Assessment of fluorescein diacetate hydrolysis as a measure of total esterase activity in natural stream sediment biofilms

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Abstract

I applied the fluorescein diacetate (FDA) hydrolysis technique as a rapid and sensitive estimator of total esterase activity in stream sediment biofilms. I investigated the effects of temperature, pH and incubation time and optimized the assay for low blanks and high fluorescein extraction. The FDA procedure was precise (c.v. = 4.15%) and could detect 10 nM fluorescein. Spatial patterns of esterase activity within stream sediment biofilms correlated with electron transport system activity, bacterial thymidine incorporation, glucosidase activity and chlorophyll a. As such, I suggest the modified and optimized technique as applicable to the investigation of total stream biofilm esterase activity. © 1997 Elsevier Science B.V.

Keywords: Fluorescein diacetate; Biofilm esterase activity; Stream sediment; Bacteria; Thymidine; Glucosidase; Electron transport system; EPS

1. Introduction

Stream biofilms represent a complex aggregation of bacteria, fungi and algae, which are embedded in a polysaccharide matrix. They constitute a major processing site of organic matter in stream ecosystems (Lock, 1993). A variety of techniques are now available to estimate biofilm metabolic (e.g. Lock and Ford, 1985; Blenkinsopp and Lock, 1990) and physiologic (Kaplan and Bott, 1985) activities. However, they either target the biofilm heterotrophic or autotrophic compartment and often require tedious laboratory work.

Fluorescein diacetate (FDA) is a non-polar esterified compound which can be hydrolysed by non-specific esterases (Guilbault and Kramer, 1964). It is generally assumed that FDA diffuses freely into intact cells (Rotman and Papermaster,
1966), where esterases hydrolyse it to the more polar fluorescein. The intracellular accumulation of fluorescein is mainly a function of its efflux and seems to be an energy-dependent process linked to a membrane potential (Prosperi, 1990). Therefore, the intracellular fluorescein accumulation renders the FDA turnover a useful indicator of cell activity.

FDA is known to be hydrolysed by bacteria (Lundgren, 1981; Chrzanowski et al., 1984), fungi (Söderström, 1977), algae (Gilbert et al., 1992) and rotifers (Moffat and Snell, 1995), and is therefore a potential indicator for the total esterase activity of stream biofilms. Microbial activity has been FDA assayed in soil (Schnürer and Rosswall, 1982), activated sludge (Fontvieille et al., 1992; Jørgensen et al., 1992; Frølund et al., 1995), in marine (Meyer-Reil, 1991; Poremba, 1995) and river sediments (Marmonier et al., 1995).

Despite this body of literature no standardized protocol exists for the FDA assay of intact, natural stream biofilms. Published techniques are mostly based on protocols from activated sludge or bacterioplankton studies. This prompted me to test and evaluate the use of the FDA hydrolysis as an estimator for esterase activity in natural stream biofilms. I experimentally checked environmental variables potentially influencing the FDA hydrolysis, measured the sensitivity and precision of the modified assay and evaluated it in a biofilm batch culture and in a field multiple assay comparison.

2. Methods

2.1. Sampling site and sampling

Sediment used throughout this study originated from Oberer Seebach (OSB) (615 m a.s.l., 47°15’N 15°04’E), a calcareous, second order mountain, gravel stream in Austria (Bretschko, 1991). Sediment and porewater samples were collected with a handpump from perforated PVC wells installed at sediment depths of 30, 60 and 90 cm in the hyporheic zone and the riparian zone. Samples were stored (4°C, in the dark) in precombusted (500°C, 4 h) borosilicate bottles and processed within 4 h after collection. I only considered sediment and particulate organic matter < 1 mm since most of the biofilm is associated with this fraction (Leichtfried, 1988). Sediment samples for the experimental trials were collected throughout 1995; the multiple assay comparison was performed on 11 March 1995.

2.2. Standard protocol followed in the trials

Sediment (0.3–0.5 g wet mass) was placed in sterile polypropylene tubes and diluted with 3 ml of 0.2-μM filtered stream water. Controls were inactivated with acetone to a final concentration of 50% (v/v) 30 min prior to the start of the assay. A working solution of FDA (Sigma Ltd.) was prepared in acetone (p.a. grade) and added to the samples to a final concentration of 200 μM. Samples were incubated for 30 min in the dark at ambient stream water temperature. Incubation was stopped with 3 ml undiluted acetone. Samples were then sonicated (30 W, 45 s) and centrifuged for 20 min at 5000 rev./min (Sigma tabletope centrifuge). Absorbance of the supernatant at 490 nm was measured spectrophotometrically against a water/acetone (50% v/v) blank. A 1-mM fluorescein stock solution was prepared from disodium fluorescein salt (Serva, research grade) and diluted with 0.2-μM filtered stream water to yield fluorescein standards ranging from 0.05 to 10 μM. FDA hydrolysis was expressed per g dry mass (DM, 80°C overnight) sediment.

2.3. Adaptation of the method

The effects of incubation time and temperature on the FDA hydrolysis were tested with time series run at ambient porewater temperature and 20°C. Substrate saturation was determined from temperature controlled incubations at substrate concentrations ranging from 2 to 620 μM FDA. Fluorescein exhibits maximum fluorescence at a pH of about eight (Guilbault and Kramer, 1964). Therefore, trials are often set to a pH of 7.6 with phosphate buffer (e.g. Fontvieille et al., 1992). Buffer addition can, however, alter the overall nutrient conditions for the microbial community and thereby its hydrolytic activity. The effect of
pH on the biofilm hydrolysis was tested with phosphate buffer (0.1 N KH$_2$PO$_4$ + 0.1 N Na$_2$HPO$_4$). To test possible effects of the buffer on hydrolysis, sediment was incubated with buffer (pH 7.6) and 0.2 μm filtered stream water, respectively.

FDA can be hydrolysed spontaneously without microbial activity (Guilbault and Kramer, 1964) thus necessating the use of killed controls. Acetone (Schnürer and Rosswall, 1982) and HgCl$_2$ (Fontvieille et al., 1992) have been suggested as inactivation agents. I tested the inactivation efficiency of acetone (50% v/v), formaldehyde (2.5% final concentration), HgCl$_2$ (0.2 mg/ml final concentration) and autoclaving (120°C, 30 min).

Fluorescein extraction from biofilms was tested by sonicating sediment samples. A Branson Sonifier 250 was equipped with a standard microtip, which was immersed 1-1.5 cm into the sample to prevent foaming and thus reduction of the ultrasonic energy. Sonication outputs of 10, 30 and 60 W were applied to four replicates and two killed controls which were chilled in an icebath to approx. 4°C. To check for sonication induced cleavage of FDA molecules, the same treatments were applied to a 200 μM FDA solution only made up in triplicate with 50% acetone.

2.4. Evaluation of the method

The relative precision of the method, defined as the coefficient of variation of replicate measurements (APHA, 1992), was calculated with 50 triplicate measurements of esterase activity from OSB hyporheic sediment. The potential sensitivity, as the lowest spectrophotometric fluorescein detection, was tested by serially diluting disodium fluorescein in 0.2-μm filtered stream water from 50 μM to 10 nM. The sensitivity was further checked by measuring hydrolysis of sediment sample sizes ranging from 30 to 900 mg dry mass.

The growth curve of biofilm generally takes a sigmoidal form (Characklis, 1989). To test whether the FDA assay is precise enough to follow this development, a batch culture was designed with approx. 150 g (wet mass) precombusted (500°C, 4 h) sediment and 2 l stream water; dissolved organic carbon concentration was 3.4 mg C/l. Stream water was prefiltered (0.5 μm) to remove major grazers. The culture was mildly aerated and stirred, and kept in the dark at room temperature in order to accelerate biofilm development. FDA hydrolysis, bacterial density and sediment carbohydrates were monitored on 12 dates over 18 days. Bacterial density was determined by staining bacteria with DAPI and epifluorescence microscopy (Velji and Albright, 1993); sediment carbohydrates were estimated by the phenol–sulfuric acid method (Dubois et al., 1956).

A multiple assay comparison was performed to analyse the relationship between FDA hydrolysis and other microbial parameters in the OSB. Sediment samples were collected from a total of 16 riparian and hyporheic sites which included sediment depths of 30, 60 and 90 cm. Parameters estimated concurrently on these samples were: ETS activity, $[^{3}H]$thymidine (TdR) incorporation, glucosidase activity and bacterial density. Biofilm ETS activity was measured via iodonitrotetrazolium (INT) (Blenkinsopp and Lock, 1990); assays were incubated with 0.02% INT for 3 h. $[^{3}H]$TdR (American Radiochemicals, 64.7 Ci mmol$^{-1}$) was used to measure TdR incorporation into bacterial DNA; the isotope was added at the saturation level concentration of 300 nM. After incubation (1.5 h), sediment was washed and frozen for subsequent alkaline extraction (0.3 N NaOH + 0.1% SDS + 25 mM EDTA) and analysis of $[^{3}H]$DNA (Findlay et al., 1984). α- and β-glucosidase activities were estimated using 4-methylumbelliferyl α-D-glucopyranoside and 4-methylumbelliferyl β-D-glucopyranoside (Hoppe, 1983). Substrate concentrations were 200 μM and 400 μM, respectively. Chlorophyll a was extracted with acetone (p.a. grade) (12 h, 4°C, in the dark) from previously frozen (liquid N$_2$) sediment and determined according to (Parsons et al., 1984).

2.5. Statistical analyses

One-way analysis of variance (ANOVA) was used to test effects among treatments. Relationships between microbial parameters were explored by least square regression analyses. The level of significance was set at $P < 0.05$ for all analyses. All values are given as mean ± S.E. Sta-
statistical analyses were performed with SYSTAT (Wilkinson, 1992). All microbial parameters are expressed per g dry mass of sediment.

3. Results

3.1. Adaptation of the method

FDA hydrolysis increased linearly with incubation time (Fig. 1A). As calculated by linear regressions, the 20°C treatment (slope 2.918 ± 0.181, \( r^2 = 0.98, P < 0.001 \)) yielded activities which were approximately twice as high as the 7°C treatment (slope 1.447 ± 0.067, \( r^2 = 0.99, P < 0.001 \)). Saturation was found between 150 and 620 \( \mu \)M for both temperature treatments (Fig. 1B). Within this saturation zone, the 20°C treatment yielded activities which were also approx. 2.2 times greater than the 7°C treatment.

The pH optimum for the FDA hydrolysis was between 7 and 7.5 (Fig. 1C). Buffer treated sediment averaged slightly lower hydrolysis (451 ± 18 \( \mu \)M FDA g\(^{-1}\) DM h\(^{-1}\)) than samples prepared with 0.2-\( \mu \)m filtered stream water (508 ± 21 \( \mu \)M FDA g\(^{-1}\) DM h\(^{-1}\)). Yet, the treatment had no significant effect (ANOVA, \( F = 4.383, P > 0.05 \)). pH remained stable in the buffer, whereas 0.2-\( \mu \)m filtered stream water pH manifested a slight decrease from 8.20 to 8.08 over a 30-min incubation period.

All four inactivation agents inhibited biofilm activity relative to the living control (Fig. 2). Acetone, however, inactivated FDA hydrolysis most efficiently and yielded an average percentage of 4.84% of the living control. Autoclaving inhibited the hydrolysis only partially; formaldehyde was the least efficient agent.

Sonication had a highly significant effect on fluorescein extraction from biofilm (ANOVA on arcsin transformed data, \( F = 34.114, P < 0.001 \)) (Table 1). Applying a 30-W sonication output for 45 s to the sample yielded almost a 430% increase.

![Fig. 1](image-url) FDA hydrolysis as a function of temperature (stream water ambient temperature and 20°C), incubation time, substrate concentration and pH. (A) Linear response of the hydrolysis rate to incubation time. (B) Saturation level for both temperature treatment was approximately 200 \( \mu \)M FDA. (C) pH optimum for FDA hydrolysis (set with phosphate buffer). Incubation time was 30 min for B and C. Given are means ± S.E. (n = 4). In panels A and D, plain dots represent the 7°C treatment, open dots the 20°C treatment.
Table 1

<table>
<thead>
<tr>
<th>Sonication output</th>
<th>Hydrolysis rate</th>
<th>% yield</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>88 ± 4 (82-96)</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>170 ± 8 (154-185)</td>
<td>193 ± 10</td>
</tr>
<tr>
<td>30</td>
<td>378 ± 13 (350-393)</td>
<td>429 ± 15</td>
</tr>
<tr>
<td>60</td>
<td>406 ± 37 (318-482)</td>
<td>461 ± 54</td>
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</table>

Sonication outputs expressed in W, hydrolysis rate in µM FDA g⁻¹·DM h⁻¹. Given are means ± S.E. and the range in parentheses, n = 4.

of fluorescein extraction relative to the control. Higher sonication output (60 W) heated the samples, yet did not increase extraction significantly (Tukey post-hoc test, P > 0.05). No sonication effect was measurable on the cleavage of FDA.

3.2. Evaluation

The relative precision ranged from 0.14 to 12.7% (c.v.) for all 50 triplicate hydrolysis measurements on hyporheic sediment; this corresponds to a standard deviation ranging from 1.41 to 68.63 µM FDA g⁻¹·DM h⁻¹. 50% of the cases had a coefficient of variation lower than 4%; the median value was 4.15%. Fluorescein absorbance could be detected to as low as a 10 nM concentration (Fig. 3A). The FDA assay was sensitive enough to produce a linear relationship between sediment sample size and hydrolysis rates across a range from 30 to 900 mg DM sediment (Fig. 3B).

Batch culture biofilm hydrolytic activity took the form of a sigmoidal curve which could best be described by the Richards growth model (Richards, 1959) (Fig. 4A). Esterase activity averaged 0.86 ± 0.04 µM FDA g⁻¹·DM h⁻¹ during the lag phase, 12.12 ± 0.57 during the log phase and increased up to 26.68 ± 2.03 µM FDA g⁻¹·DM h⁻¹ during the stationary phase. Esterase activity was correlated with carbohydrates (Fig. 4B) and, more weakly with bacterial density (Fig. 4C). Carbohydrate concentration varied from 58.5 ± 3.9 to 187.6 ± 20.7 µg g⁻¹·DM and bacterial density between 0.8 × 10⁸ and 2.5 × 10⁸ cells g⁻¹·DM.

The multi assay comparison detected hyporheic esterase activity declining over 30-, 60- and 90-cm depths from 1342 ± 53 over 533 ± 30 to 172 ± 4 µM FDA g⁻¹·DM h⁻¹, respectively. Average hyporheic biofilm hydrolysis was significantly (ANOVA, F = 9.413, P < 0.01) higher (596 ± 124 µM FDA g⁻¹·DM h⁻¹) than average riparian biofilm hydrolysis (202 ± 31 µM FDA g⁻¹·DM h⁻¹). Hydrolytic activity correlated with the ETS activity, [³H]TdT incorporation and glucosidase activities; good correlation was found with chlorophyll a (Table 2). Biofilm ETS activity showed similar yet less pronounced correlations. None of the variables measured, except [³H]TdT incorporation, correlated with bacterial density (Table 2).

4. Discussion

4.1. Adaptation

The applicability of the FDA assay to the assessment of natural stream biofilm hydrolysis has been studied. The Q₁₀, as calculated from hydrolysis kinetics at 7°C and 20°C (Fig. 1A), averaged 1.83. This is close to the Q₁₀ of 1.96 ± 0.13
FDA hydrolysis was saturated at a final substrate concentration of approximately 200 µM (Fig. 1B), which agrees with the substrate concentration used by (Marmonier et al., 1995) for the Rhône sediment. Pocemba (1995), however, found marine sediment hydrolytic activity saturated at 100 µM, and also Frølund et al. (1995) for activated sludge. I suspect that this difference may be due to the absence of photoautotrophs in deep-sea marine sediment and in activated sludge. Yet, it remains questionable whether hydrolysis saturation is a result of esterase saturation or FDA solubility (Breeuwer et al., 1995). FDA is, in fact, poorly soluble in water, which causes precipitation at higher concentration levels and thus lower concentrations of free FDA (Breeuwer et al., 1995).

In this study, the filtered stream water treatment yielded hydrolytic activities of almost 13% higher than the phosphate buffer treatment. However, the difference was not statistically significant. This supports the assumption of a change of the overall nutrient conditions. In filtered stream water, pH decreased only by 1.5% over a 30 min incubation period, which largely remains within the region where FDA hydrolysis is independent of pH (see (Fontvieille et al., 1992)). This may be attributed to the natural buffering capacity of the calcareous OSB water.

Achievement of accurate blank controls was not easy. Acetone inhibited the hydrolytic activity most efficiently with an almost 5% yield relative to the living control. Acetone was also found to be an efficient inactivation agent for soil microorganisms (Schnürer and Rosswall, 1982). By contrast, autoclaved sediment yielded absorbance signals that were almost 27% of the signal for live samples. This may be attributable to heat induced changes of the sediment chemical and physical properties (Meyer-Reil, 1991). Tuominen et al. (1994) found that autoclaved sediments released considerable amounts of DOC and soluble reactive phosphorus, thus being a good environment
Fig. 4. (A) Hydrolytic activity of batch cultured biofilm develops over a sigmoidal curve. The curve was fitted by the Richards growth model \( y = 25.812 / \left[ 1 + 137.210 \times e^{-0.024x} \right] 1.463, r^2 = 0.98, P < 0.001 \). Relationships of sediment carbohydrates (B) \( y = 1.358 \times 10^{-7} x^{3.662}, r^2 = 0.95, P < 0.001 \) and (C) bacterial density \( y = 0.676 x^{4.009}, r^2 = 0.68, P < 0.01 \) and biofilm hydrolytic activity. Given are means ± S.E. \( (n = 4) \).

for viable spores. However, considering the relative short incubation time of the FDA assay, I suspect non-biological cleavage of FDA molecules by altered sediment structure more likely. Fontvieille et al. (1992) and Marmonier et al. (1995) inactivated esterase activity with HgCl₂. I found the HgCl₂ treatment highly inefficient for the OSB sediment, which is also true for marine sediments (Meyer-Reil, 1990). It seems, that mercuric ions inhibit only arylesterases, do not influence carboxylesterases, yet can even activate acetylene-sterase (Heymann 1980 as in (Moffat and Snell, 1995)). Least effective was formaldehyde, probably because of its influence on sediment pH (see Tuominen et al. (1994)). An alternative to killed controls are time-course experiments (Meyer-Reil, 1991), which, however, can be labour-intensive. Acetone inhibited controls allow the analyst to process large numbers of samples in a relatively short time.

The extraction of fluorescein with 50% (v/v) acetone and subsequent sonication was highly efficient. Acetone alters the structure of the biofilm matrix carbohydrates and solubilizes cell membranes thus facilitating fluorescein efflux (Schnürer and Rosswall, 1982). Disruption of the biofilm matrix by sonication liberates microbial cells and can damage their membranes. This suggests that biofilm esterases are, at least partially, extracellular, either attached to the matrix sur-
Table 2
Pearson's correlation coefficients between biofilm and bacterial parameters in the hyporheic and riparian sediments from the Oberer Seebach

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± S.E. (Range)</th>
<th>Hydrolytic activity</th>
<th>ETS</th>
<th>[^{3}H]TdR incorporation</th>
<th>[^{3}H]TdR incorporation</th>
<th>[^{3}H]TdR incorporation</th>
<th>Bacterial Chlorophyll a</th>
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<tr>
<td>Hydrolytic activity</td>
<td>399 ± 80 (107–1342)</td>
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<tr>
<td>((\mu)M FDA g(^{-1}) DM h(^{-1}))</td>
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<tr>
<td>ETS ((\mu)M INT g(^{-1}) DM h(^{-1}))</td>
<td>41.3 ± 3.6 (21.7–74.0)</td>
<td>0.67**</td>
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<td>[^{3}H]Tdr incorporation</td>
<td>33.5 ± 3.6 (12.7–54.3)</td>
<td>0.69**</td>
<td>0.50*</td>
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<td>(DPM × 10(^{3}) g(^{-1}) DM h(^{-1}))</td>
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<tr>
<td>(\alpha)-Glucosidase</td>
<td>24.8 ± 4.6 (5.2–67.4)</td>
<td>0.73**</td>
<td>0.55*</td>
<td>0.68**</td>
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<tr>
<td>((\mu)M MUF g(^{-1}) DM h(^{-1}))</td>
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<tr>
<td>(\beta)-Glucosidase</td>
<td>73.2 ± 14.2 (8.3–179.7)</td>
<td>0.66**</td>
<td>0.49**</td>
<td>0.56*</td>
<td>0.47**</td>
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<tr>
<td>Bacterial density</td>
<td>3.7 ± 0.3 (1.3–6.0)</td>
<td>0.20**</td>
<td>0.03**</td>
<td>0.52*</td>
<td>0.10**</td>
<td>0.42**</td>
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<tr>
<td>(cells × 10(^9) g(^{-1}) DM h(^{-1}))</td>
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<tr>
<td>Chlorophyll a</td>
<td>15.1 ± 4.6 (0.79–61.4)</td>
<td>0.93**</td>
<td>0.68**</td>
<td>0.59*</td>
<td>0.69**</td>
<td>0.79**</td>
<td>0.14**</td>
</tr>
<tr>
<td>((\mu)g g(^{-1}) DM)</td>
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Values of \(r\) significant at: *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\) \((n = 16)\). Four analytical replicates plus duplicate blanks were used for all activity measurements.

face and/or embedded within the matrix (see also (Meyer-Reil, 1991)). This is also supported by (Frølund et al., 1995) who, using cation exchange for the extraction of extracellular polymeric substances (EPS) from activated sludge, found a large proportion of esterases to be loosely attached to the matrix.

4.2. Evaluation

The adapted FDA assay is reasonably precise with a median coefficient of variation of 4.15%. Higher coefficients of variation may be caused by sediment grain size heterogeneity. The assay was also sensitive enough to respond linearly to fluorescein formation across a range of low sediment sample sizes. Furthermore, incubation times of only 15–60 min were sufficient for obtaining easily measurable fluorescence signals. Hydrolysis rates were linear within this period (Fig. 1A), which suggests that neither inhibition nor induction of esterases took place during incubation. Keeping the incubation time low can be important in order to minimize changes in the microbial community. Further, a relatively high number of replicates can be run in order to take account of the heterogeneity of sediment biofilm activity.

Esterase activity of the batch cultured biofilm reached maximum values during the stationary phase. Sediment carbohydrates correlated highly with FDA hydrolysis and increased by a factor of 3.2. Whereas, bacterial density correlated less with esterase activity and increased only 2.5 times. This suggests (a) that esterases are predominantly associated with the biofilm extracellular polymeric substances (EPS) rather than with bacterial cells and (b) that organic molecules with ester bounds are accumulated in the biofilm matrix. The biofilm EPS can, in fact, store bacterial extracellular enzymes and organic molecules (Lock, 1993).

The multiple assay comparison convincingly demonstrated that the FDA assay is sensitive enough to detect spatial patterns of biofilm hydrolytic activity. Further, they clearly correlate with those found from other measurements targeting the algal and bacterial biofilm compartments.

In conclusion, the FDA assay is a sensitive, rapid and inexpensive method for surveying total esterase activity and for identifying active biological zones in stream bed sediment. A most stringent point of concern remains the localization of
FDA hydrolysing esterases in biofilms. Concomitantly with this goes a better understanding of the biochemical pathways of the enzymatic systems which hydrolyze FDA. Addressing these points will be crucial to fully understand the function of hydrolytic enzyme systems in sediment biogeochemistry.

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References


