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Microbial Exopolymers Provide a Mechanism for Bioaccumulation of Contaminants

G.M. Wolfaardt,[1,2] J.R. Lawrence,[1] J.V. Headley,[1] R.D. Robarts,[1] D.E. Caldwell[2]

[1]National Hydrology Research Institute, 11 Innovation Boulevard, Saskatoon, Saskatchewan, Canada, S7N 3H5

[2]Department of Applied Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0

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Abstract. Scanning confocal laser microscopy was used to directly visualize accumulation of the herbicide diclofop methyl and its breakdown products by a degradative biofilm community, cultivated in continuous-flow cell cultures. Some bacterial cells accumulated these compounds. However, most accumulation occurred in cell capsules and certain regions of the exopolymer matrix. Mass spectroscopic analysis of the biofilm material confirmed accumulation of the parent compound and its breakdown products in the biofilms. Lower molecular weight degradation products were found in the effluent, indicating mineralization of diclofop by the flow cell cultures. Grazing protozoa feeding on the biofilms nonselectively ingested cell capsules and exopolymers, suggesting direct transfer and accumulation of the contaminants in protozoa. These findings demonstrated that microbial exopolymers can play an important role in the bioaccumulation of contaminants in natural systems.

Introduction

Accumulation and transfer of organic contaminants through food chains in natural systems is a widespread problem. The mechanisms involved, particularly those responsible for the incorporation of contaminants into the food chain, remain unclear [1]. Most of our knowledge of these mechanisms stemmed from relatively few laboratory studies that have addressed the role of microbial cultures in the bioaccumulation of organic molecules. Examples of these studies include work done by Leshniowsky et al. [21], who reported on the ability of two isolates from a floc-forming bacterial community to concentrate and accumulate the pesticide aldrin from solution; Geller [15], who studied sorption of atrazine by three bacterial

Correspondence to: J.R. Lawrence.

isolates; and Grimes and Morrison [16], who also studied accumulation of chlorinated hydrocarbons by isolated cell lines. The last study suggested that cell surface area played an important role in microbial sorption of pesticides.

Accumulation of contaminants by microbial biomass, especially cell aggregates and sessile microorganisms growing attached to surfaces in biofilms, plays an important role in the functioning of biological waste-water treatment systems. Tsezos and Bell [31] termed this accumulation biosorption, and noted that the process responsibe for this accumulation is a complex combination of various mechanisms, involving among others, adsorption to cell surface molecules, and absorption into various, unidentified components of microbial cells. These studies focused primarily on measuring the amounts of organics accumulated by the bulk microbial biomass in water treatment systems, differentiation between sorption to live and dead biomass, and determining sorption to cell wall preparations [4,30].

Microbial biofilms, which represent the lowest trophic level of the food chain, constitute a significant portion of the carbon assimilated by organisms in higher trophic levels, thereby playing an important role in nutrient cycling and energy flow [12,25]. Several workers have demonstrated that bacterial extracellular polymers and other highly reactive cell components are involved in the binding of metals by complexation of metal ions [7, 14, 18, 29, 33], and by ion exchange between the metals and polyanions of the cell wall [24]. Incorporation of the accumulated metals in food chains, and transfer to higher organisms by biomagnification, have also been discussed [22, 23]. It is possible that the chemically diverse exopolymeric matrix in which biofilm cells are embedded is in a similar way responsible for the accumulation of significant amounts of organic contaminants, and the subsequent transfer of these compounds to higher trophic levels in natural ecosystems. In fact, based on the observation that dissolved organic material competed with trace metals for binding sites on organic ligands, it was suggested by others that the binding mechanisms of organic compounds and metals to microbial exopolymers were similar [12].

Studies focusing on the concentration of organic contaminants by pure cultures referred to above, and other related studies [1, 3], have demonstrated that microorganisms are involved in the bioaccumulation of organics. In addition, studies on accumulation of contaminants by microbial biomass in biological waste-water treatment systems provided adsorption data that enabled evaluation of the efficiency of pollutant removal from waste-water streams. However, information on the sites where sorption occur, the relative importance of sorption to extracellular polymers (EPS) or absorption into cells, and the ecological significance of this phenomenon have largely remained an area of speculation [3, 12, 32]. The lack of evidence for the role of EPS in the accumulation of organic contaminants, or their role as a vector introducing contaminants into the food chain, was probably due to the unavailability of techniques allowing direct observation of these compounds when concentrated in microbial biomass such as EPS. The development of scanning confocal laser microscopy (SCLM), which allows direct, nondestructive observations of biological materials [19], provides a technique to address this limitation, enabling a better understanding of the structure and function of biofilms.

Fluorescence can be utilized to view samples with SCLM. The laser produces a high-intensity illumination, and since the returning signal is processed point-by-point, even low levels of fluorescence can be imaged with a sensitive photomulti-

plier [6]. This high sensitivity, and the capability to observe samples in situ, render SCLM suitable to demonstrate the presence and distribution of fluorescent molecules in biological material such as biofilms. Many of the common organic contaminants fluorescence when excited with light of the appropriate wavelength, due to the presence of aromatic rings in their chemical structures. This property can be exploited to locate the accumulation of aromatic contaminants in biofilms.

SCLM and mass spectroscopy (MS) were utilized in the present study to view and quantify the accumulation of diclofop methyl by a degradative biofilm community.

Materials and Methods

Isolation of a Diclofop Methyl-Degrading Microbial Community

A degradative microbial consortium, capable of utilizing the herbicide diclofop methyl as sole carbon source, was isolated from the A-horizon of a meso-scale model aquifer [20] and maintained in a 250-ml continuous culture system on a minimal salts solution [10] amended with 14 $\mu g\ ml^{-1}$ diclofop methyl (Riedel-de Haën, Caledon Laboratories, Georgetown, Ontario; 99% purity).

Continuous-Flow Culture Chambers

Flow cells [10] were connected in closed loops to the continuous culture to facilitate microscopic observations of biofilms formed by the degradative microbial community. This community was also cultivated in separate flow cells, irrigated with the diclofop-minimal salts medium, or the minimum salts medium plus either 2,4 dichlorophenol, 1,3 dichlorobenzene, or 4-[2,4-dichlorophenoxy] phenol, or with 0.05% tryptic soy broth (TBS; Difco Co., Detroit, MI). The construction and maintenance of the flow cells are described elsewhere [34].

Microscopy

Laser microscopy was performed using a Bio-Rad MRC-600 scanning confocal laser, mounted on a Nikon Microphot-SA microscope. The microscope was equipped with a ×60, 1.4 numerical aperture oil immersion lens. Negative staining of the biofilm was done using fluorescein [9]. Optical sectioning of biofilms was done following the method described by Lawrence et al. [19]. Accumulation and spatial distribution of diclofop and other aromatic compounds in the biofilm matrix were determined by examining optical thin sections of unstained biofilms when the molecules of these compounds, or their aromatic breakdown products, were excited with the argon laser beam (maximum emission lines at 488 and 514 nm, and a number of smaller peaks from 274 to 528 nm). At least a 50% transmission setting for the laser intensity was required to obtain the desired level of fluorescence by diclofop methyl. Gain and black levels were manually set at 10 and 5, respectively, and the pinhole was 50% closed. Images of unstained TSB-grown biofilms were also collected to determine the laser and photo-multiplier settings at which fluorescence in the TSB grown biofilms was not observed. These settings allowed a detection threshold to be set which discriminated between the background fluorescence and that originating from diclofop and its degradation products. Fluorescence intensity was measured using the "stats" command of the Bio-Rad software. This program involves measuring the minimum, maximum, mean, and standard deviation of the grey values within an arbitrary rectangular area. Acquisition of all images was done with the ×60 oil immersion lens and a ×1.5 electronic zoom.

Fluorescence intensity was measured in protozoa feeding on diclofop or TSB-grown biofilms. XY optical thin sections were collected through amoebae feeding on these biofilms, and the Bio-Rad

software was utilized to measure the average fluorescence gray-value in individual cells. Fifty cells were analyzed for each substrate. An additional experiment was carried out to verify utilization of diclofop-related compounds sorbed to EPS. In this experiment, degradative biofilms were cultivated on glass beads in a simplified perfusion column (Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE, unpublished data). The glass beads were removed from the column, thoroughly rinsed with the minimal salts solution to remove any unbound diclofop, and subsequently exposed to mild sonication for 5 min to disrupt the EPS, but not the bacterial cells. A cell-free solution was prepared from this mixture by filtration through a 0.2 μm filter. This cell-free solution was used as the substrate to cultivate the bacterial consortium. The average fluorescence gray-value in predators feeding on this biofilm was determined as described above.

Mass Spectrometry

Flow cells containing mature biofilms (older than 21 days) cultivated on diclofop were opened and samples of the biofilm material collected and analyzed for the parent compound and its breakdown products. Samples of the sterile influent and the effluent of flow cells irrigated with the diclofop-minimal salts medium were also analyzed. A VG Autospec-Q mass spectrometer, equipped with a 4100-60 VAX data system and Opus 2.1 software, was used for this analysis, following the procedure described by Headley et al. (Headley JV, Peru K, Lawrence JR, Wolfaardt GM, unpublished data). In essence, this method involves direct insertion of a 2-μl sample for instrumental analysis with no sample extraction, cleanup, or preconcentration step. All samples were analyzed within 5 min after collection, thereby minimizing transformation during storage and analysis. Samples were introduced via a direct insertion probe into the mass spectrometer (MS). The MS was operated under electron impact ionization conditions, with source temperature 250°C, 70 eV, trap current 200 μA, mass resolution 1300, scan speed 1 decade/s, and mass range 50–600 amu.

Fluorometry

A Turner Designs Model 10 fluorometer, equipped with a light source and filters recommended for fluorescein (excitation and emission maxima = 495 and 515, respectively; these maxima are close to that of the argon laser), was utilized to determine the relationship between the concentration of diclofop methyl and fluorescence intensity. Excitation and emission maxima for diclofop methyl and two of its known one-ring breakdown products (2,4 dichlorophenol and 1,3 dichlorobenzene) were determined using a Perkin Elmer LS50 spectrophotometer.

Measurement of Accumulation and Disappearance of Diclofop Methyl in Biofilms

Flow cells irrigated with the diclofop-minimal salts medium were inoculated with the degradative microbial community, and the change in fluorescence intensity recorded for 21 days. The diclofop medium was then replaced with the minimal salts solution without diclofop for a further 35 days before irrigation with the diclofop medium was resumed until termination of the experiment (day 67). The intensity of fluorescence caused by diclofop in unstained biofilms was used as an indication of the relative amount of this compound, or its metabolites, accumulated in the biofilm matrix. Images of the unstained biofilms were digitized and fluorescence intensity measured on a 0–255 gray-value scale [8], using the Bio-Rad software. The mean gray-value of 20 images, randomly collected along the entire length of the flow cell, was determined for each sampling date.

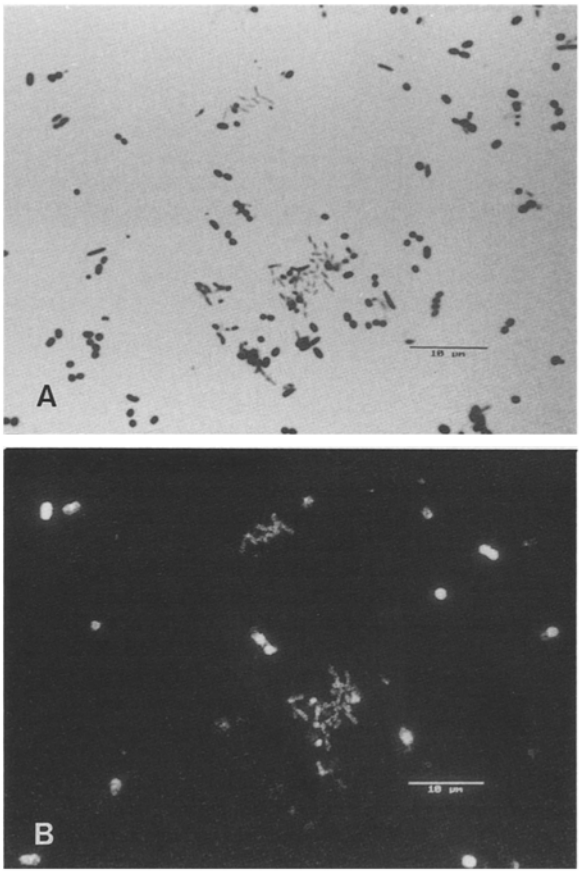

Fig. 1. A scanning confocal laser microscope was used to visualize accumulation of chlorinated organics in biofilms. Shown here are photomicrographs of a 24-h-old degradative biofilm. In **A,** fluorescein was used to negatively stain all microorganisms. The fluorescent signal of diclofop and its breakdown products were digitized in **B** to detect bacteria with high concentrations of these compounds adsorbed to the cell surface. Laser intensity was set at a threshold value at which background fluorescence by bacteria grown on TSB was negligible. The difference between the total number of cells and the number that accumulated the diclofop and its breakdown products suggests differential adsorption of these compounds by the members of this degradative consortium.

Results

Microscopic Examination of the Degradative Community

Flow cells connected to the continuous culture provided a simple way to examine biofilms formed by the degradative microbial community. This community consisted of at least nine bacterial species [35] and a variety of protistans. The fluorescence intensity of diclofop-grown biofilms, measured at a 100% transmission setting for laser intensity, gain and black levels manually set at 10 and 5, respectively, and the pinhold 50% closed, was 8.5 times higher than that of the TSB-grown biofilms (the TSB concentration used provided 7.8 $\mu g\ ml^{-1}$ carbon, the same amount of carbon provided by the 14 $\mu g\ ml^{-1}$ diclofop medium). These settings allowed observation of diclofop fluorescence within the optimum detection limits of the equipment, while fluorescence by TSB-grown biofilms was negligible. Diclofop methyl, at the concentration applied in this study, could not be detected in free solution at these settings. However, certain cells (Fig. 1), exopolymers (Fig.

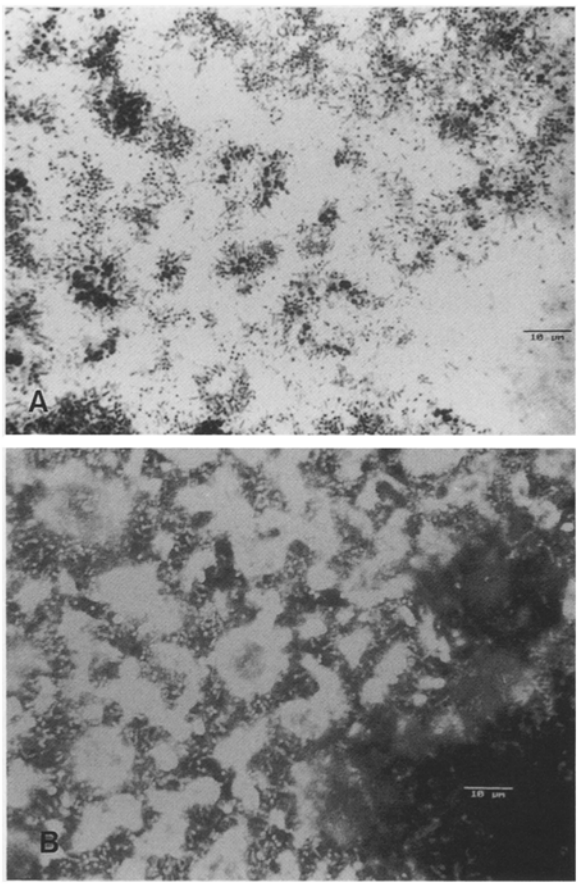

Fig. 2. **A** An optical thin section, collected at a depth 3 μm from the base of a 21 day old, negatively stained biofilm showing all the cells in a microscope field. **B** An image of the same field, before negative staining was applied, to show accumulation of diclofop methyl by EPS. Laser intensity was set at a threshold value at which background fluorescence was negligible.

2), and cell capsules (Fig. 3) became fluorescent within a few days of growth when diclofop methyl was provided as the sole carbon source, suggesting that diclofop, and/or its degradation intermediates, were selectively concentrated in the biofilms.

A variety of protistans, including flagellates, ciliates, and amoebae, were observed feeding on the diclofop-grown bacterial biofilms. The increase in fluorescence intensity observed in exopolymers and cell capsules of diclofop-grown biofilms was accompanied by an increase in fluorescence of these protistans feeding on the biofilms. SCLM optical thin sections revealed the presence and location of fluorescent regions, corresponding to intracellular food vacuoles of the predators (Figs. 4 and 5), suggesting the transfer of fluorescent ring compounds between the trophic levels. A similar observation was made in the predators feeding on an EPS (cell-free) solution. In contrast, protistans feeding on TSB-grown biofilms did not show such an increase and there was a significant difference ($P \leq 0.05$) between these two groups. For instance, the mean gray-value per cell in amoebae feeding on diclofop-grown biofilms was 90.4 (SD = 21.9) and those feeding on TSB-grown biofilms was 14.8 (SD = 4.2).

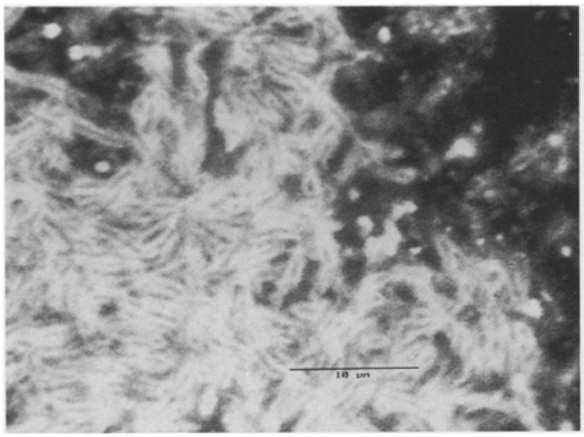

Fig. 3. An optical thin section of an unstained biofilm, electronically zoomed to illustrate accumulation of diclofop methyl in cell capsular and exopolymer material.

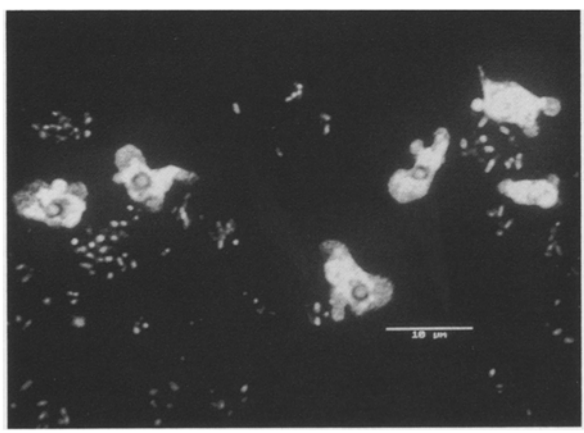

Fig. 4. Predators ingested cells and exopolymer of the bacterial consortium grown on diclofop methyl, resulting in accumulation of this compound and its aromatic breakdown products in the food vacuoles of the predators. An optical XY thin section through amoebae feeding on a diclofop-grown biofilm shows accumulation of the fluorescent compounds in cells and food vacuoles. Laser intensity was set at a threshold value at which background fluorescence was negligible.

MS Analysis

The MS data confirmed accumulation of the parent compound diclofop methyl and its aromatic breakdown products in the biofilm material. The breakdown products identified were 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-phenoxy-phenol. This result was verified in a subsequent study utilizing probe MS/MS (Headley JV, Peru K, Lawrence JR, Wolfaardt GM, unpublished data). The presence of these breakdown products in the exopolymer, but not in the influent (except 4-phenoxy-phenol), also suggested mineralization of diclofop methyl.

Fluorometry

Figure 6 shows that there was a linear relationship between the concentration of diclofop methyl and fluorescence intensity. The exitation and emission maxima of

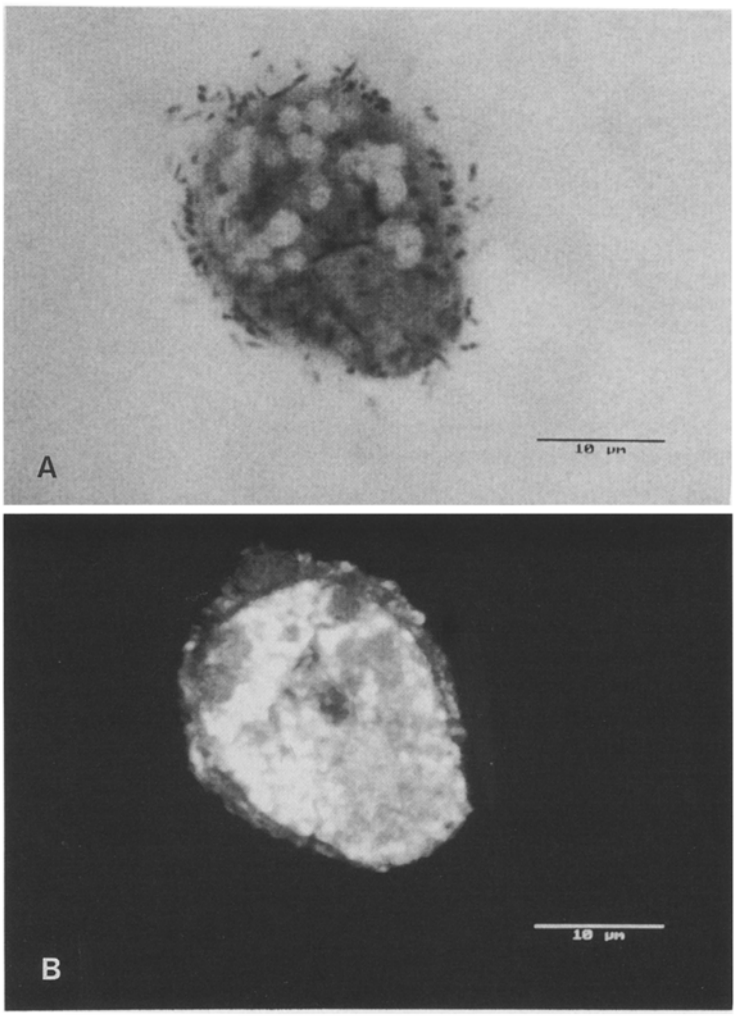

Fig. 5. **A** An optical XY thin section through a negatively stained flagellate feeding on the diclofop-grown degradative biofilm. **B** An optical section at the same focal plane, without negative staining shows accumulation of diclofop in food vacuoles of the predator.

diclofop methyl, 2,4 dichlorophenol, and 1,3 dichlorobenzene are shown in Table 1.

Changes in Fluorescence Intensity over Time in the Presence and Absence of Diclofop Methyl

Biofilm development in flow cells, irrigated with the diclofop-minimal salts medium and inoculated with the degradative microbial community, was accompanied

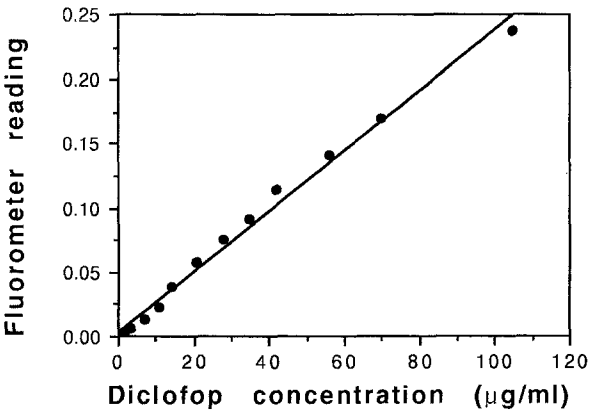

Fig. 6. Relationship between the concentration of diclofop and fluorescence intensity, measured with a fluorometer equipped with a light source and filters recommended for fluorescein.

Table 1. Excitation and emission maxima of diclofop-methyl and two of its one-ring breakdown products

Chemical	Excitation	Emission
Diclofop methyl	420	504
2,4 dichlorophenol	255	313
1,3 dichlorobenzene	313	376

by an increase in fluorescence intensity with time (Fig. 7). This increase in fluorescence intensity was typically sustained for 14–21 days, with little or no change thereafter, even if the biofilm community was maintained for an additional 90 days. However, the intensity decreased when the diclofop-minimal salts medium was substituted by a minimal salts-only medium. Subsequent reintroduction of diclofop methyl was followed by a rapid increase in fluorescence intensity until the same maximum values, recorded before the switch to minimal salts without diclofop methyl, were reached (Fig. 7). A switch from TSB to diclofop as the sole carbon source also resulted in a rapid increase in fluorescence intensity until values similar to those measured in mature diclofop-grown biofilms were reached.

Discussion

Bacterial extracellular polymers can act as ion-exchange resins [13] and they may be sinks for organic materials and metals [5, 11]. However, the involvement of these polymers in the concentration of organic contaminants or their metabolites during degradation is largely unknown. In the present study SCLM, used to analyze biofilms in flow cells connected to a continuous culture, provided a simple technique to study the fate of the chlorinated ring compound diclofop methyl in biofilms

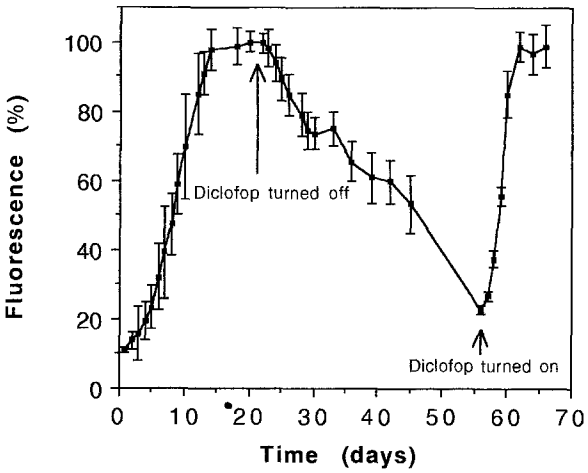

Fig. 7. Accumulation of diclofop methyl in the biofilm matrix resulted in an increase in fluorescence intensity with time. The gradual decrease in fluorescence after diclofop supply was turned off suggested either desorption of bound diclofop, or mineralization by the degradative consortium.

formed by a degradative microbial community. This technique required minimal preparation, yet it provided excellent visualization of the accumulation of contaminants in the biofilm matrix. Additional advantages of this approach were the nondestructive nature of SCLM analysis, giving real-time observations over an extended time period, the ability to analyze information in a single optical section, and three-dimensional localization of the accumulated compounds.

The increase in fluorescence with time in biofilms cultivated on a medium containing diclofop as the sole carbon and energy source suggested that accumulation of the parent compound and/or its aromatic degradation products were responsible for the fluorescence. The excitation maxima of these compounds fall within the range of the argon laser. Complexation, i.e., between an aromatic compound and EPS, may result in a spectral shift [27] which may render these compounds more fluorescent, and thus easier to detect, when sorbed to EPS. Use of MS techniques confirmed accumulation of diclofop methyl and its known breakdown products. These metabolites may be further transformed by hydroxylation, followed by dehydrogenation to form catechols [23]. In fact, the total ionic chromatograph obtained with the MS indicated the presence of 1,2-benzenediol, a catechol, in addition to the breakdown products listed above. It has been suggested in other studies (e.g., [2]) that the oxidative polymerization of catechols leads to the formation of humus-like material. The latter may also have contributed to the fluorescence observed in the present study when the consortium was grown on diclofop as the sole carbon source. The chemical structure of diclofop methyl favors hydrolysis when mixed with water to form diclofop acid. The acid undergoes decarboxylation to form a two-ring phenol, followed by further degradation to form smaller (one-ring) breakdown products, and finally CO_2 [28]. Hydrolysis of diclofop methyl to the acid form in the minimal salts solution was shown to be prevented by the phosphate buffer, which would explain the absence of diclofop acid in the influent.

Based on the known relationship between fluorescence intensity and the amount of aromatic compound (Fig. 6), we were able to record the accumulation and disappearance of diclofop and its aromatic breakdown products in the biofilm

matrix (Fig. 7). Fluorescence intensity in the biofilms typically reached maximum values within 14 to 21 days when the influent contained diclofop, with no further increase in intensity with time, suggesting that all the binding sites for this compound in the biofilm matrix were occupied. It was shown earlier that this bacterial consortium formed near steady-state biofilms in 14–21 days [34], the same time required to achieve maximum accumulation of aromatic compounds. This suggests that most accumulation takes place only in the presence of certain cell types and EPS, and that the rate of accumulation was dependent on the establishment of these cells and EPS in the biofilm. Fluorescence intensity decreased with time when diclofop methyl and other carbon sources were excluded from the influent. A possible explanation for this phenomenon is that the bound aromatic compounds are partly degraded outside the cells by extracellular enzymes before they are taken up by the cells for further, complete mineralization (complete mineralization of ^{14}C-labeled diclofop methyl to $^{14}CO_2$ by this degradative consortium has been reported) [35]. It can therefore be argued that after this equilibrium was reached (when diclofop was provided as the sole carbon source in the influent), the rate of accumulation into biofilm exopolymers was equal to the rate of removal by degradation. The rapid increase in fluorescent intensity measured in mature biofilms after the reintroduction of diclofop provided further support for the suggestion that the rate of accumulation was dependent on the presence of certain types of cells and EPS.

Transfer of contaminants in food chains, and concern for the resulting accumulation and adverse implications for organisms at higher trophic levels, have been the subjects of many studies. For example, Gossett et al. [17] demonstrated the accumulation of a variety of contaminants in marine sediments and animals near the discharge zone of a waste water treatment plant. The same authors noted that the uptake of organic contaminants by organisms at higher trophic levels occurred primarily via their food supply and not by dermal absorption. It was subsequently suggested that accumulation of contaminants in these organisms may be dependent on the availability of the contaminants to the base of the food web. However, it was unclear from their study how the contaminants entered the food chain. Other studies in marine ecosystems have indicated that the cellular biomass of bacteria is insufficient to support grazer populations; therefore, it has been suggested that bacterial exopolymers may comprise more than 50% of the carbon assimilated by organisms in higher trophic levels [12]. The observations made in the present study suggest that ingestion of microbial exopolymers, and not only cells per se, is an important mechanism for direct transfer of contaminants in microbial communities. The inverse relationship we observed between the relative abundance of diclofop and its breakdown products in biofilm material and the effluent indicated selective concentration of these compounds in biofilms. Similar observations were made when other chlorinated ring compounds (2,4 dichlorophenol, 1,3 dichlorobenzene, and 4-[2,4-dichlorophenoxy] phenol) were provided as sole carbon source (data not shown), demonstrating the generality of these observations. Amoebae, ciliates, and flagellates became fluorescent when feeding on diclofop-grown biofilms. These predators were six times less fluorescent when TSB was provided as the substrate. This difference suggested accumulation of the fluorescent compounds in the predators (Figs. 4 and 5). Previous observations by Sherr [26] suggested direct protistan feeding on high-molecular-weight compounds. It thus seems possible that bacterial

exopolymers provided a mechanism for the direct transfer of diclofop and its aromatic breakdown products to protistans.

Assessment, control, and monitoring of agricultural and industrial chemicals in the environment require a clear understanding of all the factors controlling their chemodynamics. The observations made in this study suggested that microbial exopolymers can play an important role in accumulation of chlorinated compounds and subsequent transfer of these contaminants through the food chain. The findings should have implications for studies on the fate of agro-industrial chemicals. Finally, additional laboratory and field studies are required to better understand the structure and function of microbial communities, their importance in the concentration and transfer of contaminants, and their potential for bioremediation.

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