 18 and 300 μg of TSB ml^{-1}, respectively, were more uniformly distributed than 21-day-old diclofop-grown biofilms (Fig. 7A).

Biofilms grown on 4-(2,4-dichlorophenoxy)phenol (Fig. 7B) were similar to the biofilms that developed when diclofop (Fig. 7A) was provided as the sole carbon source and, like the diclofop grown-biofilms, formed clusters (average distance between the clusters in mature biofilms was 12.0 μm; standard deviation, 3.3 μm). Clusters were also observed when 1,3-dichlorobenzene (Fig. 7C) was the carbon source. In contrast, 2,4-dichlorophenol-grown biofilms (Fig. 7D) developed relatively few clusters during a 21-day period, were more diffuse than biofilms grown on the other chemicals, and had a general appearance more similar to TSB-grown biofilms.

Vertical organization of biofilms. Stable diclofop biofilms had a 2- to 4-μm-thick base, consisting of rod-like cells, each cell surrounded by a dense exopolymeric capsule (Fig. 8A and B). The capsules were also visible in transmission electron microscope images (Fig. 8C). These encapsulated cells were usually not observed during the first 7 days. Sagittal (*xz* plane) SCLM images showed that the cell clusters developed outside this basal layer (Fig. 4B). Mounds developed above the clusters, projecting up to 30 μm beyond the surrounding biofilm (Fig. 4B).

TSB-grown biofilms were thinner (<5 μm) and lacked the vertical differentiation observed in the diclofop-grown biofilms (Fig. 6A and C). Biofilms grown on 4-(2,4-dichlorophenoxy) phenol (Fig. 7B) and 1,3-dichlorobenzene (Fig. 7C) had vertical arrangements similar to that of diclofop-grown biofilms. In contrast, few mounds developed in biofilms grown on 2,4-dichlorophenol (Fig. 7D).

FIG. 5. (A) SCLM through-view image (0 to 5 μm) of a 1-day-old biofilm grown on 18 μg of TSB ml^{-1} showing that cells were more evenly distributed and developed confluent layers in a much shorter time than those in diclofop-grown biofilms (Fig. 2). (B and C) Stereo pairs of mature biofilms grown on 300 μg of TSB ml^{-1} (panel B) and 14 μg of diclofop ml^{-1} (panel C) showing the differences in biofilm architecture. Each stereo pair consists of a pair of through-view images projected side by side. Three-dimensional images were obtained by offsetting successive focal planes of the images on the right side of each pair by 1 μm. Viewing of the images to visualize the three-dimensional effect can be done with or without stereo-glasses.

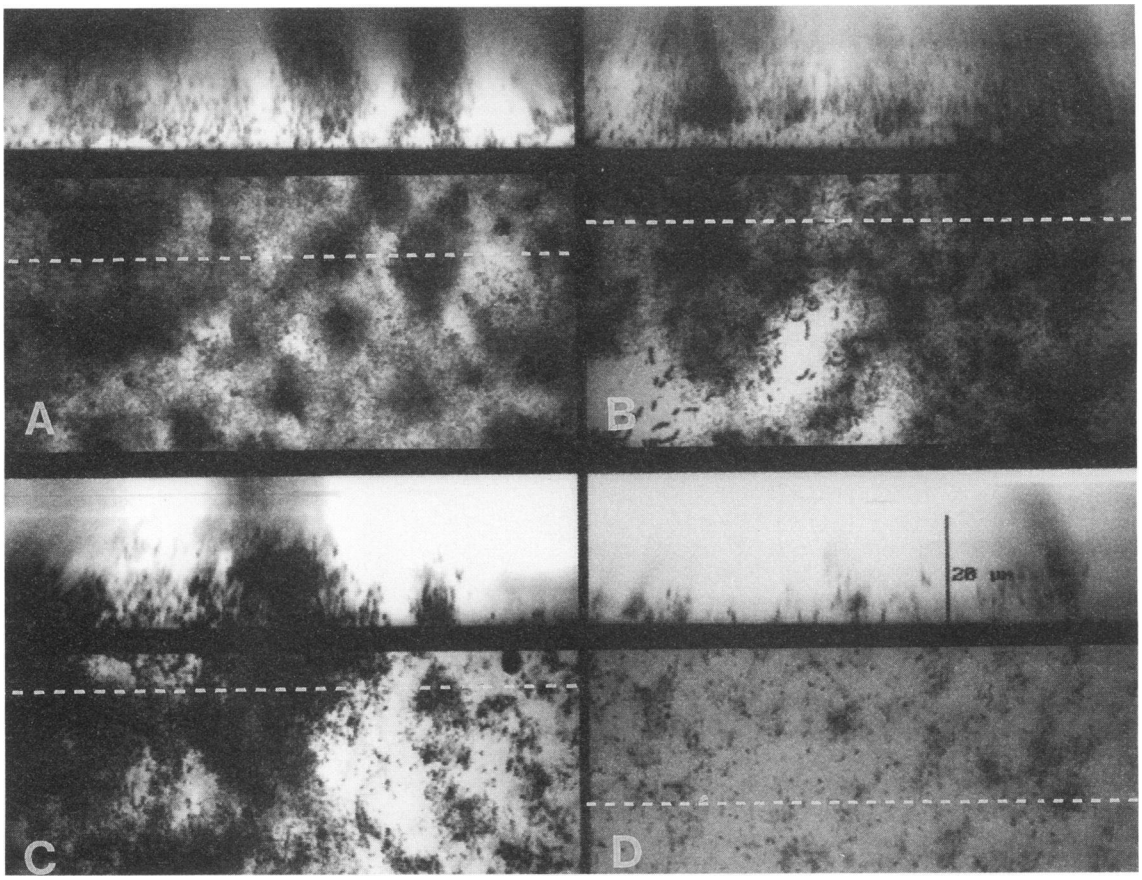


FIG. 7. SCLM images of negatively stained biofilms illustrating differences in thickness and vertical arrangement in 21-day-old biofilms of the same degradative consortium growing on different carbon sources. Each image shows an *xy* through-view image with the corresponding *xz* image, collected at the dotted line, directly above it. Biofilms were grown on diclofop (A), 4-(2,4-dichlorophenoxy)phenol (B), 1,3-dichlorobenzene (C), and 2,4-dichlorophenol (D).

Cell density and biofilm thickness. The average area covered with cells at different depths in biofilms originating from the same inoculum but grown on different carbon sources varied considerably (Fig. 3A and B). The average thickness of mature biofilms was also variable for the different carbon sources (Table 1). The development of mounds gave biofilms grown on diclofop, 4-(2,4-dichlorophenoxy)phenol, and 1,3-dichlorobenzene an irregular surface topography (Fig. 7A to C), resulting in large standard deviations in measured values for biofilm thickness (Table 1).

Cell size and morphology. Cells growing in diclofop biofilms were smaller than the cells growing on 300 μg of TSB ml^{-1} but were the same size as cells growing on 18 μg of TSB ml^{-1} (Fig. 9). A number of different cell morphotypes could be distinguished in diclofop-grown biofilms. In contrast, cells in biofilms growing on either TSB concentration were apparently homogeneous. Diclofop-grown biofilms always had the following cellular forms: (i) rods with dense capsules at the base, (ii) large coccoid cells concentrated in clusters, (iii) rod-shaped cells positioned around clusters, and (iv) smaller cocci and rods between clusters.

The horizontal and vertical arrangement, as well as the cell density and biofilm thickness, of biofilms developing in flow cells irrigated with a medium containing both diclofop and TSB were similar to those of TSB-grown biofilms (data not shown).

Biofilm responses to a switch in carbon source. TSB-grown biofilms responded rapidly to a substitution of TSB with diclofop as the carbon and energy source. Spatial orientation and cell morphology changed within 2 days from the more uniform arrangement, typical of TSB growth, where cells were evenly distributed through biofilms, to the nonuniform patterns of diclofop-grown biofilms (Fig. 6).

Total planktonic counts. Diclofop supported smaller numbers of planktonic bacteria than did TSB with a comparable carbon content. TSB flow cells had 22 times more microbial cells per milliliter of effluent than did the diclofop flow cells after 2 days (Table 2). Planktonic cell numbers in the diclofop flow cells increased until day 5 and then remained constant for the duration of the experiment (21 days). Numbers in the TSB flow cells decreased with time and were only 1.8 times higher than the diclofop flow cells at the end of the experiment.

Community-level carbon source utilization. The microbial community from the continuous culture, used as the inoculum for experimentation, utilized 39% of the 95 carbon sources in the BIOLOG microplates (Table 3). The similarity between the continuous-culture community and those maintained in flow cells on the chlorinated ring compounds varied between 84% [4-(2,4-dichlorophenoxy)phenol] and 89% (diclofop). In contrast, the community from the TSB flow cell could utilize only 57% of the carbon sources used by the continuous-culture community.

TABLE 1. Average thickness of mature biofilms grown on TSB and phenolic compounds

Carbon source	Thickness (mean ± SD, n = 50)
Diclofop	23.6 (15.1)
TSB (300 μg ml^{-1})	4.5 (0.76)
TSB (18 μg ml^{-1})	4.4 (0.86)
4-(2,4-Dichlorophenoxy)phenol	19.7 (10.4)
2,4-Dichlorophenol	4.5 (2.3)
1,3-Dichlorobenzene	13.0 (6.9)

TABLE 2. Total planktonic cell numbers in the effluents of flow cells irrigated with 14 μg of diclofop ml^{-1} and 18 μg of TSB ml^{-1}

Day	No. of cells (10^8) grown on carbon source:	
	Diclofop	TSB
2	0.58	13.0
4	5.7	11.0
5	3.4	10.1
7	3.4	10.0
14	3.4	7.2
21	2.7	4.8

DISCUSSION

We have observed a number of differences between biofilms starting from the same inoculum but grown on diclofop or TSB. These included differences in initial attachment and growth rates, biofilm architecture and topography, biofilm thickness and cell density, planktonic cell numbers, and community-level metabolic fingerprints. From these observations we hypothesized that the spatial arrangement of cells in degradative biofilms allows close associations between members of the consortium and that these associations are obligatory for the utilization of diclofop or other chlorinated organic compounds as the sole carbon source.

The degradative consortium formed biofilms in a much shorter time when grown on TSB than when diclofop was provided as the sole carbon source (Fig. 2, 3, and 5). TSB-grown biofilms did not show much variability in thickness, spatial orientation of cells, cell density, and cell morphology. In contrast, diclofop-grown biofilms had an irregular surface topography and thickness, multicellular arrangements, and a larger variety of morphological cell types. Increases in biofilm depth, as well as the appearance of encapsulated cells at the base of diclofop biofilms, occurred only after formation of clusters consisting of large coccoid cells. Total planktonic cell counts in diclofop flow cells were also low initially; the TSB flow cells had 22 times more cells after 1 day. However, this difference decreased to 1.8 times at the end of the experiment (Table 2). It thus appears that growth and development of biofilms on diclofop as the sole carbon source were dependent on the spatial orientation and cooperation between members in the biofilm consortium.

Examples of interrelated groups of bacteria, such as dechlorinating groups, H_2 consumers, and sulfate reducers, have been described by others (see, e.g., references 30 and 35). By using SCLM, we were able to visualize cellular arrangements that suggested interspecies metabolic interactions in degradative biofilms. It seems possible that SCLM, in conjunction with gene probes (15), will in future provide an accurate tool to elucidate metabolic communications in degradative biofilms.

Knackmuss (18) demonstrated that it is important for cells to avoid accumulating toxic intermediates, such as chlorocatechols, during the degradation of chlorobenzenes. He suggested that this can be achieved by the establishment of certain catabolic functions rather than the selection of specific organisms. For example, in our study it is possible that cells growing in the clusters were responsible for the removal of toxic metabolites, a rate-limiting step which regulated further degradation of diclofop. Another possible explanation for the relatively long initial period of limited biofilm growth is that a longer time was required to establish anaerobic zones for reductive dechlorination. Häggblom (14) noted that cleavage of the carbon-halogen bond is the critical step in the degradation of chloroaromatic compounds. This step is not needed for the degradation of labile compounds such as TSB; therefore a higher rate of initial biofilm development was observed for TSB-grown biofilms.

The mean cell sizes in biofilms growing on diclofop and 18 μg of TSB ml^{-1} were virtually the same (Fig. 9). Bengtsson (3) reported a doubling in cell size of groundwater bacteria when the concentrations of dissolved organic carbon and phosphate were increased 10-fold. This increase was attributed to the possibility that the small size of bacteria in pure groundwater improved nutrient uptake efficiency. This may explain differences in cell size observed in this study between cells growing on 300 μg of TSB ml^{-1} and those in the diclofop and 18-μg ml^{-1} TSB flow cells. We concluded that the type of carbon source did not have an effect on cell size.

It has often been reported that microorganisms derive certain benefits from growing at surfaces (28). It has also been noted that sessile growth is stimulated by carbon limitation (16). A lack of carbon might be an explanation for the thicker biofilms when diclofop was the sole carbon source (Table 1). However, since the carbon contents in the diclofop and 18-μg ml^{-1} TSB flow cells were equal, we concluded that diclofop required additional chemical reactions to be mineralized, rendering it more resistant to microbial degradation than a complex growth medium such as TSB.

There was no significant difference in biofilm thickness between flow cells irrigated with 300 and 18 μg of TSB ml^{-1} (mean biofilm thicknesses, 4.5 ± 0.76 and 4.4 ± 0.86 μm, respectively). This provides further support for the suggestion that the thicker, more variable biofilms in diclofop flow cells were not a function of total carbon in the growth medium. Nevertheless, it does not rule out the possibility that the amount of available carbon was smaller in diclofop medium. If this was the case, adaptations, such as the formation of aggregates to allow close juxtaposition of interrelating organisms, could have been required to make the carbon available for metabolism.

The role of adaptation in the development of structural differences between biofilms grown on labile complex media, and those grown on more recalcitrant xenobiotic compounds is largely unknown. Degradation rates were often used to assess microbial adaptations to the introduction of xenobiotic compounds, and enhanced biodegradation of pesticides in fields with previous applications was attributed to microbial adaptation (26). Lee and Ward (24) used the term "acclimated microorganisms" to describe organisms capable of enhanced degradation as a result of prior exposure to contaminants, whereas adaptation was described by Wilson et al. (37) as a phenomenon caused by several mechanisms such as growth of active organisms, enzyme induction, mutation, or genetic changes. Our observations suggested that adaptation also includes the development of different cellular arrangements within microbial communities.

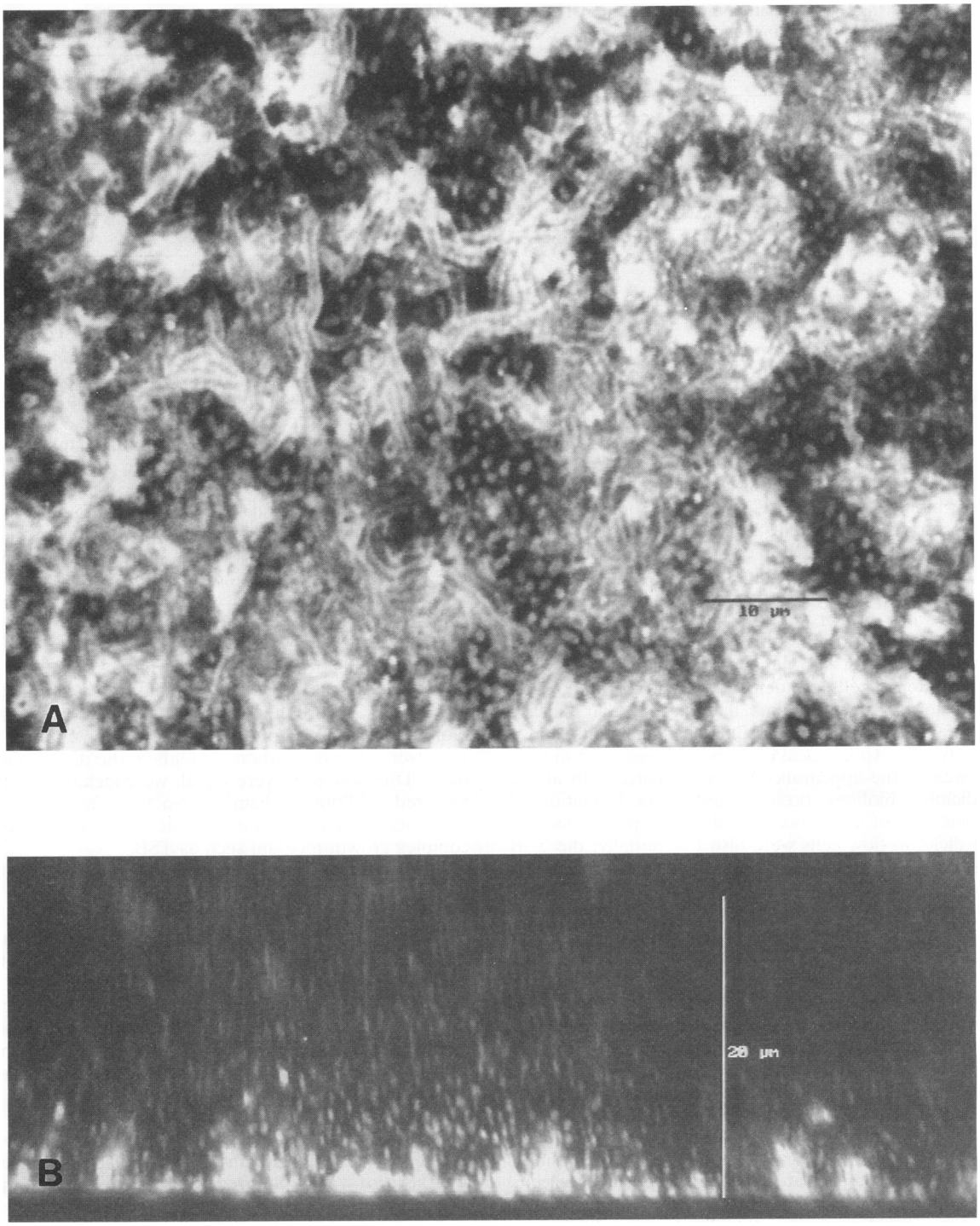


FIG. 8. Diclofop-grown biofilms developed a few layers consisting of encapsulated cells, near the glass surface. The dye Nile Red, specific for hydrophobic compounds, bound to these capsules. (A) SCLM thin section, collected 1 µm from the base of a biofilm stained with Nile Red, shows the horizontal distribution of these encapsulated cells. (B) Vertical distribution of the encapsulated cells. (C) Transmission electron micrograph of these cells showing the density of the capsules. Bar, 1.0 µm.

Flow cells were inoculated with the content of a continuous culture, maintained on diclofop as the sole carbon and energy source for more than 12 months. It can be expected that the microbial population in the continuous culture developed the ability to utilize diclofop. This degradative consortium consisted of at least nine different members, which were isolated on TSA plates in separate experiments. So far, no pure culture isolated from the degradative consortium has shown the ability to convert any significant amount (>0.5% after 7 days) of [^{14}C]diclofop into $^{14}CO_2$. In contrast, the continuous culture

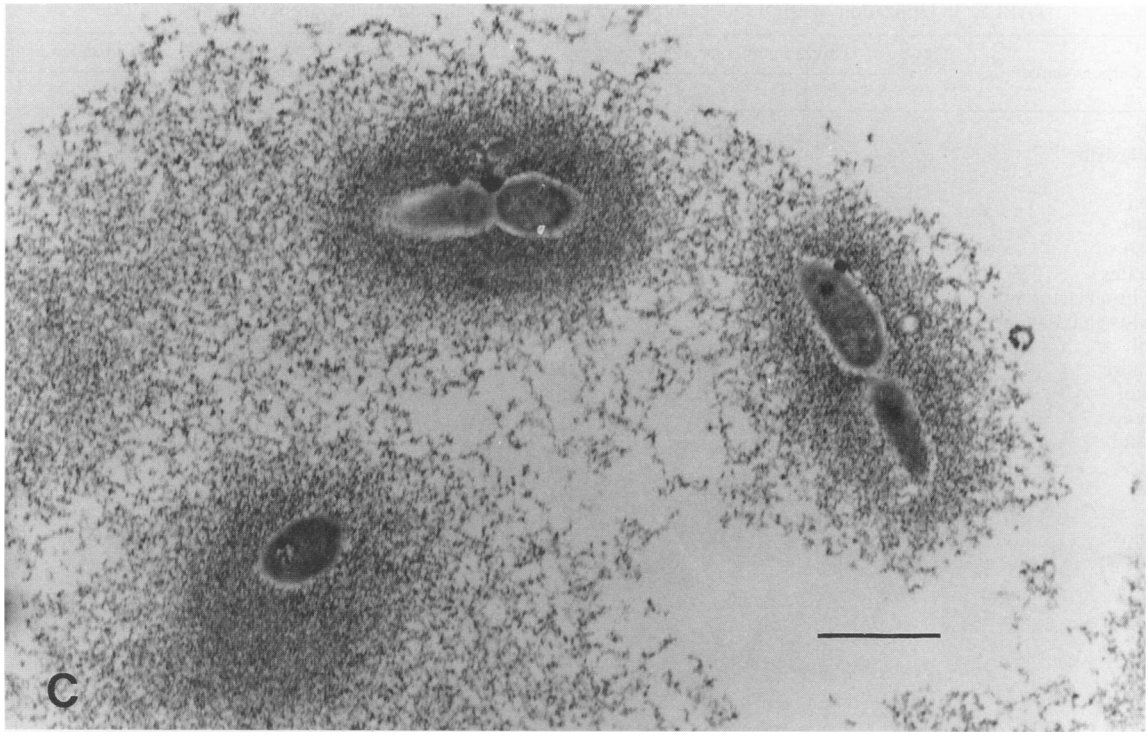


FIG. 8—*Continued.*

population converted more than 60% of the label into $^{14}CO_2$ after 7 days (38).

The rapid response to form a typical diclofop biofilm structure when this consortium, after being maintained on TSB for 21 days, was reintroduced to diclofop as sole carbon source (Fig. 6B and D) suggested that besides selection, there was a genetic basis for development of this degradative consortium.

It appears that genes responsible for structural changes in

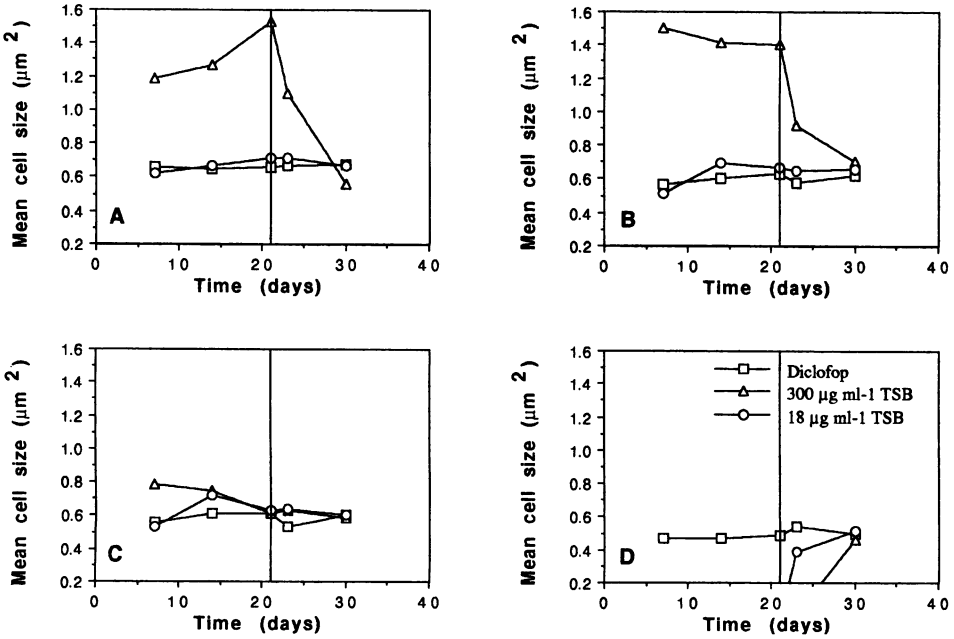


FIG. 9. Comparison of the mean cell size (square micrometers) at 0 μm (A), 2 μm (B), 4 μm (C), and 8 μm (D) from the base of biofilms grown on diclofop and TSB. Biofilms were cultivated for 21 days (indicated by a vertical line on each graph) on diclofop, 300 μg of TSB ml^{-1}, and 18 μg of TSB ml^{-1}, respectively, before the diclofop was replaced with 300 μg of TSB ml^{-1} and both TSB concentrations were replaced with diclofop. Note that the larger cells at 0- and 2-μm depths in the biofilm grown on the higher TSB concentration were replaced by smaller cells after the switch to diclofop, whereas the mean size of cells grown on diclofop and 18 μg of TSB ml^{-1} was not affected by the switch in carbon source.

TABLE 3. Utilization of BIOLOG carbon sources by microbial communities from continuous culture[a]

Carbon source	Utilization by inoculum					
	A	B	C	D	E	F
Polymers						
α-Cyclodextrin	−	−	−	−	−	−
Dextrin	−	−	−	−	−	−
Glycogen	−	−	−	−	−	−
Tween 40	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+
Carbohydrates						
N-Acetyl-D-galactosamine	−	−	−	−	−	−
N-Acetyl-D-glucosamine	−	−	−	−	−	−
Adonitol	−	−	−	−	−	−
L-Arabinose	−	−	−	+	−	−
D-Arabitol	−	−	−	−	−	−
cellobiose	−	−	−	−	−	−
l-Erythritol	−	−	−	−	−	−
D-Fructose	−	−	−	−	−	−
L-Fucose	−	−	−	+	−	−
D-Galactose	−	−	−	−	−	−
Gentiobiose	−	−	−	+	−	−
α-D-Glucose	−	+	+	+	+	−
m-Inositol	−	−	−	−	−	−
α-Lactose	−	−	−	−	−	−
Lactulose	−	−	−	−	−	−
Maltose	+	+	−	+	+	−
D-Mannitol	+	+	−	−	−	−
D-Mannose	+	+	+	+	+	−
D-Melibiose	−	−	−	−	−	−
β-Methyl-D-glucoside	−	−	−	−	−	−
Psicose	−	−	−	−	−	−
D-Raffinose	−	−	−	−	−	−
L-Rhamnose	−	−	−	−	−	−
D-Sorbitol	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−
D-Trehalose	−	−	−	−	−	−
Turanose	−	−	−	−	−	−
Xylitol	−	−	−	−	−	−
Esters						
Methyl puryvate	−	+	−	+	+	−
Monomethyl succinate	−	−	−	−	−	−
Carboxylic acids						
Acetic acid	−	+	+	+	−	+
cis-Aconitic acid	+	+	+	+	+	+
Citric acid	+	+	+	+	+	+
Formic acid	−	−	−	−	−	−
D-Galactonic acid lactone	−	−	−	−	−	−
D-Galacturonic acid	+	+	+	+	+	+
D-Gluconic acid	+	+	+	+	+	−
D-Glucosaminic acid	−	−	−	−	−	−
D-Glucuronic acid	−	+	+	+	+	+
α-Hydroxybutyric acid	−	−	−	−	−	−
β-Hydroxybutyric acid	+	+	+	+	+	+
γ-Hydroxybutyric acid	+	+	+	+	+	+
p-Hydroxyphenylacetic acid	+	+	+	+	+	−
Itaconic acid	−	−	+	+	+	+
α-Ketobutyric acid	−	−	−	−	−	−
α-Ketoglutaric acid	+	+	+	+	+	+
α-Ketovaleric acid	−	−	−	−	−	−
D,L-Lactic acid	+	+	+	+	+	+
Malonic acid	−	−	+	+	+	−
Propionic acid	−	+	+	+	−	+
Quinic acid	+	+	+	+	+	+
D-Saccharic acid	+	+	+	+	+	+
Sebacic acid	−	−	−	−	−	−
Succinic acid	+	+	+	+	+	+
Brominated chemicals						
Bromosuccinic acid	+	+	+	+	+	+
Amides						
Succinamic acid	+	−	−	+	−	−
Glucuronamide	−	+	−	+	−	−
Alaninamide	−	−	−	+	−	−
Amino acids						
D-Alanine	−	+	+	+	+	+
L-Alanine	+	+	+	+	+	+
L-Alanyl-glycine	+	+	−	+	−	−
L-Asparagine	+	+	+	+	+	+
L-Aspartic acid	+	+	+	+	+	+
L-Glutamic acid	+	+	+	+	+	+
Glycyl-L-aspartic acid	−	−	−	−	−	−
Glycyl-L-glutamic acid	−	−	−	−	−	−
L-Histidine	+	+	+	+	+	−
Hydroxy-L-proline	+	+	+	+	+	−
L-Leucine	−	−	−	−	−	−
L-Ornithine	−	+	+	−	−	−
L-Phenylalanine	−	−	−	−	−	−
L-Proline	+	+	+	+	+	+
L-Pyroglutamic acid	+	+	+	+	+	+
D-Serine	+	−	+	+	+	−
L-Serine	+	+	+	+	+	+
L-Threonine	−	−	−	−	−	−
D,L-Carnitine	−	−	−	+	+	−
γ-Aminobutyric acid	+	+	+	+	+	−
Aromatic chemicals						
Urocanic acid	+	+	+	+	+	−
Inosine	+	+	+	+	+	−
Uridine	−	−	−	−	−	−
Thymidine	−	−	−	−	−	−
Amines						
Phenylethylamine	−	+	+	+	−	−
Putrecine	+	+	+	+	+	−
2-Aminoethanol	+	+	+	−	−	+
Alcohols						
2,3-Butanediol	−	−	−	−	−	−
Glycerol	+	−	−	−	−	−
Phosphorylated chemicals						
D,L-α-Glycerol phosphate	−	−	−	−	−	−
Glucose-1-phosphate	+	−	−	−	−	−
Glucose-6-phosphate	−	−	−	−	−	−

[a] BIOLOG GN microplates were used to compare patterns of sole-carbon-source utilization by flow cell communities. The metabolic fingerprint of the continuous culture, maintained on 14 μg of diclofop ml^{-1} and used to inoculate flow cells, is represented by A. Flow cells were irrigated with diclofop (B), 4-(2,4-dichlorophenoxy)phenol (C), 2,4-dichlorophenol (D), 1,3-dichlorobenzene (E), and TSB (F). All solutions were prepared to provide the same amount of carbon as 14 μg of diclofop ml^{-1}.

the biofilm architecture, when the consortium was grown on diclofop, were expressed as a result of the absence of other labile carbon sources rather than the presence of diclofop, since biofilms in flow cells irrigated with a mixture of diclofop and TSB resembled those in flow cells with only TSB. Utilization of other organic compounds by degradative organisms in preference to contaminants is a common phenomenon (12).

Biofilms grown on diclofop usually require at least 14 days to reach the mature stage. However, it took TSB-grown biofilms only 2 days to acquire the typical diclofop-grown biofilm structure after being switched to diclofop, suggesting that cells carrying degradative genes were present in TSB biofilms in large numbers and that these genes were expressed soon after the switch to diclofop. The diclofop-degrading ability was thus not lost during the 21 days of growth on TSB.

Determination of community composition by analyzing spe-

cific signatures such as the fatty acid content (9) of the whole community, instead of attempting to isolate and characterize individual members of the community, is now well established. We used the BIOLOG system to determine metabolic fingerprints of flow cell communities on the basis of their sole-carbon-source utilization (10). The notable difference in the number of positive reactions between the microbial communities maintained in flow cells on TSB and the aromatic compounds (Table 1) demonstrates the metabolic difference between communities grown on labile and more recalcitrant carbon sources. It is also interesting that the TSB community was unable to utilize any of the four aromatic chemicals on the BIOLOG plate, whereas two of them were utilized by all the flow cell communities grown on aromatic compounds.

We used other chlorinated ring compounds to establish whether structural changes in biofilms grown on diclofop were unique to this compound or whether it was a general phenomenon in response to growth on recalcitrant compounds. The structure and surface topography of biofilms grown on 4-(2,4-dichlorophenoxy)phenol corresponded to those of diclofop-grown biofilms (Fig. 7A and B). The distribution and density of clusters were also similar in biofilms grown on diclofop and 4-(2,4-dichlorophenoxy)phenol. The latter compound is formed when diclofop acid undergoes decarboxylation to form a phenyl ether which in turn is transformed into the phenol (34). Thus, the structure of this phenol metabolite resembles the structure of diclofop methyl (a two-ring compound), except for the slight modification in the aliphatic side chain. Microbial behavior could therefore be expected to be similar when grown on either one of these two compounds. It was suggested by Smith (34) that further degradation would involve ring fission to form, among others, 2,4-dichlorophenol, one of the other compounds used in this study.

Alexander and Aleem (1) related resistance of aromatic herbicides to biodegradation to the position of the halogen on the aromatic nucleus and by the linkage and type of aliphatic side chain. The differences observed between biofilms grown on 2,4-dichlorophenol and 1,3-dichlorobenzene (Fig. 7C and D) reflect this difference in their chemical stability. On the basis of these criteria, 2,4-dichlorophenol is a less recalcitrant compound than 1,3-dichlorobenzene. Overall, the behavior of microorganisms when grown on the other organic compounds confirmed the generality of observations made when diclofop was provided as the sole carbon source.

Finally, our observations suggest that changes in biofilm architecture and spatial orientation may facilitate utilization of xenobiotic compounds by degradative microbial communities. Even though the implications of structural differences, in particular the cellular composition and spatial organization of cells, observed between degradative biofilms grown on different carbon sources are largely unknown, we agree with Dworkin that the time has come to abandon the traditional notion that cell-cell interactions between bacteria are restricted to a small, unusual group of organisms (8). It seems certain that, with the implementation of new technologies (see, e.g. reference 27), more evidence will become available implying metabolic communication between members of degradative biofilm consortia. A better understanding of these interactions is also important for the development of improved bioremediation techniques.

ACKNOWLEDGMENTS

Financial support was provided by The National Hydrology Research Institute, Environment Canada.

George D. W. Swerhone constructed the flow cells.

REFERENCES

1. **Alexander, M., and M. I. H. Aleem.** 1961. Effect of chemical structure on microbial decomposition of aromatic herbicides. J. Agric. Food Chem. **9:**44–47.
2. **Atlas, R. M.** 1984. Diversity of microbial communities. Adv. Microb. Ecol. **7:**1–47.
3. **Bengtsson, G.** 1989. Growth and metabolic flexibility in groundwater bacteria. Microb. Ecol. **18:**235–248.
4. **Caldwell, D. E., D. R. Korber, and J. R. Lawrence.** 1992. Confocal laser microscopy and digital image analysis in microbial ecology. Adv. Microb. Ecol. **12:**1–67.
5. **Caldwell, D. E., D. R. Korber, and J. R. Lawrence.** 1992. Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. J. Microbiol. Methods **15:**249–261.
6. **Caldwell, D. E., and J. R. Lawrence.** 1986. Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **12:**299–312.
7. **Caldwell, D. E., and J. M. Tiedje.** 1975. A morphological study of anaerobic bacteria from the hypolimnia of two Michigan lakes. Can. J. Microbiol. **21:**363–376.
8. **Dworkin, M.** 1991. Introduction, p. 1–6. *In* M. Dworkin (ed.), Microbial cell-cell interactions. American Society for Microbiology, Washington, D.C.
9. **Federle, T. W., M. A. Hullar, R. J. Livingston, D. A. Meeter, and D. C. White.** 1983. Spatial distribution and biochemical parameters indicating biomass and community composition of microbial assemblies in estuarine mud flat sediments. Appl. Environ. Microbiol. **45:**58–63.
10. **Garland, J. L., and A. L. Mills.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. **57:**2351–2359.
11. **Geesey, G. G., and J. W. Costerton.** 1986. The microphysiology of consortia within adherent bacterial populations, p. 238–242. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Mladinska Knjiga, Ljubljana, Slovenia.
12. **Goldstein, R. M., L. M. Mallory, and M. Alexander.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. **50:**977–983.
13. **Grotenhuis, J. T. C., F. P. Houwen, C. M. Plugge, and A. J. B. Zehnder.** 1986. Microbial interactions in granular sludge, p. 163–168. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Mladinska Knjiga, Ljubljana, Slovenia.
14. **Häggblom, M.** 1990. Mechanisms of bacterial degradation and transformation of chlorinated monoaromatic compounds. J. Basic Microbiol. **30:**115–141.
15. **Holben, W. E., B. M. Schroeter, V. G. M. Calabrese, R. H. Olsen, J. K. Kukor, V. O. Biederbeck, A. E. Smith, and J. M. Tiedje.** 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. **58:**3941–3948.
16. **Kjelleberg, S.** 1984. Effects of interfaces on survival mechanisms of copiotrophic bacteria in low-nutrient habitats, p. 151–159. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.
17. **Klinger, J. M., R. P. Stowe, D. C. Obenhuber, T. O. Groves, S. K. Mishra, and D. L. Pierson.** 1992. Evaluation of the Biolog automated microbial identification system. Appl. Environ. Microbiol. **58:**2089–2092.
18. **Knackmuss, H.-J.** 1984. Biochemistry and practical implications of organohalide degradation, p. 687–693. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.
19. **Kolenbrander, P. E.** 1989. Surface recognition among oral bacteria: multigeneric coaggregations and their mediators. Crit. Rev. Microbiol. **17:**137–159.
20. **Korber, D. R., J. R. Lawrence, L. Zhang, and D. E. Caldwell.** 1990. Effect of gravity on bacterial deposition and orientation in laminar flow environments. Biofouling **2:**335–350.
21. **Lamont, H. C., W. B. Silvester, and J. G. Torrey.** 1987. Nile red fluorescence demonstrates lipid in the envelope of vesicles from N_2-fixing cultures of *Frankia*. Can. J. Microbiol. **34:**656–660.

22. **Lang, E., H. Viedt, J. Egestorff, and H. H. Hanert.** 1992. Reaction of the soil microflora after contamination with chlorinated aromatic compounds and HCH. FEMS Microbiol. Ecol. **86:**275–282.
23. **Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell.** 1991. Optical sectioning of microbial biofilms. J. Bacteriol. **173:**6558–6567.
24. **Lee, M. D., and C. H. Ward.** 1985. Biological methods for the restoration of contaminated aquifers. Environ. Toxicol. Chem. **4:**743–750.
25. **MacLeod, F. A., S. R. Guiot, and J. W. Costerton.** 1990. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Appl. Environ. Microbiol. **56:**1598–1607.
26. **McCuster, V. W., H. D. Skipper, J. P. Zublena, and D. T. Gooden.** 1988. Biodegradation of carbamothioates in butylate-history soils. Weed Sci. **36:**818–823.
27. **Nichols, P. D., J. M. Henson, J. B. Guckert, D. E. Nivens, and D. C. White.** 1985. Fourier transformed-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteria-polymer mixtures and biofilms. J. Microbiol. Methods **4:**79–94.
28. **Parkes, R. J., and E. Senior.** 1988. Multistage chemostats and other models for studying anoxic environments, p. 51–71. *In* J. W. T. Wimpenny (ed.), Handbook of laboratory model systems for microbial ecosystems, vol 1. CRC Press, Inc., Boca Raton, Fla.
29. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
30. **Reineke, W., and H.-J. Knackmuss.** 1988. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. **42:**263–287.
31. **Rozgaj, R., and M. Glancer-Soljan.** 1992. Total degradation of 6-aminonaphthalene-2-sulphonic acid by a mixed culture consisting of different bacterial genera. FEMS Microbiol. Ecol. **86:**229–235.
32. **Schmidt, S. K., R. Smith, D. Sheker, T. F. Hess, J. Silverstein, and P. M. Radehaus.** 1992. Interactions of bacteria and microflagellates in sequencing batch reactors exhibiting enhanced mineralization of toxic organic chemicals. Microb. Ecol. **23:**127–142.
33. **Sjollema, J., H. C. van der Mei, H. M. Uyen, and H. J. Busscher.** 1990. Direct observations of cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria. FEMS Microbiol. Lett. **69:**263–270.
34. **Smith, A. E.** 1977. Degradation of the herbicide diclorfop-methyl in Prairie soils. J. Agric. Food Chem. **25:**893–898.
35. **Takeda, K., K. Ito, S. Amatani, and Y. Tokiwa.** 1992. Bacterial composition of flocs and aqueous phase in a methanogenic digester. Bull. Jpn. Soc. Microb. Ecol. **7:**27–37.
36. **Thiele, J. H., M. Chartrain, and J. G. Zeikus.** 1988. Control of interspecies electron flow during anaerobic digestion: role of floc formation in syntrophic methanogenesis. Appl. Environ. Microbiol. **54:**10–19.
37. **Wilson, J. T., J. F. McNabb, J. W. Cochran, T. H. Wang, M. B. Tomson, and P. B. Bedient.** 1985. Influence of microbial adaptation on the fate of organic pollutants in ground water. Environ. Toxicol. Chem. **4:**721–726.
38. **Wolfaardt, G. M., J. R. Lawrence, J. V. Headley, R. D. Robarts, and D. E. Caldwell.** Submitted for publication.