APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1995, p. 152–158 Vol. 61, No. 1
0099-2240/95/$04.00+0

Bioaccumulation of the Herbicide Diclofop in Extracellular Polymers and Its Utilization by a Biofilm Community during Starvation

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Received 4 April 1994/Accepted 19 September 1994

Continuous-flow cell systems were used to cultivate a degradative biofilm community with the herbicide diclofop methyl as the sole carbon and energy source. The aromatic character of this compound and its breakdown products enabled direct visualization of their accumulation in the biofilm matrix. This accumulation could be inhibited by addition of a more labile carbon source to the culture medium or by inhibition of cell activity. The fluorescence of diclofop-grown biofilms remained constant after 14 to 21 days but decreased with time when diclofop was omitted from the irrigation solution. However, this decrease was inhibited by cyanide, indicating either utilization or release of accumulated diclofop when the cells were viable. Subsequent experiments with [^{14}C]diclofop also indicated that decreased fluorescence in the absence of an exogenous carbon source resulted from degradation of adsorbed diclofop and its breakdown products by the biofilm bacteria. These results demonstrate that biofilm exopolymers can facilitate storage of nutrients for subsequent mineralization during periods of carbon limitation.

Bacteria often improve their chances of reproductive success and survival by the production of extracellular polymeric substances (EPS). The functional roles of EPS and their involvement in attachment, microcolony formation, floc formation, protection against heavy metals, protection against predation and environmental fluctuations, increased resistance against antimicrobial agents, and localization of extracellular enzymes are well described in the literature (10–12, 24, 30).

One functional aspect of microbial behavior which is not clearly understood is the involvement of EPS in the accumulation and subsequent utilization of nutrients. Many natural environments contain low nutrient concentrations, and to survive under such conditions it is necessary for microbial communities to adapt. Accumulation of growth-limiting nutrients by EPS may be an adaptive strategy for survival in environments where these nutrients are available at levels below threshold concentrations (17, 25) required by microorganisms to remain viable.

It has been suggested that nutrients are accumulated in EPS by the same mechanisms responsible for the accumulation of metals (11). Metal binding is a common phenomenon in wastewater treatment, involving complexation of EPS with metals (28), and in ion exchange, mostly via carboxyl groups on uronic acids (11). However, Christensen and Characklis (8) reasoned that although most EPS have cation-exchange properties which may enable bacteria to use EPS as a nutrient trap under oligotrophic conditions, it is unlikely that they can function effectively as a trap for soluble nutrients in environments where divalent cations are abundant. This is in contrast with the results of Costerton (9) and others (see, e.g., references 10 and 15), who proposed that the EPS matrix of biofilms can act as an ion-exchange resin which accumulates nutrients. Various studies suggesting the potential of EPS to sequester and accumulate nutrients extracellularly were cited by Decho (11). However, these studies did not present direct evidence for the mechanisms involved. The relative importance of extracellular sorption to EPS in relation to absorption into cells or adsorption onto cells is also not known (3).

Bacteria may adhere to surfaces and grow as sessile biofilm communities in response to oligotrophic (low-nutrient) conditions (18, 26). Low nutrient concentrations may also stimulate production of EPS by both sessile and planktonic cells (4). The attached cells are usually associated with greater amounts of EPS than are planktonic cells (4, 37). Vandevivere and Kirchman (33) demonstrated a fivefold-greater production of EPS by attached cells than free-living cells of the same bacterium. It is possible that the increased production of EPS by attached cells improves the efficiency of biofilms in trapping nutrients. EPS may play an additional role in the utilization of trapped nutrients by the localization of extracellular enzymes which hydrolyze high-molecular-weight organic matter into a more utilizable form before it enters the cell (11). The extent to which the nutrients accumulated in EPS can be utilized during periods of nutrient limitation is not known.

Patel and Gerson demonstrated the capacity of a *Rhizobium* strain to reutilize its own EPS and suggested that the enzymes involved were extracellular enzymes (27). However, only a limited number of organisms appear to possess the enzymes required to depolymerize their own EPS (12, 27), suggesting that the majority of EPS-producing organisms are unable to utilize their own EPS as carbon sources. It was therefore suggested that EPS generally do not serve as reserve sources of carbon and energy (12, 27).

Organic contaminants, such as industrial and agricultural chemicals, enter the environment in various ways. Natural microbial communities play an important role in determining the fate of many of these contaminants by utilizing them as a source of carbon and energy (1, 19). It is possible that these

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xenobiotic compounds are accumulated in EPS by the same mechanisms responsible for the accumulation of naturally occurring nutrients. Baughman and Paris (2), in a review of bioaccumulation of organic contaminants in aquatic systems, pointed out that little effort has been made to elucidate the role of microorganisms in the bioaccumulation of organic contaminants. The fate of organic contaminants once they have been accumulated is also largely unknown.

In a previous study, we suggested, on the basis of scanning confocal laser microscopy (SCLM) and probe mass-spectral analyses, that the accumulation of the herbicide diclofop methyl, a chlorinated two-ring compound, and its aromatic breakdown products occurred in cell capsules and the EPS matrix of a degradative biofilm community (36). The objective of the present study was to use direct measurements by SCLM and radioisotopic techniques to investigate the potential for subsequent utilization of the stored contaminants as a carbon source during periods of carbon limitation.

MATERIALS AND METHODS

Inoculum, culture conditions and culture medium. Continuous-flow cells were used to cultivate degradative biofilms. Inocula for all experiments were obtained from a degradative microbial consortium maintained in a 250-ml continuous-culture system, with diclofop methyl, the methyl ester of 2-[4-(2,4-dichlorophenoxy)phenoxy]methyl propanoic acid, as the sole carbon source.

Construction of flow cells (35) and maintenance of a nine-membered bacterial community in continuous culture (34) were described previously. The flow cells were surface sterilized with a 6% hypochlorite solution and irrigated with the growth medium at a flow rate of 0.2 mm s^{-1} using a Watson Marlow 201Z peristaltic pump. A single 0.5-ml pulse of the degradative microbial community was added to each flow cell, with the pump turned off, for 1 h before flow was resumed. All experiments were conducted at room temperature (23 ± 2°C).

The culture medium consisted of a minimal salts solution amended with 14 mg of the test compound $liter^{-1}$ as the sole source of carbon and energy. Diclofop methyl (Riedel-de Haën; 99% purity) was used as the model contaminant. The composition of the minimal salts solution (per liter) was as follows: 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 solution A; and 4.24 g of Na_2HPO_4 and 2.7 g of KH_2PO_4 for solution B. A and B were autoclaved separately and mixed, and sterile diclofop was added after the mixture had cooled to room temperature.

Microscopy. A Bio-Rad MRC-600 scanning confocal laser, mounted on a Nikon Microphot-SA microscope, was used to directly visualize accumulation of the test compounds in biofilms (36). In essence, this method involves excitation of the aromatic molecules with the argon laser beam (maximum emission lines at 488 and 514 nm, and a number of smaller peaks from 274 to 528 nm). The resulting fluorescent signal is processed point by point with a photomultiplier, digitized, and analyzed with the accompanying software. Fluorescence intensity, measured on a 0 to 255 gray-value scale (6), was used as an indication of the relative concentration of the fluorescent compound by following the procedure described earlier (36). A laser intensity with a transmission setting of at least 50% was required to obtain the desired level of fluorescence by diclofop methyl. The pinhole opening and the gain and black levels were manually set to allow a detection threshold which discriminated between background fluorescence and that originating from diclofop methyl and its degradation products, collectively referred to as diclofop in the following discussion.

Accumulation of diclofop in the presence of a labile carbon source, and the effect of a switch in carbon source on accumulation. A mixture containing the diclofop methyl-minimal salts medium and 300 mg of tryptic soy broth (TSB; Difco) $liter^{-1}$ was used to determine the accumulation of diclofop with time in the presence of a complex culture medium. Fluorescence intensity in flow cells irrigated with 300 mg of TSB $liter^{-1}$ was also measured. Flow cells were irrigated with the diclofop methyl-TSB mixture and with TSB for 21 days, and the irrigation solutions were replaced with the diclofop methyl-minimal salts medium to determine the ability of biofilms to accumulate diclofop and related molecules after cultivation on a complex growth medium. Flow cells irrigated with the diclofop methyl-minimal salts medium were switched to TSB after 21 days to determine the fate of the accumulated aromatic molecules in the presence of a labile carbon source. Optical thin sections in the *xy* plane, as well as *xz* sagittal images (20), of unstained biofilms were examined by SCLM to visualize and quantify accumulation of the fluorescent compounds.

FRAP. Fluorescence recovery after photobleaching (FRAP) was used to measure differences in the accumulation of diclofop in viable biofilms and biofilms inhibited with 0.65 g of KCN $liter^{-1}$. Fluorescent molecules in defined rectangular areas of biofilms cultivated with diclofop as the sole carbon source were irreversibly bleached with the laser beam. Fifteen consecutive scans at maximum intensity were used for this purpose. Recovery of fluorescence in the bleached areas (compared with the unbleached areas) was subsequently monitored at a lower laser intensity in the KCN-inhibited and untreated biofilms. Bleaching was performed with the electronic zoom set at 3, resulting in a field area of 400 by 270 μm. Fluorescence recovery was measured at zoom 1.5 (field area, 800 by 560 mm).

Displacement of accumulated diclofop from the biofilm matrix by ion exchange. Biofilms were cultivated in flow cells for 21 days with diclofop methyl as the sole carbon source. The average fluorescence intensity, measured at 20 different locations in each flow cell, was determined on the 0 to 255 gray-value scale, and then the diclofop methyl-minimal salts medium was replaced with various concentrations of NaCl (0.6, 5.8, 14.5, and 29.0 g $liter^{-1}$) for 24 h. Fluorescence intensity was subsequently measured to observe any effect of the NaCl on fluorescence intensity.

Release of accumulated diclofop from biofilm material with organic solvents. Biofilms were cultivated as for the ion-exchange experiment. They were then bathed in various methanol concentrations (20, 50, and 80%) for up to 12 h and well rinsed with the minimal salts solution to remove the methanol. Fluorescence intensity, which is used as an indication of the relative amount of diclofop accumulated in the biofilms, was measured by the same procedure as for the ion-exchange experiment, to detect release of diclofop from the biofilm matrix by the solvent. This procedure was also used when biofilms were bathed in other solvents (pesticide-grade hexane and diethyl ether) for up to 20 min.

Biofilm thickness, measured along vertical transects in *xz* sagittal sections, and spatial arrangements of cells in biofilms before and after treatments were used to determine the effect of these treatments on the structural integrity of biofilms. Optical thin *xy* sections (20) of negatively stained biofilms (7) were collected at 1.0-μm intervals before and after treatments and were electronically superimposed to observe changes in the spatial arrangements of cells in the biofilm matrix.

Change in the relative concentration of accumulated diclofop in the absence of a carbon source in the irrigation solution. Biofilms were cultivated in flow cells for 21 days with diclofop methyl as sole carbon source, and the average gray value was measured by the same procedure as for the ion-exchange experiment. Microbial activity was then inhibited with 0.65 g of KCN $liter^{-1}$ in three of the flow cells. These flow cells and three control flow cells without inhibition with KCN were subsequently irrigated with the diclofop-free minimal salts solution, and the fluorescence intensity was measured at regular intervals for 25 days. A single KCN dose per 48 h was used to prevent recovery of cells in the inhibited biofilms.

Radioisotopic experiments to assess the fate of accumulated diclofop. Biofilms were cultivated with the diclofop methyl-minimal salts medium for 21 days. [^{14}C]diclofop methyl (uniformly labeled on the nonchlorinated [dioxyphenyl] ring; specific activity, 0.02 μCi ml^{-1}) in minimal salts was then used as the growth medium for the next 7 days to allow accumulation of labeled diclofop in the biofilm matrix. The supply of the labeled diclofop methyl was then terminated, and the flow cell was irrigated with the minimal salts medium without a carbon source. The effluent from the flow cell was analyzed daily for 25 days. Hydrochloric acid (0.1 N) was added to the effluent to drive off $^{14}CO_2$ evolved from the labeled diclofop. A stream of air, moistened and stripped of CO_2 with 0.5 N NaOH, was used to carry the $^{14}CO_2$ to a series of three CO_2 traps (each trap being a standard 20-ml scintillation vial) containing 10 ml of 0.5 N NaOH. The CO_2 traps were removed, and 10 ml of fluor (Aquasol-2; Du Pont-NEN, Boston, Mass.) was added. Radioactivity, corrected for sample quenching and machine efficiency with an external standard, was determined with a Packard Tri-Carb 1900CA scintillation counter.

RESULTS

Accumulation of aromatic compounds in biofilm EPS and the effect of an alternative carbon source. EPS in biofilms cultivated with diclofop methyl as the sole carbon and energy source accumulated fluorescent compounds, as indicated by a steady increase in fluorescence intensity with time (Fig. 1). This increase was not observed when the community was incubated with nonfluorescent TSB as the carbon source. The nature of the fluorescent compounds bound by the EPS of the biofilm community has been examined in a number of experiments reported in this paper and in reference 36. We have established by fluorimetry that the excitation and emission maxima for diclofop and two of its known metabolites (2,4-dichlorophenol and 1,3-dichlorobenzene) were 462 and 504 nm, 255 and 313 nm, 313 and 376 nm, respectively. The major lines of the scanning confocal laser microscope are 488 and 514 nm, with secondary lines from 275 through 528 nm; thus, the most likely candidate for detection by SCLM is diclofop, although some contribution from other ring compounds cannot

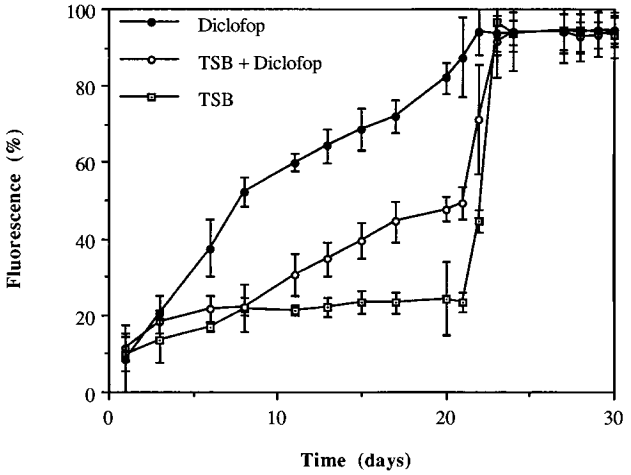

FIG. 1. Accumulation of diclofop and metabolites in biofilm material was estimated by SCLM as the change in fluorescence intensity on a 0 to 255 gray-value scale. Shown here are the percent changes in fluorescence (where 0 = 0% and 255 = 100%) with time in biofilms cultivated on diclofop, a diclofop-TSB mixture, and TSB. The TSB and TSB-diclofop mixture respectively were replaced with the diclofop-minimal salts solution on day 21, while flow cells irrigated with the diclofop-minimal salts medium for the first 21 days were switched to TSB on day 21. No significant decrease in fluorescence was observed after the switch from diclofop to TSB, indicating persistence of the accumulated fluorescent molecules in the biofilm matrix.

be completely discounted. The interpretation of the fluorescent signal may be further complicated by the effect on the fluorescent nature of ring structures of binding to other molecules (29). The probe mass spectrometry studies (36) carried out with washed EPS from the community showed the presence of the parent compound diclofop methyl and its aromatic breakdown products identified as diclofop acid, 4-(2,4-dichlorophenoxy)phenol, 2,4-dichlorophenol, 2-chlorophenol, 1,3-dichlorobenzene, 4-(2,4-dichlorophenoxy)dehydrophenetole, 4-(2,4-dichlorophenoxy)phenetole, and 4-phenoxyphenol. These metabolites were not present in the irrigation medium, indicating that they arose through degradation in the biofilm.

The increase in fluorescence observed in this study was typically sustained for 14 to 21 days, after which no further accumulation was observed. This period corresponds to the time noted previously (35) for the community to reach apparent steady state. In contrast, only a small amount of background fluorescence was observed when the same microbial community was cultivated on TSB (an 8.5-fold-higher fluorescence intensity was observed in the diclofop methyl-grown biofilms than in TSB-grown biofilms) when measured on a 0 to 255 gray-value scale. Biofilms cultivated on a mixture of diclofop methyl-minimal salts medium and 300 mg of TSB liter^{-1} also accumulated less diclofop, based on differences in fluorescence intensity (2.2-fold), than did those cultivated on the diclofop methyl-minimal salts medium (Fig. 1). A switch in irrigation solution from either TSB or the TSB-diclofop methyl mixture to the diclofop methyl-minimal salts solution was followed by a rapid increase in fluorescence intensity (Fig. 1), implying diclofop accumulation. Replacement of the diclofop methyl-minimal salts medium with TSB was not followed by a decrease in fluorescence intensity (Fig. 1), suggesting that the accumulated diclofop methyl (or aromatic breakdown products) was not released or utilized in the presence of the labile carbon source.

FRAP in viable and KCN-treated biofilms. Measurement of fluorescence recovery after photobleaching (Fig. 2) showed a more rapid recovery in untreated biofilms than in biofilms inhibited with KCN. The viable biofilms recovered their original fluorescence intensity within 72 h. The corresponding recovery in inhibited biofilms during this period was only 60% of the original value (Fig. 3).

Release of diclofop from biofilm material by ion exchange and with organic solvents. No detectable decrease in fluorescence intensity was observed after biofilms were irrigated with NaCl or exposed to methanol, hexane, or diethyl ether, implying that these treatments did not effect displacement or release of bound diclofop and its breakdown products from the biofilm matrix. These treatments also did not affect the structural integrity of biofilms.

Change in the concentration of accumulated diclofop in the absence of a carbon source in the irrigation solution. Replacement of the diclofop methyl-minimal salts medium with the minimal salts solution without a carbon source was followed by a decrease in fluorescence (>90% loss after 20 days) in viable biofilms, indicating a decrease in the concentration of diclofop methyl and its aromatic breakdown products accumulated in the biofilm matrix. In contrast, only a small decrease in fluorescence (<10% loss after 20 days) was observed in the biofilms treated routinely (every 48 h) with KCN (Fig. 4A and 5), suggesting that compounds which accumulated in EPS were not released from the EPS when metabolic activity was inhibited.

Growth of degradative biofilms on [^{14}C]diclofop methyl resulted in the accumulation of the labeled molecules in the biofilm matrix. Subsequent irrigation of the flow cell with the carbon-free minimal salts solution resulted in the production of $^{14}CO_2$ for up to 21 days after the switch (Fig. 4B). A relatively small amount of $^{14}CO_2$ (<1% of total label applied) evolved during the first 4 days after the switch, indicating a lag in degradation of the labeled compound. In addition, very little non-CO_2 labeled material (i.e., other metabolites) was detected in the effluent stream.

DISCUSSION

The present study provides evidence for the accumulation of diclofop methyl and its aromatic breakdown products in biofilm EPS when it was provided as the sole carbon source. This accumulation occurred in cell capsules as well as the EPS matrix. The presence of labile carbon resulted in a 2.2-fold reduction in this apparent accumulation as indicated by fluorescence intensity in the biofilm (Fig. 1), indicating that sorption was suppressed by the more labile nutrient. In previous batch culture experiments, we attributed increased degradation of radiolabeled diclofop methyl in the presence of TSB (34) to cometabolism (degradation of a compound when other organic material acts as the primary carbon source) and to the fact that TSB may serve as a noncompetitive substrate for enzyme systems involved in diclofop degradation, similar to the role of formate during cometabolism of trichloroethylene described elsewhere (23). In these batch systems, the requirement for extracellular accumulation of diclofop to facilitate its degradation and the production of the sorptive exopolymer would probably not exist.

Differences in biofilm thickness and cellular organization between biofilms of the same degradative consortium grown on TSB versus diclofop methyl have been discussed previously (35). It was shown that the time required for biofilms grown on diclofop methyl to reach maturity was between 14 and 21 days (35). In the present study, fluorescence of the diclofop methyl-grown biofilms typically reached a maximum value after 14 to 21 days, and this value then remained relatively constant (even when the flow cell community was maintained for months),

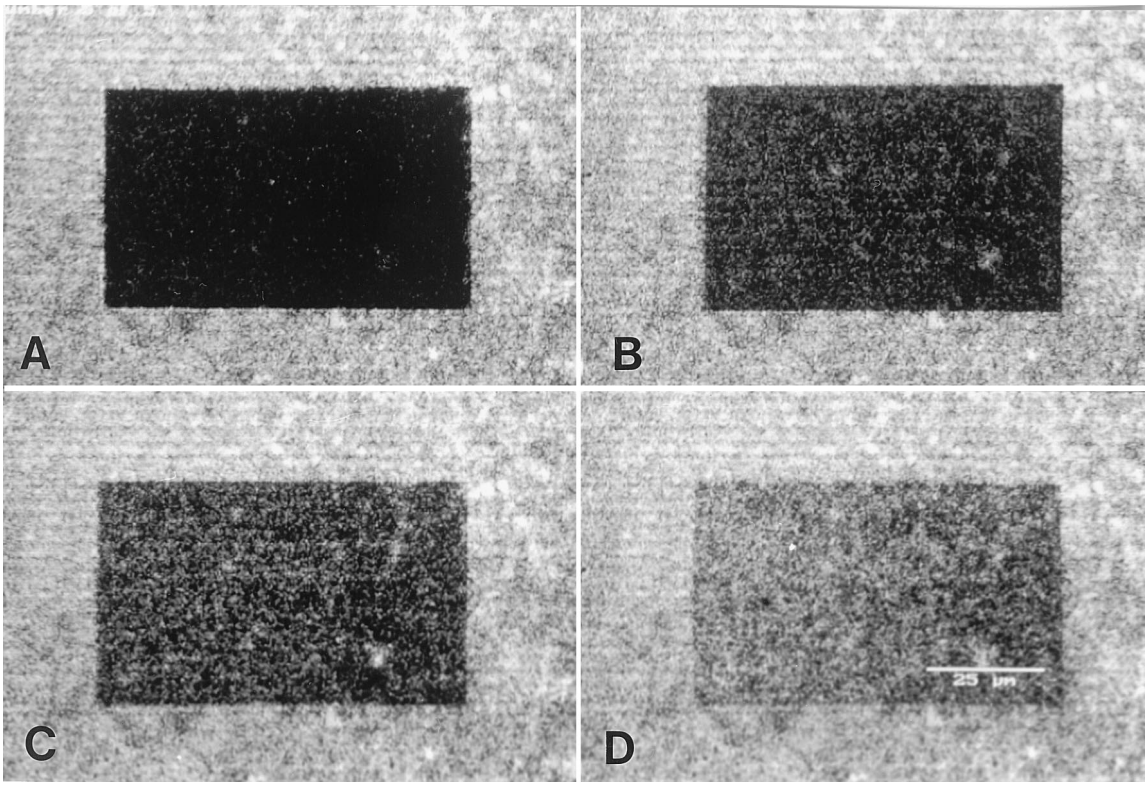

FIG. 2. FRAP was measured to compare the rates of accumulation in viable and KCN-inhibited biofilms. Shown here is a typical example of fluorescence recovery in a viable biofilm immediately (A), 3 h (B), 6 h (C), and 24 h (D) after photobleaching.

suggesting either (i) that the accumulation rate of diclofop methyl and ring compounds formed during partial degradation of diclofop methyl equaled the rate at which the ring structures of these compounds were degraded and that there was no new net growth of polymers or (ii) that all binding sites were occupied by immobilized diclofop compounds, with no subsequent utilization. The fact that no decrease in fluorescence intensity was observed after substitution of the diclofop methyl-minimal salts solution with TSB implied that the accumulated molecules were not utilized as a carbon source (when another carbon source was available). In contrast, the rapid decrease in fluorescence intensity in living biofilms after replacement of the diclofop methyl-minimal salts solution with the minimal salts solution without a carbon source suggested subsequent utilization of the accumulated compounds. The minimal change in fluorescence after inhibition of the biofilm bacteria (Fig. 4A) provided additional support for the assumption that the accumulated diclofop was utilized by biofilm bacteria. However, desorption of diclofop from biofilm EPS might also have played a role in this decrease in fluorescence; therefore, additional experiments were required to verify this assumption. The objective of the ensuing experiments was to study the role of microbial activity in the accumulation of diclofop methyl and its breakdown products and to determine whether the accumulated compounds were available for subsequent utilization as a carbon source.

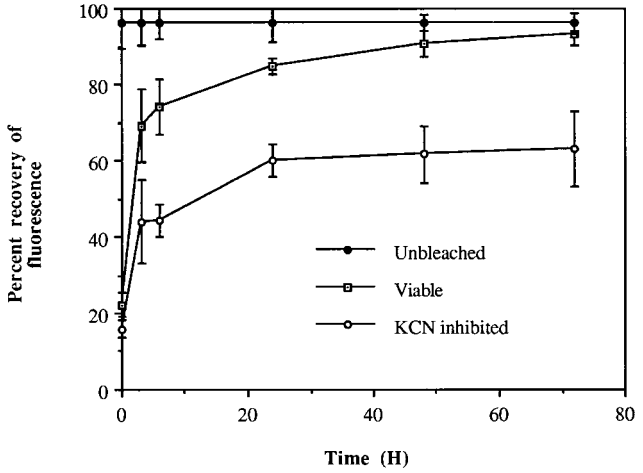

FIG. 3. Profiles of FRAP in viable and inhibited biofilms. The fluorescence intensities in photobleached areas are represented as percentages of the average intensity in unbleached areas.

Efforts were made to displace the accumulated fluorescent compounds from the biofilm exopolymers. The rationale was to displace the accumulated compounds and then measure the rate of reaccumulation by the EPS of active and inhibited biofilm communities, thereby evaluating the role of microbial activity in the accumulation process. Close associations which developed between certain bacteria when this nine-membered biofilm consortium was cultivated on chlorinated ring compounds such as diclofop methyl gave rise to the development of a highly irregular biofilm topography (35) and subsequently to much variation in measured values of biofilm thickness (23.6 ± 15.1 μm). Comparison of optical thin sections collected at selected points in both the *xy* and *xz* planes, before and after treatments, was therefore the preferred method to evaluate the

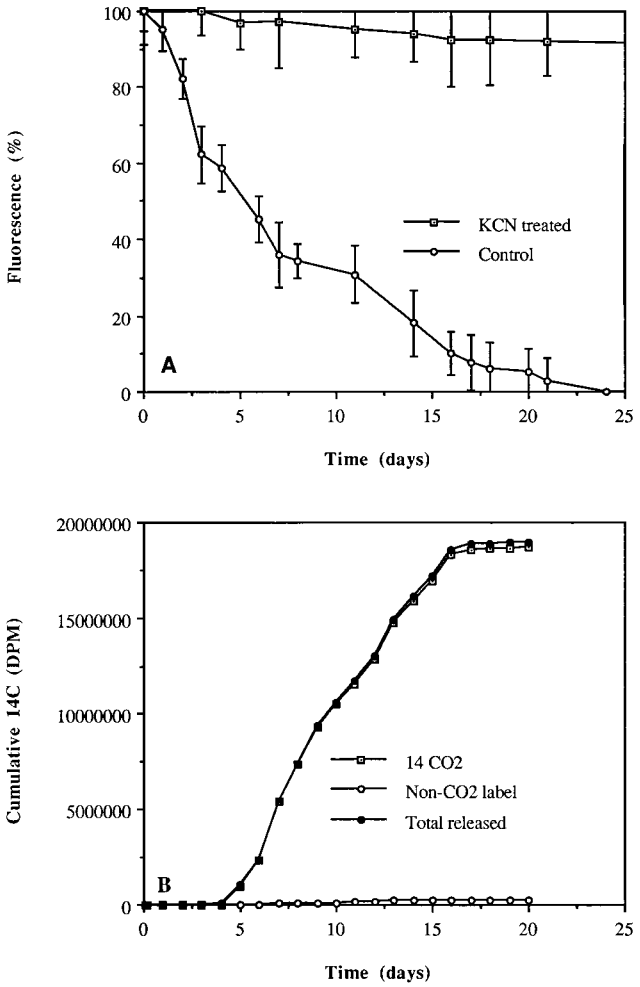

FIG. 4. (A) Degradation of sorbed compounds as indicated by change in fluorescence intensity. Biofilms were cultivated with diclofop as the sole carbon source for a minimum of 21 days to allow sorption of the diclofop and metabolites by EPS. The biofilms were subsequently irrigated with a minimal salts solution without a carbon source. Fluorescence was measured to determine the fate of the sorbed compounds during the absence of a carbon source in the irrigation solution. A decrease in fluorescence in viable but not cyanide-inhibited biofilms suggested that microbial metabolism was responsible for the decrease. (B) Growth of the biofilm community on [^{14}C]diclofop followed by measurement of $^{14}CO_2$ evolved during irrigation with the carbon-free minimal salts solution confirmed mineralization of the sorbed compounds. Note also the relatively small amount of label recovered as non-CO_2 products in the effluent.

effect of the treatments on accumulated diclofop and biofilm structure.

The efforts at elution with NaCl or with organic solvents were unsuccessful. Similar observations were made by Grimes and Morrison (14), who reported difficulty in removing chlorinated insecticides from bacterial cells when washing them with deionized water, acetone, or phosphate buffer. However, Geller (13) observed rapid desorption of atrazine from three bacterial isolates after washing them with phosphate buffer. Both studies investigated rapid adsorption (up to 4 h) to suspended cells in pure culture. EPS were unlikely to play such an important role as in the present study, which focused on accumulation in biofilms containing large amounts of EPS, formed over weeks. The treatments used in the present study also did not affect the structural integrity, such as the thickness of biofilms, demonstrating the stability of the EPS matrix in these mixed-species biofilm communities. This is in contrast to results obtained by Marshall et al. (22), who reported that biofilm polymers of *Pseudomonas fluorescens* contracted when treated with electrolytes such as NaCl, with the simultaneous reduction in biofilm thickness.

Failure to remove fluorescent compounds from mature biofilms by NaCl or organic solvents was followed by the utilization of FRAP to alter the fluorescent character of accumulated molecules. FRAP is generally used to measure diffusion-dominated transport in liquid systems (5). Typically, a fluorescent, mobile probe is irreversibly bleached in a defined region of the biological matrix under investigation with a high-intensity light source, and the rate at which the nonfluorescent molecules are replaced by fluorescent molecules is measured to indicate the mobility of the probe (21). Nonreactive probes which do not interact with the medium and which have a strong fluorescent signal are ideal for this purpose. The fluorescent signal of diclofop methyl or its degradation products in solution was not strong enough at the concentrations used to be detected by SCLM, although they could be measured by fluorimetry. However, accumulation of these compounds in biofilm material, and possibly a shift in excitation and emission spectra of the sorbed molecules, resulted in a stronger fluorescent signal which could easily be detected by SCLM. Photobleaching of the accumulated molecules rendered them nondetectable. Replacement of the bleached molecules, presumably by diclofop from the irrigation solution, was accompanied by fluorescence recovery, providing an indication of the turnover rate, probably controlled by the degradation rate, of the accumulated molecules.

The higher rate of fluorescence recovery after photobleaching in untreated biofilms than in biofilms in which the cells were inhibited by KCN (Fig. 3) indicated that microbial activity was responsible, at least in part, for the accumulation of diclofop in biofilms. Tsezos and Bell (32) observed that the amount of organic pollutants accumulated by dead microbial biomass was greater than accumulation by living microorganisms, as determined by measuring the remaining concentrations of the pollutants in solution, and cited other investigators who made similar observations. The compounds used by these authors were presumably not degraded by the microorganisms used in their studies (31). No comparison was made in the present study between the amounts of diclofop accumulated by living and dead biofilms. However, on the basis of the assumption that photobleaching affects only the fluorescent characteristics of diclofop, it was shown that microbial activity increased accumulation rates of organic molecules by biofilm material. Although the FRAP data could not indicate whether this effect was direct or indirect (the latter as a result of biodegradation which frees binding sites), it indicated that this accumulation is not entirely passive as suggested for the accumulation of trace metals such as mercury (16).

The release of $^{14}CO_2$ from the biofilm containing accumulated [^{14}C]diclofop methyl for 21 days when the carbon source was excluded from the irrigation solution indicated that the compound or products of its partial degradation were indeed stored in biofilm EPS and acted as a carbon reserve for subsequent mineralization during nutrient limitation (Fig. 4B). This result supported the earlier suggestion (based on the observed decrease in fluorescence intensity in living biofilms but not in inhibited biofilms without an exogenous carbon source) that such a mechanism does exist. It also supports the contention that release of the contaminant occurs via degradation, not desorption, as does the comparably small amount of label recovered in the effluent stream as non-$^{14}CO_2$ materials (Fig. 4B). The observed lag in the appearance of $^{14}CO_2$

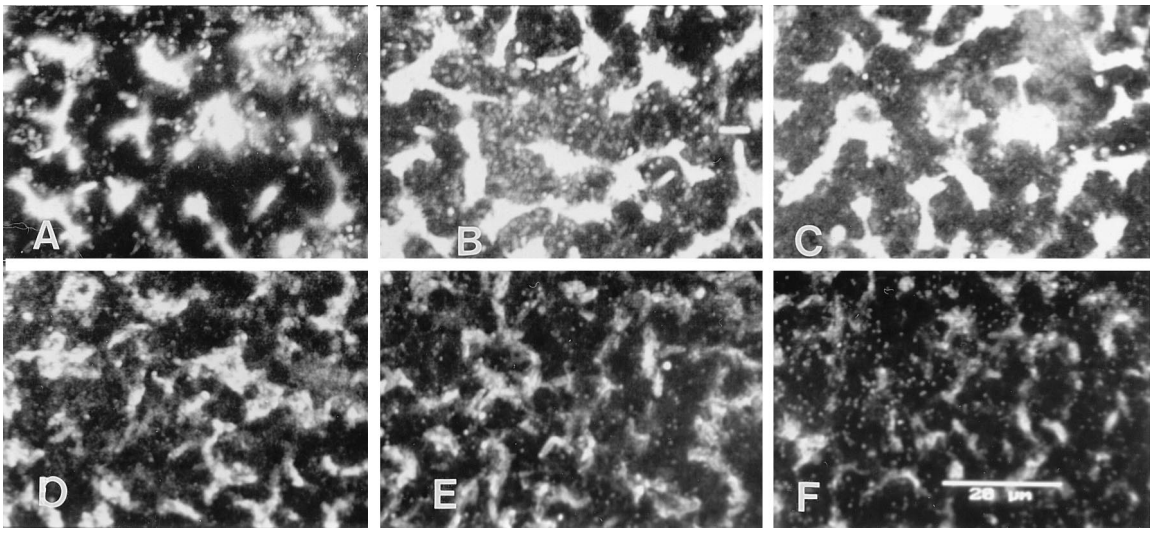

FIG. 5. SCLM images of unstained biofilms showing no decrease in fluorescence intensity with time in biofilms inhibited with KCN after 14 days (A), 21 days (B), and 28 days (C). However, significant decreases in accumulated compounds were observed in viable biofilms after 14 days (D), 21 days (E), and 28 days (F), respectively. Also compare with profiles in Fig. 4A.

relative to decline in fluorescence signal (Fig. 4A and B) is consistent with the fact that loss of fluorescence is due to ring cleavage whereas production of detectable $^{14}CO_2$ occurs after ring cleavage in the degradation process.

The arguments against a role for EPS in bacterial nutrition are based primarily on the absence of enzymes which would enable bacteria to depolymerize their own EPS (12, 27) and the physical and chemical properties of biofilms which should make it unlikely for EPS to function effectively as a nutrient trap (8). However, although these arguments are relevant to pure cultures, they may not be applicable to heterogeneous communities, such as those used in the present study and found in natural systems, in which utilization of the EPS produced by other bacteria of the same community may be possible.

In conclusion, our results indicated that the accumulation of diclofop methyl and its breakdown products by EPS may be an active mechanism which enabled the bacterial community to optimize utilization of these compounds as carbon sources. The results contradicted the contention (8) that EPS do not play a role in the storage of carbon sources. These mechanisms may be essential for microbial communities to function in oligotrophic environments and may play an important role in the attenuation of agricultural or industrial pollutants in pristine environments.

ACKNOWLEDGMENTS

This work was supported by a PestPlan, Environment Canada grant (to J.R.L.).

Hoechst (AgrEvo) Canada is acknowledged for providing labeled diclofop methyl. George D. W. Swerhone and Kerry Peru are acknowledged for technical support.

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