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Modified enzyme activity assay to determine biofilm biomass

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Abstract

An assay of potential exoproteolytic enzyme activity was modified to quantitatively measure the biomass of attached biofilm. The assay utilized the nonfluorescent compound L-leucine-β-naphthylamide (LLβN) that becomes fluorescent when bacterial exoenzymes break the peptide bond, releasing the fluorochrome β-naphthylamine. Fluorescence development was measured by pumping the liquid phase of a biofilm sample through a fluorescence detector and recording the detector output using a personal computer. A significant linear relationship was shown to exist between the rate of fluorescence development and the biofilm's biomass as carbon, determined using total direct cell counts, measured cell volumes and an existing relationship between cell volume and cell carbon. The technique was used to measure biofilm biomass for experiments where iron oxides were the substratum. Biofilm biomass measurements made using heterotrophic plate counts (HPCs) on R2A medium were shown to correlate well to biomass measurements made using the modified enzyme assay. The technique was shown to be sufficiently sensitive to measure biomass on samples containing little biofilm biomass, such as those exposed to free chlorine. While granular and porous media were used for the experiments presented, small biofilm coupons could easily be used to measure biofilm biomass, expanding the number of possible applications for the enzyme assay technique.

Keywords: Biofilm; Biomass; Exoproteolytic activity; Porous media

1. Introduction

The measurement of biomass in a biofilm is required for numerous activities related to the study and prediction of biofilm behavior. In biological filtration studies, biofilm biomass measurements made at various media depths can be used in the development of models for the filter's intended purpose. Similarly, biofilm biomass in a reactor or pipe loop is often used to make comparisons between different disinfectants or antimicrobial agents for control of biofilm.

Biofilm activity and biomass measurements are also necessary to determine kinetic parameters such as specific growth and nutrient uptake rates. These kinetic parameters are used in predictive mathematical models for a variety of applications including drinking water (Dukan et al., 1996; Servais et al., 1995).

A review of biofilm biomass measurement techniques (Lu and Huck, 1993) indicated the wide range of methods available and the variable results that can be obtained depending on the method of measurement. It would appear that most biomass measurement techniques depend upon the type of experiment being performed and the equipment available to the researchers.

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Measurement of biofilm biomass requires that either the biofilm cells be removed from the substratum they are attached to or the measurement be made in-situ, without removal of the biofilm. Arguments can be made and supported for either method and are not presented herein. Heterotrophic plate counts (HPCs) on R2A agar are commonly used to determine viable cells within the biofilm removed from the substratum (Camper et al., 1996). In-situ biomass measurement becomes particularly attractive when the substratum is fragile or easily disaggregated. For example, iron oxides resulting from corrosion of ferrous metals interfere with microscopic techniques used to estimate biomass, particularly those techniques relying upon biofilm removal from the substratum and some form of cellular stain such as acridine orange (Hobbie et al., 1977), 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980), or live–dead stains (Lisle et al., 1998). Techniques to remove biofilm from the iron oxide surface also remove iron oxide particles. The small iron oxide particles greatly hinder the ability to count cells microscopically since their number typically exceeds that of the cells.

This paper describes a method used to assess biofilm biomass in-situ where the substratum was crushed ferrous metal corrosion products or iron oxides deposited on glass beads. These substrata were used to study the interactions among factors that influence biofilm, bacterial regrowth and corrosion in drinking water distribution systems, the details of which can be found elsewhere (Butterfield et al., submitted for publication). The method we are reporting consisted of modifying the potential exoproteolytic activity (PEPA) method presented by Billén (1991) and Laurent and Servais (1995) to measure biomass in an in-situ biofilm. Exoproteolytic activity was related to microbial biomass by several researchers (Somville and Billén, 1983; Laurent and Servais, 1995). Adopted from a clinical procedure presented by Roth (1965) for fluorometric assay of leucine aminopeptidase, the nonfluorescent compound L-leucine-β-naphthylamide (LLβN) is added in millimolar concentrations to the liquid phase surrounding bacteria. When the peptide bond within LLβN is broken by the action of bacterial exoenzymes, the fluorochrome β-naphthylamine is cleaved from the L-leucine molecule. Measurement of the fluorescence in the liquid phase indicates the amount of β-naphthylamine hydrolyzed from the original LLβN. The rate at which the LLβN is hydrolyzed is used to measure the exoproteolytic activity of the bacteria within the sample.

In earlier work reported by Somville and Billén (1983) and Laurent and Servais (1995), there was shown to be a linear relationship between the rate of exoenzymatic activity and the measured bacterial biomass as carbon. Bacterial cells in suspension were used to measure the PEPA and develop its relationship to bacterial biomass. The method reported herein differs in that the relationship between exoproteolytic activity and bacterial biomass was developed using attached, in-situ biofilm. In addition, this new technique utilized a continuous flow-through fluorescence detector and a personal computer to continuously record the development of fluorescence in the liquid phase of the biofilm samples. In this paper, we present a description of the method, the results for calibration to biofilm biomass and measurement of biomass in samples containing iron oxide substrata.

2. Methods and materials

2.1. Biofilm development

It was desired to correlate PEPA to the amount of biomass present on a given substrate for a project that involved the study of biofilm on iron oxide surfaces. One set of experiments (Biotic 1) utilized ~0.5-mm-diameter spherical glass beads (Biospec Products, Bartlesville, OK) coated with iron oxides (IOCBs—iron oxide-coated beads). The other experiment (Biotic 2) consisted of crushed and sized corrosion products (CPs) from an unlined section of cast iron pipe removed from a drinking water distribution system. The purpose of the experiments was to evaluate the effects of different corrosion control treatment methods on the growth and development of biofilms on iron oxide surfaces using humic substances as the source of organic carbon.

Removal of biofilm from iron oxide coatings or corrosion products introduces particles that interfere with any microscopic techniques that might be employed to determine biomass. Therefore, calibration of the enzyme assay was performed on samples that con-

tained plain, uncoated glass beads that had received the same feed solution as used in the two biotic experiments, thus allowing microscopic techniques to be used to estimate biofilm biomass on the uncoated glass beads. Each of the two biotic experiments included two columns that contained uncoated glass beads, and designated as the negative Fe columns (column groups 13 and 22, see Table 1). In addition, two uncoated glass bead columns were operated separate to the two biotic experiments to provide additional data points for calibration. The experimental setup is described in detail elsewhere (Butterfield et al., submitted for publication) and will only be briefly described in this paper.

Small columns packed with media and operated in an upflow mode were utilized for the experiments. The types of experimental treatments examined are shown in Table 1. Each column group consisted of two replicate columns. Columns containing uncoated glass beads represented the negative iron control and were used to calibrate biofilm biomass to PEPA rate. The reactor columns consisted of 1-cm-diameter PTFE tube columns with an approximate volume of 8 cm^3. End fittings and retaining screens were stainless steel. All feed solutions, tubing, fittings and columns were initially sterile. To prevent disruption of the iron oxides, the IOCBs and CPs were not autoclaved and therefore not sterile. Feed solutions consisted of humic substances derived from Eliot Silt Loam Soil (International Humic Substances Society BS102M) diluted in reagent-grade water (Nanopure, Barnstead/Thermolyne, Dubuque, IA). The feed concentration of the humic substances was ~2.85 mg/l as non-purgeable organic carbon measured using a Shimadzu TOC-5000A (Shimadzu Scientific Instruments, Columbia, MD). Feed solutions were amended with sodium bicarbonate for alkalinity and sufficient nitrate/phosphate to create carbon limiting growth conditions based on stoichiometric calculations. The free chlorine residual in the effluent of the chlorinated columns was maintained at 0.15–0.2 mg/l. Phosphoric acid was added at a dose of 3.0 mg/l as phosphate to the columns that received this form of corrosion control. All experiments were performed at room temperature (20–22 °C).

Table 1
Treatment types and column group numbers for the biotic experiments

Experiment/treatment type	IOCBs Biotic 1 column group number	CPs Biotic 2 column group number
Time zero, +Fe	12	21
Negative Fe, +humics, pH 7.3[a]	13	22
+Fe, negative humics, pH 7.3	14	23
+Fe, +humics, pH 7.3	15	24
+Fe, +humics, pH 8	16	–
+Fe, +humics, pH 9	17	25
+Fe, +humics, Cl_2	18	26
+Fe, +humics, Cl_2, PO_4	19	27
+Fe, +humics, PO_4	20	28
Negative Fe, +humics, pH 7.3[a]	–	30

[a] Indicates columns used for biofilm biomass calibration.

Prior to inoculation, the media in the columns was "loaded" with a saturating humic substances solution with the goal of adsorbing as much humic substances as possible to the media surfaces, providing similar initial conditions for all columns with respect to adsorbed humic substances. Saturation of the media surfaces with humic substances was followed by column inoculation with a mixed population of heterotrophic bacteria from the effluent of a biologically active carbon (BAC) filter column treating dechlorinated drinking water. The BAC effluent was pumped through the packed columns for 24 h without recycle. The packed column reactors were operated for a minimum of 56 days before sampling for biofilm biomass.

2.2. Biofilm sampling

Glass beads were aseptically removed from approximately the top and bottom 1.5 cm of the columns for the enzyme assay, heterotrophic plate counts (HPCs), total direct cell counts (TDCs) and sizing of bacterial cells. Samples were split evenly in the vertical direction, or along the column's axis, taking an equal amount of beads for the enzyme assay and an equal amount for HPCs and TDCs. Columns were removed from the feed solution only long enough to do the sampling of the top or bottom. After a sample was removed, the column was reassembled and placed back on-line with the feed solution continuing to go through the column until the opposite end of the column was sampled. Approximately 1 g of beads was placed into a sterile, 20-ml EPA glass vial (Qorpak, Pittsburgh, PA) containing 3 ml of filter-

sterilized reverse osmosis water. All tubing and vials used for the assay were autoclaved prior to use. Glass beads removed for HPCs and TDCs were placed into 9 ml of sterile reverse osmosis water for subsequent analysis.

2.3. Biofilm quantification

The number of biofilm cells on the uncoated glass beads was determined using HPCs and TDCs. After the glass beads were placed into 9 ml of sterile water, the sample was placed in ice and sonicated for 1 min using a probe sonicator (Tekmar Model TM50, 50 W, amplitude 100), followed by vortex mixing for 1 min. Split-samples were serially diluted and three spread plates per dilution using R2A medium (Difco, Detroit MI) were used for determining HPCs. The undiluted sample was fixed using filter-sterilized formaldehyde (~0.07% final concentration) and subsequently sampled for TDCs by filtering the appropriate volume through 0.2-μm black polycarbonate filters (Osmonics, Livermore, CA), stained for a minimum of 30-min using 4′,6-diamidino-2-phenylindole (DAPI, Sigma) stain (Porter and Feig, 1980) and the number of cells determined by epifluorescent microscopy. Twenty fields or 200 cells minimum were counted per sample.

The filtered and stained samples used to determine TDCs were utilized to determine cell sizes by collecting sufficient digital images of the stained cells to provide a minimum of 200 cells per sample. Images were recorded using an Olympus BH-2 microscope and a charge-coupled device (CCD) camera (Optronics) and Image-Pro Plus™ software. Cell sizes were determined using the free software ImageTool, version 2.00, developed at the University of Texas Health Science Center at San Antonio, TX. Calibration was performed using images of a graduated micrometer slide. Cell length (L) and width (W) were used to calculate cell volume using the formula $(\pi/4)W^2(L-W/3)$, where W is the cell width (minor axis length) and L is the cell length (major axis length) (Bratbak, 1985). The mean cell volume was converted to cell carbon using a cell carbon to cell volume correlation developed for biofilm in similar environmental conditions (Butterfield, 1998). The relationship between cell volume (X) and cell carbon per cell volume (femtograms of carbon per μm^3) was developed using a Generalized Pareto function (Aroner, 1996; Hosking and Wallis, 1987), described as follows:

$$\alpha^{-1}\lfloor 1-\kappa(X-\beta)/\alpha\rfloor^{1/\kappa-1}$$

$$\alpha = 0.0078;\ \beta = -0.0122;\ \kappa = 0.4992;$$

$$r^2 = 0.91;\ \text{Fit Std Error} = 4.18;$$

where X is the cell volume (μm^3), and α, β and κ are the function parameters.

For this work, the mean cell volume was 0.231 μm^3 (S.D. = 0.378, $n = 1561$) and the resulting mean carbon per cell was 37.53 fg (S.D. = 18.82, $n = 1561$). TDCs for each biofilm sample were multiplied by the carbon per cell value to obtain the estimated biofilm biomass per unit weight of column media or per gram of glass beads for calibration. Biofilm biomass using HPCs was determined by multiplying the colony forming units per unit weight of media by the carbon per cell value.

2.4. Enzyme assay

The apparatus used to perform the enzyme activity assay consisted of a continuous flow fluorescence detector connected to a personal computer for data collection and analysis. Small diameter PTFE tubing extended through holes in the PTFE-lined, silicon septum in the lid of the sample vials. A positive displacement tubing pump (Gilson Model 312 with 10-roller-head, Middleton, WI) was used to pump liquid at a rate of 3.5 ml/min from the sample vial through the fluorescence detector and back to the sample vial. The sample vial was placed on a rotating shaker table to provide gentle mixing of the sample's liquid phase. The shaking frequency was adjusted such that the sample media was not agitated. The fluorescence detector (Jasco Model FP-920) was set at an excitation wavelength of 340 nm and an emission wavelength of 410 nm. The fluorescence response was logged continuously on a personal computer using Dionex PeakNet software, release 4.30 (Dionex, Sunnyvale, CA). The result was a continuous reading of the sample's fluorescence response.

Calibration of the fluorescence response to the concentration of β-naphthylamine was performed by preparing standards with known concentrations of β-

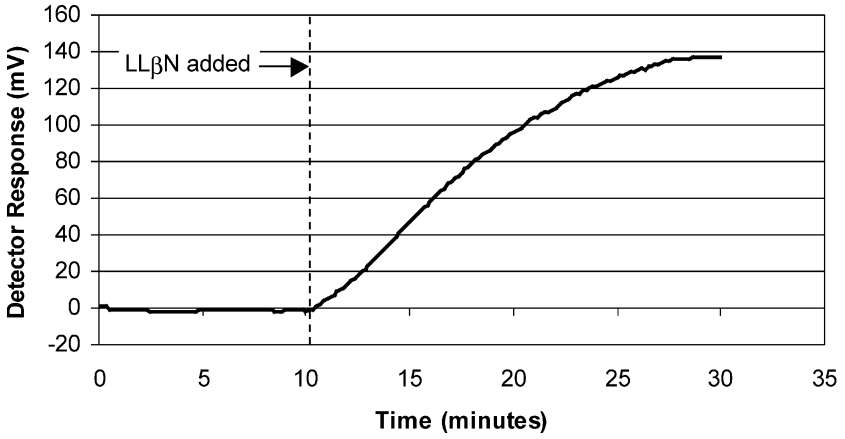

Fig. 1. Response curve from fluorescence detector for biofilm sample from column 13-2, bottom.

naphthylamine (Sigma) and determining a fluorescence response for each known concentration and at different settings for the instrument gain. Calibration utilized a once-through mode for pumping the standard through the fluorescence detector. The resulting relationship between detector response units and β-naphthylamine concentration was linear for each gain setting.

The enzyme assay began by initially pumping only the liquid phase of the sample through the fluorescence detector and establishing a baseline. Once the baseline was established, the detector output was set to zero and LLβN (prepared fresh each day using filter-sterilized reverse osmosis water) was added to the sample at a concentration of 1 mM, a concentration that has been found to be saturating (Laurent and Servais, 1995). Once the LLβN was added to the sample, the resulting fluorescence was measured over a period of 20–30 min. Trial experiments demonstrated that this length of time was required for the rate to deviate from an initial linear rate to a curve approaching a saturation point. Data collected by the computer were then used to determine the rate of enzyme activity. The fluorescence response in millivolts (mV) was converted to nM β-naphthylamine using the calibration curve. Table Curve 2D software, version 4 (SPSS) was used to perform the linear regressions on the data. The initial 10 min of data was used to establish the PEPA rate (nmol β-naphthylamine/l/min). An example of the output from the fluorescence detector as logged by the computer is shown in Fig. 1. Correlation was performed using the software Minitab™, version 13.3.

3. Results

3.1. PEPA–biomass calibration

Results of the biomass calibration to PEPA using biofilm derived from uncoated glass beads and TDCs to estimate biomass are shown in Fig. 2. There was a significant linear relationship between PEPA and biofilm biomass ($p = 0.000$). The slope of the regression line was 0.02876 nmol of β-naphthylamine

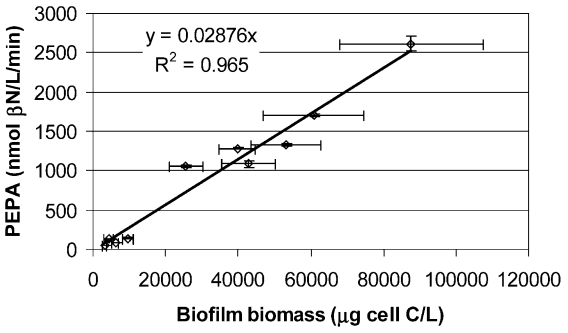

Fig. 2. Calibration relationship to convert PEPA to bacterial biomass using biofilm from uncoated glass bead samples. All error bars represent 95% confidence limits. Horizontal error bars for biofilm biomass are based on the 95% confidence limits for the TDC data. βN = β-naphthylamine.

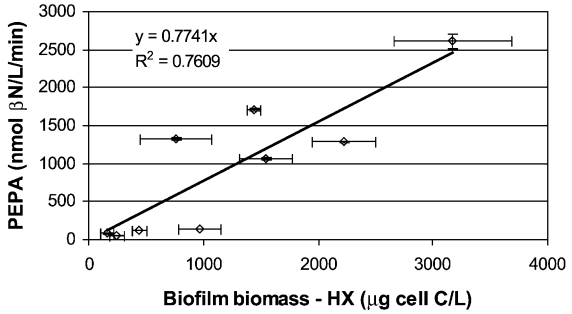

Fig. 3. Relationship between PEPA and bacterial biomass estimated using HPC data for biofilm from uncoated glass bead samples. Vertical error bars represent 95% confidence limits. Horizontal error bars for biofilm biomass are based on the standard deviation for the HPC data. βN = β-naphthylamine.

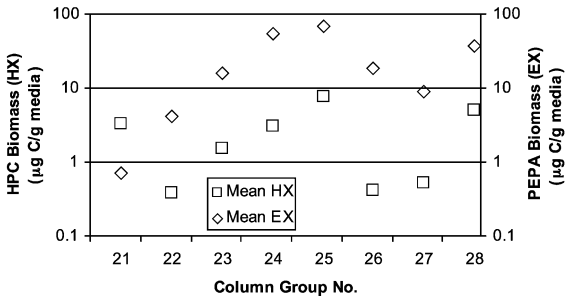

Fig. 4. Biofilm biomass at the top of the columns (mean for two replicates) measured using HPCs (HX) and PEPA (EX) for the CPs Biotic 2 experiment.

produced per minute per μg of biofilm biomass as carbon.

A similar calibration was developed using biofilm HPC data in lieu of TDC data to estimate biomass (Fig. 3). Since there was a significant difference between TDC values (total cells/g) and HPC values (cfu/g), there was also a significant difference in the slope of the correlation line. Using the HPC data to estimate biomass (HX), the calibration slope was 0.774 nmol βN/μg of biofilm biomass as carbon ($R^2 = 0.761$ and $p = 0.000$). The correlation between biofilm biomass and PEPA was better when TDC data was utilized, indicating that PEPA is a better representation of total biomass, at least to the extent of the methods used to determine TDCs.

Table 2
Correlation between biofilm biomass measured using HPCs (HX) and PEPA (EX)

Experiment	HX–EX	Log HX–log EX
Biotic 1, top	0.485 ($p = 0.041$)	0.896 ($p = 0.000$)
Biotic 1, bottom	0.583 ($p = 0.011$)	0.927 ($p = 0.000$)
Biotic 1, all top and bottom[a]	0.686 ($p = 0.000$)	0.902 ($p = 0.000$)
Biotic 1, geometric mean[b]	0.572 ($p = 0.021$)	0.963 ($p = 0.000$)
Biotic 2, top	0.893 ($p = 0.000$)	0.881 ($p = 0.000$)
Biotic 2, bottom	0.860 ($p = 0.000$)	0.944 ($p = 0.000$)
Biotic 2, all top and bottom[a]	0.848 ($p = 0.000$)	0.916 ($p = 0.000$)
Biotic 2, geometric mean[b]	0.946 ($p = 0.000$)	0.944 ($p = 0.000$)

[a] All top and bottom measurements combined.
[b] Geometric mean of the top and bottom measurements, used to estimate total biomass in the column.

3.2. Results from experiments

The biofilm biomass that developed on IOCBs and CPs were measured using HPCs and the PEPA method. Ideally, there should be a correlation between biofilm biomass measured using the two methods. Table 2 presents the results of correlation between the two methods for data from both experiments. All correlations were positive and significant ($p < 0.05$). Correlations of $\log_{10}$ transformed data showed a stronger correlation than did the nontransformed data for the IOCBs Biotic 1 experiment.

An interesting example of the biomass measurements (data for top of columns from the CPs Biotic 2 experiment) is presented in Fig. 4. Note that at time zero in column group (CG) 21, there was more HX biomass than EX biomass. These biofilm cells had not yet been acclimated to the nutrient feed (humic substances) and showed little exoproteolytic enzyme activity, even though there were biofilm cells that were culturable on R2A. This result indicates the PEPA method may be sensitive to factors related to the biofilm's environment, requiring calibration of the method for different environmental conditions.

4. Discussion

Laurent and Servais (1995) used the PEPA technique to estimate biofilm biomass, but the method presented in this paper was unique and has certain

advantages and multiple applications. A unique aspect of this technique was that a continuous measurement of PEPA was made with minimal disturbance of the biofilm sample, allowing the researcher to monitor the enzyme activity of the biofilm during the assay. The technique used in this work was found to be applicable to biofilm on granular media, but could just as well have been used for biofilm on a flat surface. Sample coupons with an established biofilm could easily be placed in the sample vial. Alternative fluorescent substrates (Hoppe, 1991) could be utilized depending upon the needs of the researcher.

As shown in Fig. 4, the PEPA method was capable of measuring biofilm biomass over a wide range of values. The biofilm biomass on the media in columns receiving chlorine was much less (2–3.5 logs) (Butterfield et al., submitted for publication) compared to the non-chlorinated columns, demonstrating the potential sensitivity of the test. For samples with little biofilm biomass, the sensitivity (gain) of the fluorescence detector was increased and a βN calibration curve developed at the increased sensitivity setting.

From results of early tests using this technique, it was discovered that the biofilm sample could not be removed from the nutrient feed stream for any significant period of time. When nutrient feed to the reactor columns was stopped for a period of approximately 30 min, the initial PEPA response was two to four times greater than for a similar sample that had only been off-line for a period of approximately 5 min, the minimum time required to sample the column media and start the enzyme assay procedure. While we did not perform experiments to verify why this occurred, we hypothesize that either the biofilm cells responded by increasing exoenzyme production or the cells continued their normal production of exoenzymes, and there was simply an increase in concentration because there was no continuous replacement of the liquid. An increase in exoenzyme production could have been for the purpose of hydrolysis of any attached humic substances to provide needed nutrients no longer supplied by the feed solution. Therefore, any application of this technique must minimize the time reactors are taken off-line to obtain the biofilm samples, and the enzyme assay must be started as soon as possible after biofilm sampling.

There were several modifications of the PEPA method as developed by Billén (1991) that increased the usefulness of the technique. One basic difference was in calibrating PEPA to biomass. The calibration constant for this work was 0.0290 nmol βN/l/min. Previous calibration work used either planktonic cells found in natural aquatic environments (Billén, 1991) or biofilm cells detached from the substratum and placed in suspension (Laurent and Servais, 1995), resulting in calibration constants from 0.15 to 0.20. The technique reported here used biofilm attached to glass beads to calibrate PEPA to biomass and resulted in a statistically significant linear calibration relationship.

Another difference in calibration techniques was the time used to determine the PEPA rate. Previous work measured the PEPA rate over a period of 30–60 min (Billén, 1991; Laurent and Servais, 1995). After review of the results from approximately 36 biofilm samples, the first 10 min of the response curve was used since the first 10 min were the most linear in response, after which the response began to curve towards a saturation point. Work by Button (1991) indicated the initial slope of a response curve represented the cells' ability to utilize substrates in an oligotrophic environment. Our work was performed at organic carbon concentrations of ~3 mg C/l, a concentration that approaches an oligotrophy. Therefore, the initial slope should be a better measurement of biomass in the oligotrophic environment used for the experiments.

A potential source of error in the method presented in this paper would be the presence of detached biofilm organisms in the fluid passing through the fluorescence detector. These cells could lead to a decrease in fluorescence due to blocking of the source light. However, if this were the case, the resulting slope of the line would be less than the slope of the calibration regression line shown in Fig. 2, increasing rather than decreasing the difference in calibration constants between our work and that of previous researchers. The presence of cells in the recirculation liquid is not seen as a problem.

Using this modified technique, it would be possible to perform the enzyme assay using an entire reactor. This would minimize any disruption of the biofilm and would provide conditions similar to those used during the experiment. Use of an entire column reactor could minimize mass transfer limitations between the biofilm and liquid phase, and provide a realistic view of the amount of active biofilm biomass in a reactor system.

5. Conclusions

This work presents a modification of an existing technique that allows measurement of biofilm biomass without removing the biofilm from its substratum. While the technique does require a fluorescence detector linked to data logging software, these particular pieces of equipment are commonly found in laboratories containing HPLC equipment. The technique does not require the removal of biofilm from the substratum and can be calibrated to biofilm developed under conditions similar to those used in the experiment where biofilm biomass measurements are desired. A continuous record of the biofilm's response to the fluorescent substrate is automatically logged and provides not only the data required to calculate PEPA rate, but also provides information regarding the early response of a biofilm to a particular fluorescent substrate. The technique is sensitive to small amounts of biofilm as can be found in samples where chlorine or other disinfectants are present. In the work reported herein, the PEPA technique correlated well to biofilm biomass measurements using HPC data. However, the PEPA technique is a better representation of total biomass than is the HPC method and is sensitive to the activity of the biofilm. Calibration of biofilm biomass to PEPA should be performed using conditions as similar as possible to those in the intended particular application.

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