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On line, non-destructive biomass determination of bacterial biofilms by fluorometry

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Summary

The lack of on-line methodology for the determination of microbial biomass and activity of attached bacteria has severely limited the study of biofilm physiology. This study showed that the fluorescent emission of aromatic amino acids in microbial biofilms can be used to determine the biomass formed on 316 stainless steel coupons. Cells resuspended from the substratum were enumerated by viable and acridine orange counts showing correlation coefficients of 0.77 and 0.98, respectively, when compared to the tryptophane fluorescence. Substrata treated with a fluorescent epoxy coating (F-150) showed no fluorescence that could be attributed to the microorganisms. Bioluminescent emission of an actively growing bioluminescent bacterium, *Vibrio harveyi*, was correlated with acridine orange counts ($r^2 = 0.95$) and fluorescence ($r^2 = 0.93$). The results of these studies suggest that fluorescence measurements can be used to monitor microbial biomass associated with various substrata. Coupled with bioluminescence measurements, this method provides information on both biomass constituents and metabolic activity, and therefore possibly an indicator of sub-lethal toxicity.

Key words: Biofilm; Bioluminescence; Flow cell; Fluorometry; NAD(P)H; On-line monitoring; Tryptophane; *Vibrio harveyi*

Introduction

Biofilms are ubiquitous throughout nature and can be found associated with many surfaces exposed to aqueous environments. Biofilms consist of microbial cells immobilized at a substratum, frequently embedded in an organic polymer matrix consisting of cellular biomass, extracellular polymeric substances, and entrained detrital matter.

Biofilm formation can be involved in beneficial processes such as the production of vinegar and the degradation of raw sewage. Alternatively, biofilms can be deleterious. Their formation on the hulls of ships and other submerged structures results in increased fuel costs, clean-up costs, and increased stress due to viscous drag. Biofilms

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have also been shown to be implicated in microbially influenced corrosion (MIC). Angell and Chamberlain [1] have shown that biofilms consisting of a consortium of *Pseudomonas* spp. are associated with the surface of copper water pipes which are considered to be a toxic surface to bacteria. It was shown that the bacterial exopolysaccharides were responsible for the copper tolerance of the bacterial consortium. Most MIC has been attributed to the action of the anaerobic sulphate reducing bacteria (SRB) [2], and recently, a community of aerobic pseudomonads have been shown to be involved in Type 1½ pitting of copper [3]. The impact of MIC world wide has been calculated to amount to several billions of U.S. dollars each year [4].

Most systems currently employed to enumerate biofilm biomass on optically opaque surfaces necessitate the destruction of the biofilm. Once the bacteria are extracted, a number of different methods exist for quantifying the bacteria including viable plate counts, acridine orange direct counts (AODC) and total lipid analysis [5]. Direct, on-line microscopic counts have been applied to biofilms, although these are best suited to translucent surfaces which allow transmitted light observations to be made. A system has recently been described using Nomarski DIC microscopy to study biofilms on optically opaque surfaces [6]. Although this method allows the visualization of biofilms, it does not allow for any quantitative analysis. Various other methods have been described for the on-line determination of biomass, these include; Fourier transformed infrared (FT-IR) spectroscopy [7] and the quartz-crystal microbalance [8]. FT-IR has proved useful for the determination of biomass, but is restrictive in that the penetration of the infrared evanescent wave is only possible in a biofilm up to a few cells thick. The quartz crystal microbalance has been shown to be capable of detecting biomass, but lacks the ability to characterize biofilm constituents. Systems have recently been developed utilizing the natural luminescence of some marine bacteria [9]. However, these studies are restricted to marine systems with just a few strains of bacteria. With the cloning of the *lux* genes, which encode for luminescence, onto convenient vectors [10,11] there is the possibility of extending these studies to other bacteria [12].

Fluorescence occurs when an excited electron returns to a lower-energy orbit emitting a photon of light. Substances which display significant fluorescence generally possess delocalized electrons formally present in conjugated double bonds. Common fluorophores include the aromatic amino-acid tryptophane. Tryptophane absorbs ultraviolet light at about 280 nm, with the excitation of some electrons to a higher-energy orbit. When an electron subsequently returns to a lower-energy orbit a photon of light is emitted with a wavelength of about 350 nm. The fluorometric emission of light at a specific wavelength following excitation is measured by a sensitive photo-multiplier tube (PMT).

The application of fluorescence measurements for the characterization of cultures was first applied by Duysens and Amesz [13], who found that the fluorescence spectra for suspensions of aerobic baker's yeast were similar to that for NAD(P)H and that addition of ethanol to the culture significantly enhanced the spectra. Zabriskie and Humphrey [14] demonstrated that a linear relationship existed between culture fluorescence and biomass for cultures of *Saccharomyces cerevisiae*, *Streptomyces* sp. and *Thermoactinomyces* sp. A multiple excitation fluorometric system has been

described which uses four key bacterial fluorophiles [NAD(P)H, pyridoxine, tryptophane and riboflavin] to monitor fermentation processes [15]. The results indicated that the fluorescence intensity and behavior of various cellular fluorophors vary significantly between different fermentation systems and that the best way to monitor cell concentrations in a bioreactor by fluorometry may be to monitor several fluorophors. The use of fluorometry to monitor fermentations has been the subject of significant research efforts over the last few decades with in excess of one hundred papers being published on the subject.

This paper evaluates the use of a fluorometer with a fiberoptic accessory to measure the fluorescence emission of tryptophane and NAD(P)H of an actively growing bacterial biofilm. The technique has been utilized for on-line measurements of biofilm biomass and metabolic activity in a laminar-flow environment.

Materials and Methods

Bacteria and medium

In these studies a naturally luminescent culture of *Vibrio harveyi* (ATCC 14126) was utilized to allow on-line light readings to be taken as a biomass indicator. An artificial seawater medium (ASW) [16] with the addition of 0.01% glycerol, 0.02% casamino acids, and 10 mM Tris buffer (Sigma Chemical, St. Louis, MO) at pH 7.5 was used throughout the experiment.

Flow cell design and operation

The laminar flow cells previously described by Mittelman et al. [9] were used which in summary measured 150 mm W × 285 mm L × 3.3 mm D, with a working volume for the flow chamber of 75 ml, into which five flush mounted coupons could be placed. The top of the flow cells had a number of removable screws (three per coupon) which contained a flush mounted quartz glass window. The coupons measured 35 mm W × 70 mm L × 3 mm H and were fabricated from 316 stainless steel polished to a 600 grit finish. Other coupons were coated with F-150 a white epoxy coating that was known to fluoresce. The coupons and flow cells were cleaned using detergent followed by a water rinse. The systems were then rinsed in the following solvents: chloroform, acetone and methanol. Sterilization of the flow cell was achieved by swabbing with ethanol followed by air drying in a laminar flow hood. The flow cells were assembled in the hood prior to being aseptically connected to the medium feeds and waste collection system.

Flow cells were inoculated from a continuous flow stirred tank reactor (CFSTR), 1 l working volume, fed with ASW medium maintaining a dilution rate of 0.1 h^{-1}, at ambient temperature (25–30°C). Aeration of the culture was achieved by stirring at approximately 300 rpm with a magnetic stir bar. The flow cells were inoculated from the CFSTR at a flow rate of 0.5 ml min^{-1} this was diluted with ASW medium maintained at a flow rate of 700 ml h^{-1}. After inoculation the flow was halted; the ASW flow was maintained to the laminar flow cell at a dilution rate of 9.33 h^{-1}. A further laminar flow cell was set up on a separate feed of medium and maintained as a sterile control.

Bioluminescence measurements

Bioluminescence was measured in situ with an Oriel (Stratford, CT) liquid light pipe-photomultiplier tube-ammeter light monitoring system through the 10 mm lumen in the polypropylene screws. The quartz glass windows were cleaned 1 h prior to bioluminescence and fluorescence readings.

Fluorometric measurements

On-line fluorometric monitoring was performed to monitor biomass, by tryptophane fluorescence, and cellular activity, by NAD(P)H fluorescence, using a SPEX Inc. (Edison, NJ) F212 Fluorolog II spectrofluorometer. The Fluorolog II (212) comprised a 450 W ozone free xenon lamp, with a double grating excitation spectrometer containing a fixed slit width of 2.5 mm. Light was directed onto the coupon via a bifurcated quartz fiberoptic bundle, which was placed in the polypropylene screws allowing the light to pace through the quartz window to the biofilm. Fluorescence was detected via the same bifurcated fiberoptic bundle and fed via a front face mirror to a double grating emission spectrometer again with a fixed slit width of 2.5 mm where it was directed onto a PMT. Readings for tryptophane were taken by exciting the biofilm at 290 nm and measuring the emission at 342 nm; NAD(P)H utilized excitation at 340 nm and emission at 425 nm. Due to the highly reflective nature of the coupons it was found that double grating spectrometers were necessary on both the excitation and emission in order to minimize the level of stray light reaching the PMT from the lamp. Excitation of 290 nm was chosen for the tryptophane determination as it has been shown that at this wavelength tyrosine will not fluoresce, but tryptophane continues to give a strong signal.

Biofilm analysis

Cells were quantitatively extracted from the coupons via a sonication procedure employing 1.131 cm^2 glass O-ring extractors (Kontes Glass, Vineland, NJ) as described previously [17]. Actual numbers of both viable cells and total cell numbers were determined using the Miles and Misra plate count technique [18] on marine agar and acridine orange direct counts of stained bacteria (AODC), respectively.

Results

Tryptophane determination

The ability of the system to detect tryptophane was demonstrated by carrying out an emission scan (exciting at 290 nm) for an active culture of *Vibrio harveyi* measured in a cuvette placed within the Fluorolog II, the resulting scan is shown in Fig. 1. A control scan for the stainless steel coupon is shown in Fig. 2, this shows that there is no peak in emission at 342 nm. However, the scan for a biofilm on the coupon is shown in the same figure which shows a similar emission peak to that detected for the broth culture. In order to determine that this peak was in fact tryptophane and not one of the other aromatic amino acids, 10^{-5} M solutions of tryptophane, tyrosine and phenylalanine were coated onto clean stainless steel coupons and place in a flow cell. Fig. 3 shows the emission scan for each of the three following excitation at 290 nm, this clearly shows that the only aromatic amino acid to fluoresce upon excitation at

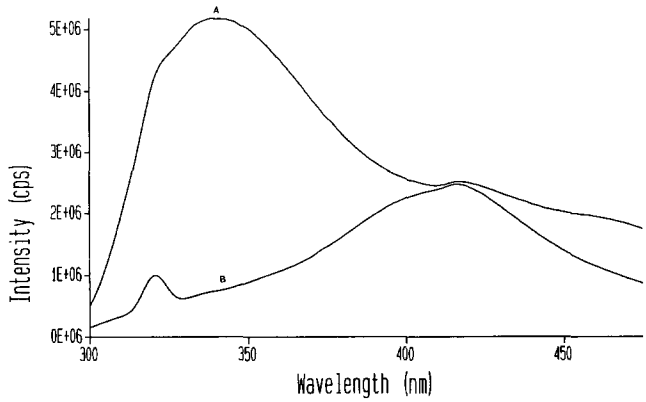

Fig. 1. Emission scans for the measurement of tryptophane in bacterial cells, (excitation 290 nm) (A) Planktonic culture of *V. harveyi* (10^8 cells/ml measured in a cuvette). (B) Artificial seawater medium blank in a cuvette (note water Raman peak at 320 nm).

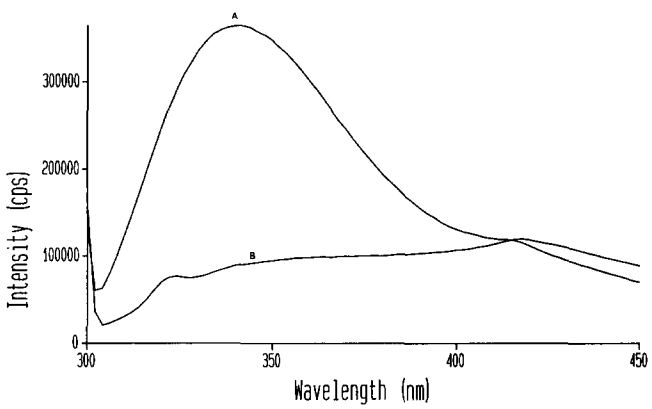

Fig. 2. Emission scans for the measurement of tryptophane in bacterial cells, (excitation 290 nm). (A) Biofilm of *V. harveyi* grown on 316 stainless steel coupon. (B) Sterile 316 stainless steel coupon (note water Raman peak from medium at 320 nm).

290 nm is tryptophane. It can therefore be concluded that the fluorescence seen from the cultures is due to tryptophane and not one of the other aromatic amino acids.

Over the time course of the experiments it can be seen that the counts for tryptophane fluorescence increase (Fig. 4) as the biofilm grows. Over the same time period there was no significant increase in the tryptophane counts from the sterile control, indicating that there was no significant adsorption of tryptophane from the medium to the stainless steel coupons.

With the white fluorescent F-150 coating no discernable trend could be detected over the time period studied. Some of the coupons showed an increase while others showed a decrease with time, this could be due in part to the build up of a biofilm on the surface, absorbing either the excitation or emitted light from the coating. Over the

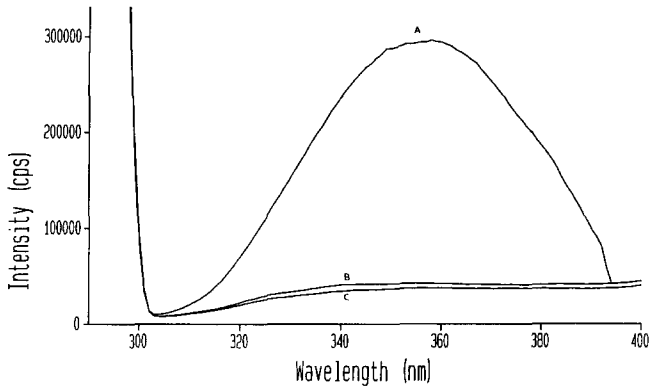

Fig. 3. Emission scans for aromatic amino acids on 316 stainless steel (excitation 290 nm). (A) Tryptophane solution (10^{-5} M). (B) Phenylalanine solution (10^{-5} M). (C) Tyrosine solution (10^{-5} M).

same time period the AODC counts also increased (Fig. 5) confirming that there had been bacterial growth on the stainless steel and F-150 coated coupons in the inoculated system, no bacteria were detected in the sterile flow cell. Fig. 6 shows a plot of the AODC values against the tryptophane fluorescence counts, showing a good positive correlation between tryptophane values and AODC counts with the r^2 coefficient of 0.987. The standard error for both methods was high with a percentage error of 60% for the acridine orange counts and 73% for the tryptophane counts, confirming the known variability for biomass determination methods [19]. A similar

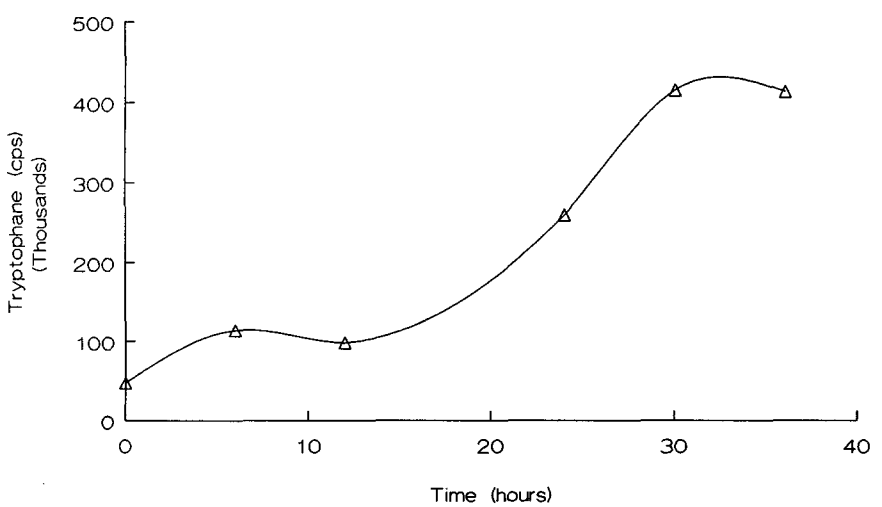

Fig. 4. Growth curve for *V. harveyi* biofilm on 316 stainless steel as determined by tryptophane fluorescence.

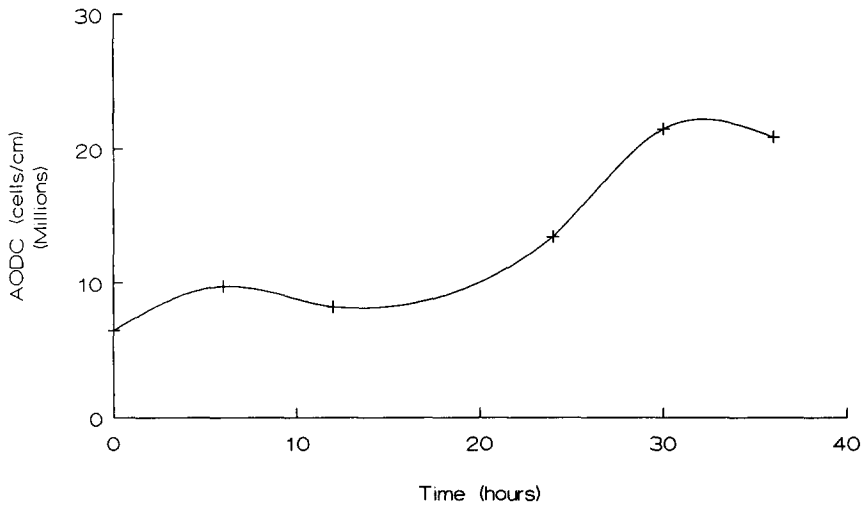

Fig. 5. Growth curve for *V. harveyi* biofilm on 316 stainless steel as determined by acridine orange counts following extraction of the biofilm.

trend was seen between viable counts and tryptophane, but as expected the correlation of 0.778 was not as high since the tryptophane method detected both viable and non-viable cells.

The bioluminescence readings also showed an increase with time up to 30 h, after

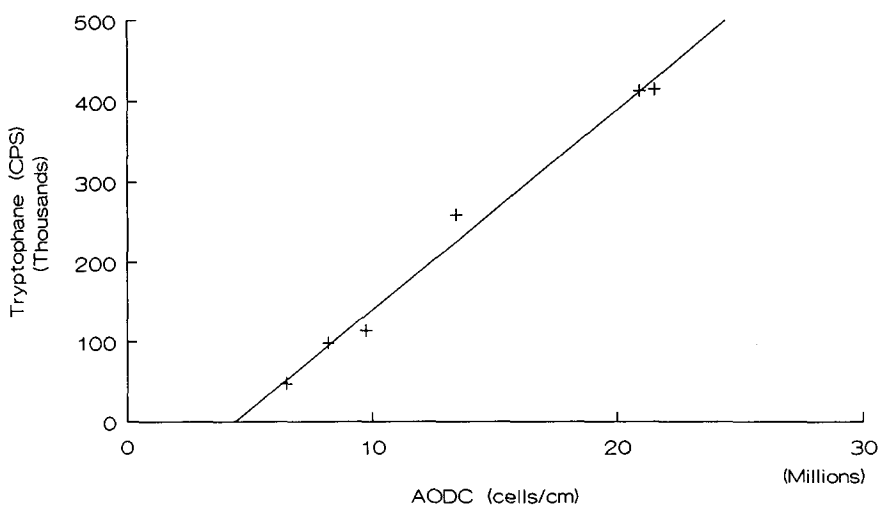

Fig. 6. Linear regression plot of tryptophane counts against acridine orange counts for a *V. harveyi* biofilm grown on 316 stainless steel ($r^2 = 0.987$).

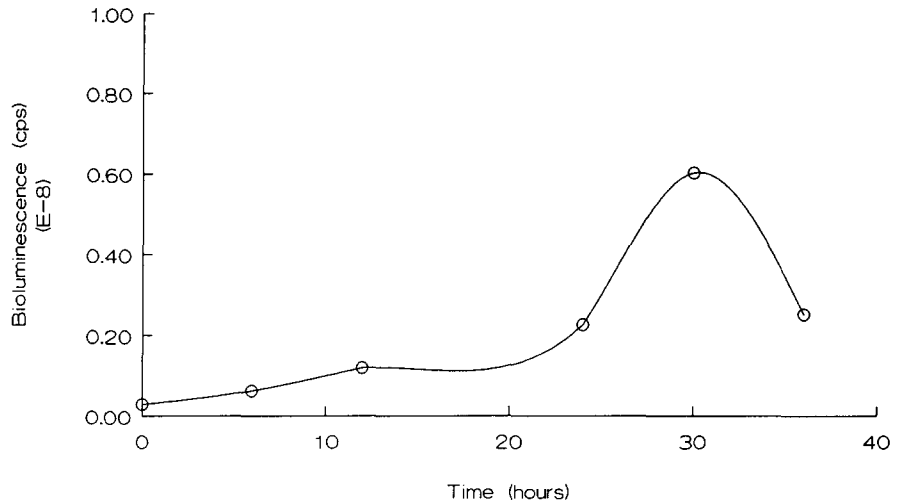

Fig. 7. Growth curve for *V. harveyi* biofilm on 316 stainless steel as determined by bioluminescence readings.

which they dropped to the 24 h value (Fig. 7). Both the tryptophane and AODC counts showed that bacterial numbers leveled out at thirty hours. The drop in bioluminescence readings may indicate a drop in metabolic activity, accompanied by a drop in cell growth. During active growth over the first 30 h of the experiment, as

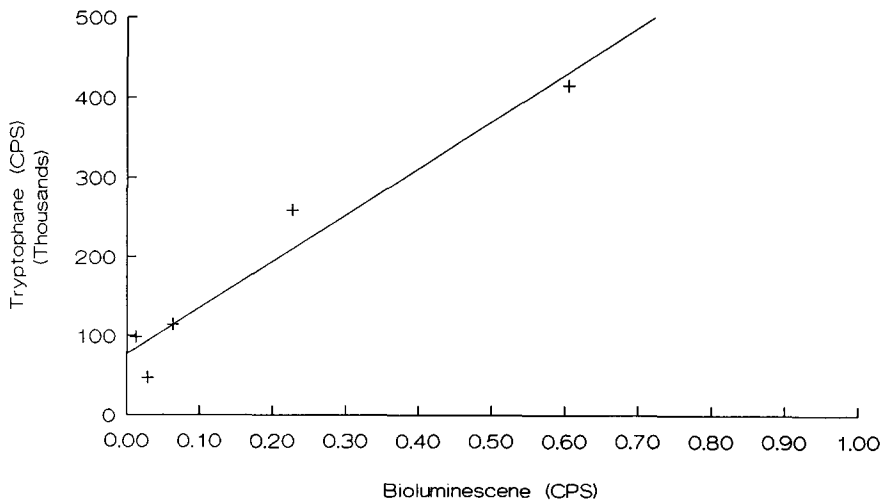

Fig. 8. Linear regression plot of tryptophane counts against bioluminescence for a *V. harveyi* biofilm grown on 316 stainless steel ($r^2 = 0.954$).

indicated by both AODC and tryptophane, bioluminescence correlates to tryptophane with a correlation coefficient of 0.954 (Fig. 8.). This relationship was not valid however if the 36 h reading was included, when active growth had stopped, when a correlation coefficient of 0.722 was obtained. This would indicate that while the bacteria are actively growing, bioluminescence is a good indicator of cell number as reported by Mittelman et al [9]. However, bioluminescence is directly affected by a number of parameters such as growth rate, oxygen concentration and nutrient concentration and indirectly affected by the presence of respiratory inhibitors. It is therefore suggested that bioluminescence is a good indicator of biomass only during active growth in defined conditions.

NAD(P)H determination

Fig. 9 shows the detection of NAD(P)H for an active culture of *Vibrio harveyi* measured in a cuvette placed within the Fluorolog II following excitation at 340 nm. Fig. 10 shows a scan taken, using the fiberoptic accessory, of a clean sterile stainless steel coupon with excitation at 340 nm, again a large emission peak is seen at 425 nm. This peak from the stainless steel is however, due to fluorescence from the fiberoptic cable used. The cable was chosen to give the least all round fluorescence, with the capability to carry u.v. light. Alternative fiberoptic cables are being sort which do not fluoresce at 340 nm excitation, with the view to using a different cable for NAD(P)H from that used for tryptophane.

Despite the known interfering fluorescence of the cable measurements were made in the hope that the NAD(P)H fluorescence could be detected above that of the cable. The results however, show that this was not the case, with the readings being maintained at the same level as the background values shown in Fig. 10. This result was similar for both the stainless steel and the fluorescent coating.

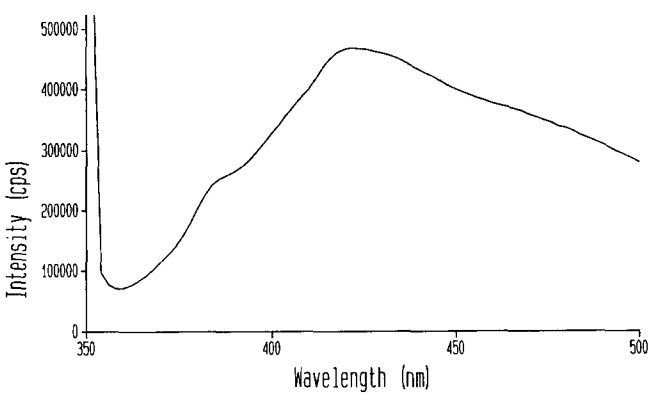

Fig. 9. Emission scan (excitation 340 nm) for an active plánktonic culture of *V. harveyi* (10^8 cells/ml) in a cuvette showing an emission peak at approximately 420 nm demonstrating the possible detection of NAD(P)H.

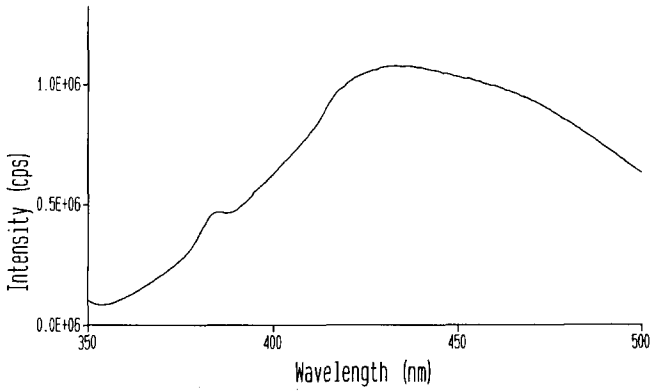

Fig. 10. Emission scan (excitation 340 nm) for a sterile 316 stainless steel coupon utilizing a fiberoptic cable demonstrates an emission peak at approximately 420 nm due to the internal fluorescence of the fiberoptic cable.

Conclusions

These experiments have clearly shown that by using a SPEX Fluorolog II, with double grating spectrometers for both excitation and emission in combination with a fiberoptic cable, it is possible to utilize tryptophane fluorescence as an on-line measurement of biomass for bacterial biofilms grown in laminar flow chambers. The technique is already being applied to study the efficacy of potential anti-fouling paints, and will be of great benefit to many biofilm studies. Future work will concentrate on the detection of other possible biomass markers such as pyridoxine and riboflavin for bacteria and chlorophyll for algae along with the detection of NAD(P)H as an indicator of cellular activity.

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