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Test systems have been developed which enable the evaluation of bacterial biofilm formation and metabolic activity under conditions simulating those of the in situ environment. A series of laminar-flow adhesion cells were constructed with provisions for on-line, non-destructive measurements of bioluminescence, fluorescence, open circuit potential, and pO_2 for monitoring colonization and succession as influenced by a systematic change in bulk-phase and substratum conditions. Bioluminescence and fluorescence by biofilms of the bioluminescent, marine bacterium, *Vibrio harveyi* were utilized as endpoints for adhesion in evaluations of antifouling (AF) ship hull coatings. Resistance to colonization of *V. harveyi* was noted in the order of F-121 (Navy)> BRA 640 (IP)> 15% DNP. Future work will evaluate changes in cellular lipid biomarkers associated with biofilms and planktonic cultures exposed to copper contained in AF coatings and in bulk-phase chemostats, respectively. The test systems and procedures developed under ONR funding will enable studies of materials compatibility, antifouling efficacy, and biocide toxicity in diverse ecosystems.

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BIOFILM ECOLOGY OF BIOLUMINESCENT BACTERIA

ONR N00014-91-J-1598

Final Report Date: August 10, 1992

Marc W. Mittelman & David C. White (PI), University of Tennessee, Knoxville, Center for Environmental Biotechnology

SUMMARY

Test systems have been developed which enable the evaluation of bacterial biofilm formation and metabolic activity under conditions simulating those of the *in situ* environment. A series of laminar-flow adhesion cells were constructed with provisions for on-line, non-destructive measurements of bioluminescence, fluorescence, open circuit potential, and pO_2 for monitoring colonization and succession as influenced by a systematic change in bulk-phase and substratum conditions. Bioluminescence and fluorescence by biofilms of the bioluminescent, marine bacterium, *Vibrio harveyi* were utilized as endpoints for adhesion in evaluations of antifouling (AF) ship hull coatings. Resistance to colonization of *V. harveyi* was noted in the order of F-121 (Navy)> BRA 640 (IP)> 15% DNP. Future work will evaluate changes in cellular lipid biomarkers associated with biofilms and planktonic cultures exposed to copper contained in AF coatings and in bulk-phase chemostats, respectively. The test systems and procedures developed under ONR funding will enable

studies of materials compatibility, antifouling efficacy, and biocide toxicity in diverse ecosystems.

BACKGROUND

Bacterial biofilm formation on inanimate substrata in freshwater, marine, and physiological environments often precedes microbially influenced corrosion and other biofouling activities. The impact, world-wide, of these activities amounts to billions of U.S. dollars each year (Dowling et al., 1991). In addition to their direct involvement in fouling and corrosion activities, bacteria--and other microorganisms--can have an impact on the settling and adhesion of macrofouling organisms to engineered surfaces. For example, Kirchman et al. (1982) Weiner et al. (1985) described specific bacteria which promoted barnacle and oyster formation on surfaces exposed to marine environments.

Colonization of and attachment to surfaces in these environments is mediated by a number of interrelated environmental factor such as fluid dynamics (Mittelman et al., 1990), bulk-phase biotic and abiotic constituents (Marshall, 1988), and the physicochemistry of the substrata (Absolom et al., 1983). To a great extent, research targeted to adhesion processes has been limited by a lack of test systems and analytical techniques for examining bacterial colonization and biofilm formation under conditions which approximate those of in situ environments.

The ability to reproducibly colonize replica test substrata with relevant bacterial populations is a necessary component of antifouling efficacy studies. Laboratory and field applications of the "Robbins Device" have been described by Ruseska et al. (1982) and Characklis et al. (1982) have developed annular-type reactors for the study of biofilm effects on fluid frictional resistance. Most of the systems developed thusfar have been designed for microscopic evaluation of bacterial colonization on glass substrata. Provisions for monitoring biofilm development on other, non-glass substrata, electrochemical/luminescent methods for studying adhesion, and quantitative assessments of biofilm biomass and metabolic activity have not been made in most of the existing systems.

Bioluminescent bacteria have been employed in a few different types of biofouling and toxicity assessments. In ONR sponsored research in this laboratory, Mittelman et al. (1992) utilized *lux* constructs of *Pseudomonas fluorescens* in an on-line assay, using bioluminescence as an endpoint for adhesion. Jassim et al. (1990) have described an *in vivo* bioluminescence technique for evaluating biocide effects on planktonic bacterial populations. King et al. (1990) utilized a bioluminescent reporter plasmid to evaluate aromatic hydrocarbon utilization in contaminated soils.

The design and application of laminar-flow adhesion cells designed for studies of bacterial biofilm formation and determinations of AF coating efficacy are described in this final report. These cells enabled determinations of bacterial colonization and succession using bioluminescence, fluorometry, and shifts in electrochemical potential as endpoints for adhesion.

MATERIALS & METHODS

Flow cell design. The flow cells consisted of an upper block of translucent, laminated Lexan and a lower block of ultra-high molecular weight polyethylene. Overall dimensions of the cells were 15.0 cm W X 28.5 cm L X 3.3 cm H. The upper block was milled to provide an 0.2 cm deep flow channel; in addition, it contained a series of removable polypropylene screws with 1.2 cm diameter quartz glass discs at their base, flush-mounted with the flow channel (Fig. 1). This arrangement enabled direct observation of a series of removable, flush-mounted coupons recessed into the bottom block. Open circuit potential (OCP) measurements were facilitated by means of a Ag/AgCl reference electrode installed in the top block. The upper block also contains provisions for oxygen monitoring via an 0.3 cm diameter semi-micro amperometric probe. Laminar flow conditions were validated in dye studies and by observing a silk thread normal to the flow channel as described by Berg and Block (1984).

Test substrata. Uncoated 316 stainless steel (SS) coupons polished to a 600 grit finish were used in validation and OCP experiments. Three free-association coatings were evaluated for antifouling efficacy: 15% (w/w) dinitrophenol (DNP), and two copper-based paints, Navy F-121 and International Paints (IP) BRA-640. All coatings were applied to an epoxy base-coat. Epoxy coatings free of any AF agents were used as experimental controls. The finished dimensions of the test substrata were 3.5 cm W X 7.0 cm L X 0.3 cm H.

Continuous culture conditions. A continuous culture of the bioluminescent bacterium, *Vibrio harveyi* (ATCC 14126), was used to colonize polymer coated test coupons with and without antifouling additives (Fig. 2). An artificial seawater medium (ASW) (ASTM, 1986) 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 experiments. A dilution rate of 0.1 h^{-1} was used in the continuous culture vessel. All experiments were performed at ambient temperature (23-25 C).

Bioluminescence measurements. Bioluminescence was measured *in situ* with an Oriel (Stratford, CT) liquid light pipe-photomultiplier tube-ammeter light monitoring system through a 1.0 cm lumen in the polypropylene screws. The quartz glass window-polypropylene screw assembly was replaced prior to bioluminescence measurement to eliminate contributions from glass-associated biofilms.

Fluorometric measurements. Preliminary on-line fluorometric monitoring of biomass and activity was performed with a Spex Instruments Fluorolog II spectrofluorometer (Edison, NJ) equipped with a quartz fiberoptic cable.

Electrochemical measurements. The OCP of uncoated SS coupons was monitored with a Keithley (Keithley Instruments, Cleveland, OH) model 706 multichannel scanner and measured on a Hewlett Packard (Palo Alto, CA) model 3458A voltmeter interfaced with a GPIB board and IBM clone personal computer. The test coupons served as the working electrodes; a SS thumbscrew provided the connection to the working electrode.

Biofilm analyses. Reproducibility of colonization was determined by direct counting of acridine orange stained bacteria (AODC) and by viable counts on marine agar. Cells were quantitatively extracted from coupon surfaces via a sonication procedure employing 1.131 cm^{-2} glass o-ring extractors (Kontes Glass, Vineland, NJ).

Bioluminescence inhibition studies. Sodium azide and carbon monoxide were used to study the response of V. harveyi biofilm and planktonic populations to metabolic inhibitors. Changes in bioluminescence of 24 h cultures as a function of 30 min exposures to 50 mL min-1 and 10 mM carbon monoxide and sodium azide, respectively, were monitored.

RESULTS & DISCUSSION

Replica experiments with Vibrio harveyi biofilms demonstrated reproducible colonization on coupons 3-5 within the laminar-flow adhesion cells (Fig. 3). The first two coupons typically showed greater numbers of cells and higher bioluminescent readings than did coupons 3-5. These differences may be due to differential substrate availability. Bioluminescence, AODC, and viable counts were reproducible for 5 mL min^{-1} flow rates. A significant positive correlation was established between bioluminescence and viable/direct bacteria counts (Fig. 4). The Lexan tops will enable fouling studies with cyanobacteria, diatoms, and other photosynthetic organisms. In addition, replica bioluminescence/ fluorescence data can be obtained from three different areas of each coated/uncoated test coupon.

Resistance to colonization of V. harveyi was noted in the order of F-121 (Navy)> BRA 640 (IP)> 15% DNP (Fig. 5, Table 1). There was good agreement between bioluminescence, viable count, and direct count data for AF coated surfaces as with the uncoated SS controls. The effect of AF compound release on non-AF containing coatings is shown in Table 1. There was decreased attachment from AF coatings release in the order of F-121>15% DNP≈BRA 640 (IP). Bioluminescent biofilm bacteria were shown to be useful indicators of AF coating efficacy under dynamic-flow conditions.

When carbon monoxide and sodium azide were used as metabolic inhibitors, significant changes in lightoutput were noted. Bioluminescence was significantly reduced in V. harveyi biofilms exposed to 50 mL min^{-1} concentrations of CO over 30 min (Fig. 6). Interestingly, increases in bioluminescence were observed when bulk-phase cultures were exposed to like concentrations of CO (Fig. 7). Ulitzer et al. (1981) suggested that some inhibitors of electron transport, e.g., cyanide, result in an increase in the expression of cellular luciferase. They postulated that this phenomenon could be explained by an increase in the intracellular levels of reduced coenzymes, which, in turn, increase flavin reduction and aldehyde production. Both of these compounds are substrates in the bacterial bioluminescence system. It is unknown at this time why a differential response to CO was seen between the biofilm and bulk-phase. In the case of 30 min exposures to 10 mM sodium azide, significant decreases in light production were noted for both biofilm and bulk-phase bacteria (Figs. 8,9).

Guckert et al. (1992) suggested that increases in the *trans*:*cis* ratio of monoenoic phospholipid fatty acids (PLFA) were indicative of organismal stress. The ratio of unsaturated:saturated monoenoic fatty acids can which provides an indication of "membrane fluidity". Analyses in-progress are determining changes in lipid biomarkers as a function of copper-induced stress for both chembstat and biofilm cultures of *V. harveyi*. Poly-B-hydroxy alkanoate (PHA) is synthesized by a number of microorganisms as a response to "unbalanced growth" conditions (Dawes, 1984). It may also prove useful as an indicator of stress in response to AF agents. Future work will incorporate PHA analysis of biofilms and bulk-phase cultures exposed to AF agents.

The SPEX system enables low-level detection of both bioluminescence (photon counting) as well as fluorescence emissions from tryptophane and other aromatic amino acids and nucleotides (Fig. 10). Tryptophane was detected in bulk-phase cultures and in situ biofilms of V. harveyi associated with 316 SS surfaces (Fig. 11). Biomass and metabolic activity can be monitored on AF coated and control surfaces in situ on a real-time basis. Several compounds show promise as biomass/metabolic activity markers within biofilms (Table 2).

OCP values, which provide an indication of surface potential, were significantly perturbed by the addition of V. harveyi; however, neither the magnitude nor the onset of the observed perturbations were diagnostic for biomass quantity or community structure (Fig. 12). Changes in potential preceded visible biofilm formation and bioluminescence production. The OCP is a net potential, describing the sum of cathodic and anodic reactions. The cathodic reaction, which predominates in stainless steels exposed to aqueous environments--contrasted with mild or carbon steels, in which the anodic reaction predominates--is described by

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$$

The OCP is primarily controlled by two parameters contained within the Nernst equation, pH and oxygen. For the cathodic reaction,

$$E= E(O_2/OH^-) + RT/nF\ (\ln[pO_2]/[OH^-]^4$$

While OCP measurements may prove useful for monitoring the onset of fouling on uncoated, metallic surfaces, electrochemical surface potentials cannot be measured on non-metallic coatings; i.e., AF or fouling release epoxy combinations. However, these measurements might prove useful in evaluations of coating integrity. If mechanical and/or biological degradation created holidays in the coatings, changes in OCP would result upon contact of seawater with the underlying metal surfaces.

Research performed under this program has resulted in the development of a new test system and analytical regime for assessing the effectiveness of AF coatings against microfouling organisms. The utility of bioluminescent bacteria in performing these studies was demonstrated. The significance of this work may be seen in the light of evidence that AF compounds targeted towards particular macrofoulants may be subject to rapid biodegradation by the *in situ* microbial population. In previous work performed in this laboratory under the DARPA program, benzoic acid contained within an AF coating was rapidly degraded by *Alteromonas* *atlantica* biofilms. While benzoic acid may inhibit larval settlement, it is clear that this compound is rendered ineffective by a naturally-occurring marine bacterium, which utilized this compound as a sole carbon source.

The multipurpose, laminar-flow adhesion cells provided a reproducible means for colonizing various surfaces and measuring biomass accumulation. Bioluminescence measurements were used to determine AF coating efficacy and the effect of coating release on sessile marine bacteria. Future research will utilize this test system for studies of sublethal toxicity effects on microbial monocultures and consortia in order to better understand the effects of AF compounds on the ecology of microfouling organisms.

Work has also been proceeding on a parallel track with engineered bioluminescent bacteria which could be utilized in AF coating and ecological studies. Several freshwater biofilm isolates from corroding pipeline surfaces probed positive for various *alg* biosynthetic genes, providing preliminary evidence of a role for alginates in adhesion/corrosion processes (Wallace et al., 1992). *Lux* fusions have been performed which enable detection of alginate formation using bioluminescence as an endpoint. Bacterial alginate production, as inferred from DNA homology studies, was associated with a majority of bacteria isolated from corroding pipeline surfaces in freshwater TVA pipelines (Wallace et al., 1992).

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Table 1. Treatment and release effects on V. harveyi colonization.

Treatment	n	Treatment Efficacy[1]		Effect of Release[1]	
		Viable	AODC	Viable	AODC
DNP	4	38 (41)[2]	[3]*64 (3.5)	*28 (25)	*48 (5.8)
IP BRA-640	3	*0.73 (0.23)	*32 (19)	*25 (39)	74 (17)
F-121	3	*<0.0003	*12 (3.8)	*11.2 (5.4)	*36 (16)

[1]expressed as a percent of control value
[2]standard deviation
[3]significantly different from the control (non-treatment) at $P<0.05$

Table 2. Examples of relevant wavelengths for fluorometry of fouled surfaces.

Biomass	Excitation	Emission
TRP; TYR; PHE	260-280	303; 348; 282
ACTIVITY		
bioluminescence	-------	490
ATP	272	380
NADH	340	460
ALGAE		
chlorophyll b	480	640

FIGURE LEGEND

Fig. 1. Laminar-flow adhesion cell. Holes drilled at entry and exit ends of the cell provide access for electrochemical monitoring.

Fig. 2. Flow diagram for studies of antifouling coating efficacy.

Fig. 3. Bioluminescence of flow cell coupons colonized with *V. harveyi*.

Fig. 4. Relationship between bioluminescence and viable and total bacteria counts on uncoated 316 SS coupons.

Fig. 5. Treatment efficacy of coatings expressed as a percentage of control bioluminescence vs. time.

Fig. 6. Effect of CO on biofilm bioluminescence.

Fig. 7. Effect of CO on bulk-phase bioluminescence.

Fig. 8. Effect of sodium azide on biofilm bioluminescence.

Fig. 9. Effect of sodium azide on bulk-phase bioluminescence.

Fig. 10. Diagrammatic representation of fluorometer application to antifouling coating efficacy studies.

Fig. 11. Detection of the aromatic amino acid tryptophane in *V. harveyi* biofilms associated with 316 SS.

Fig. 12. OCP of *V. harveyi* biofilms associated with 316 SS.

PUBLICATIONS RESULTING FROM PROGRAMMATIC RESEARCH

Mittelman, M.W., J. Packard, A.A. Arrage, S.L. Bean, P. Angell, and D.C. White. 1992. Bioluminescent and fluorometric detection of biofilm bacteria in evaluations of antifouling coating efficacy. Biofouling (submitted).

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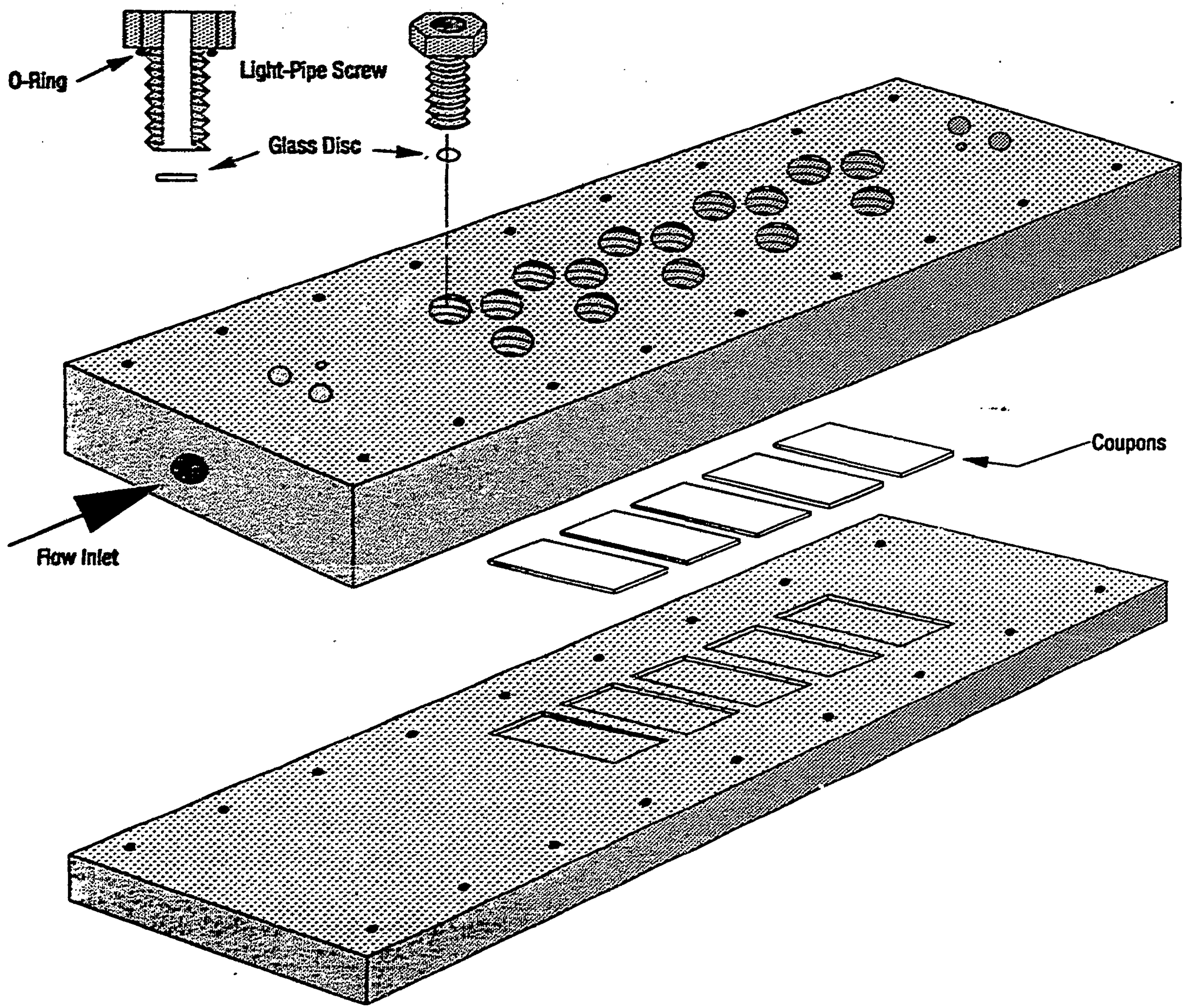
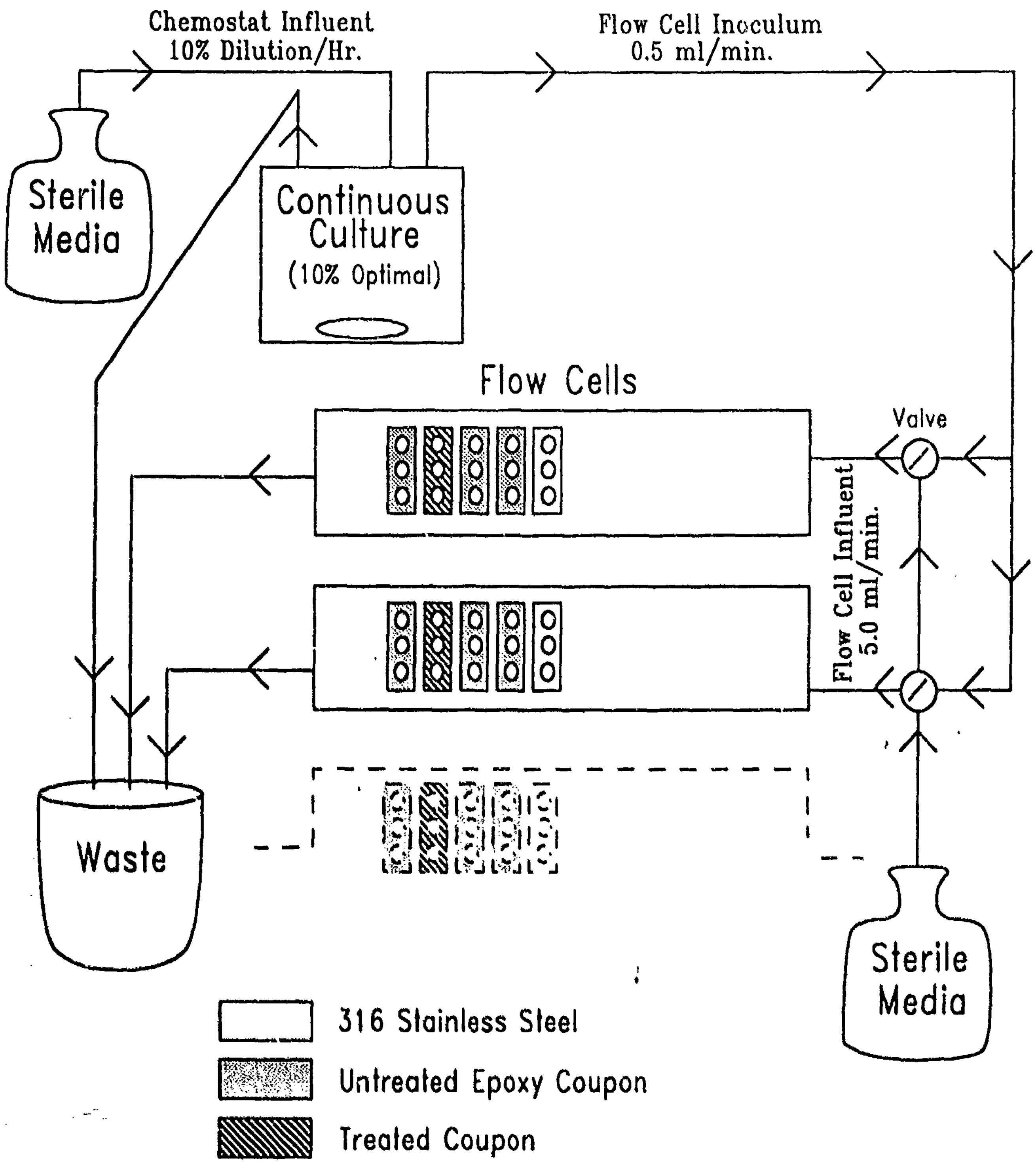
PATENTS PENDING/FILED.

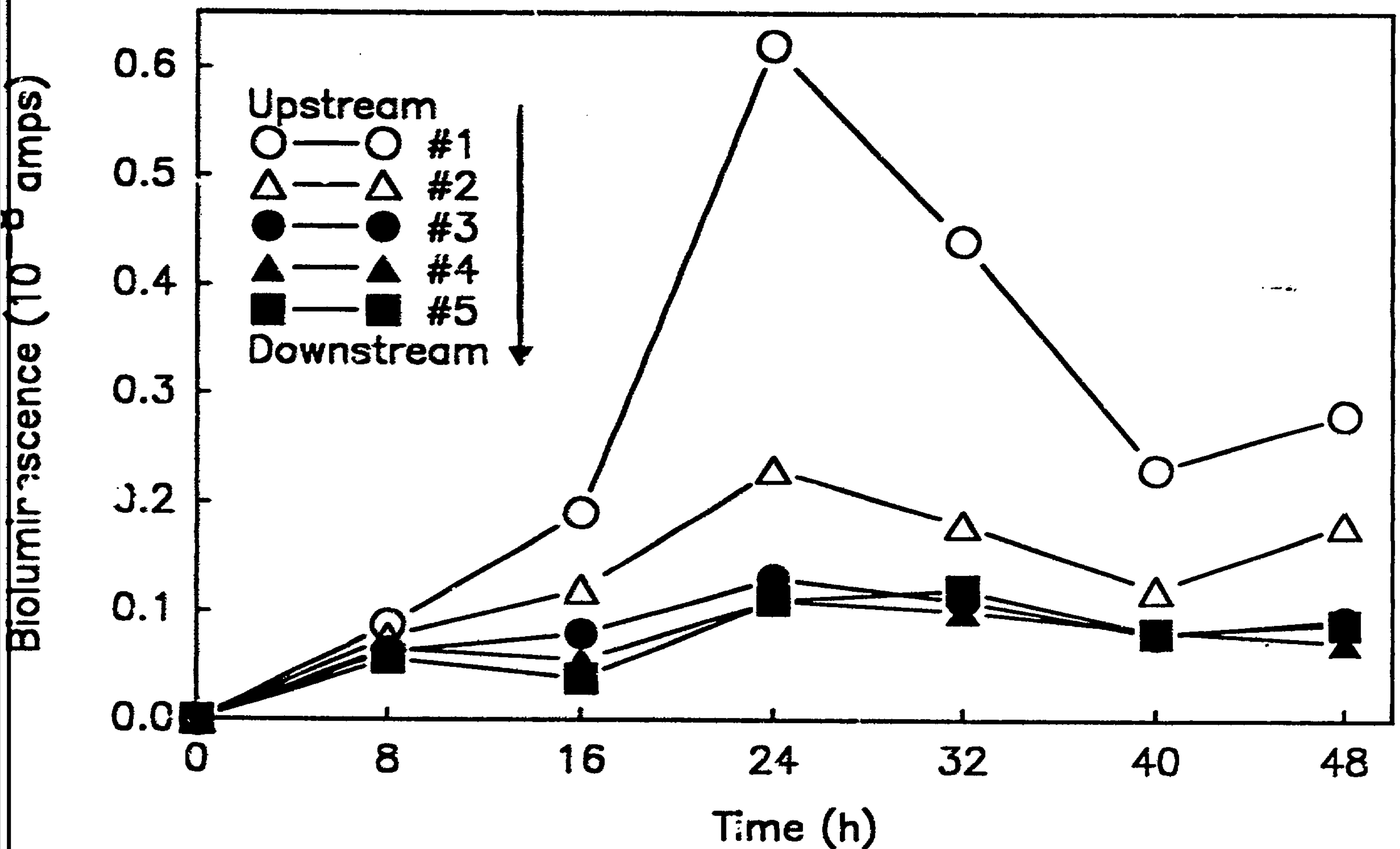
No patents were filed resulting from this research.


O-Ring
Light-Pipe Screw
Glass Disc
Flow Inlet
Coupons

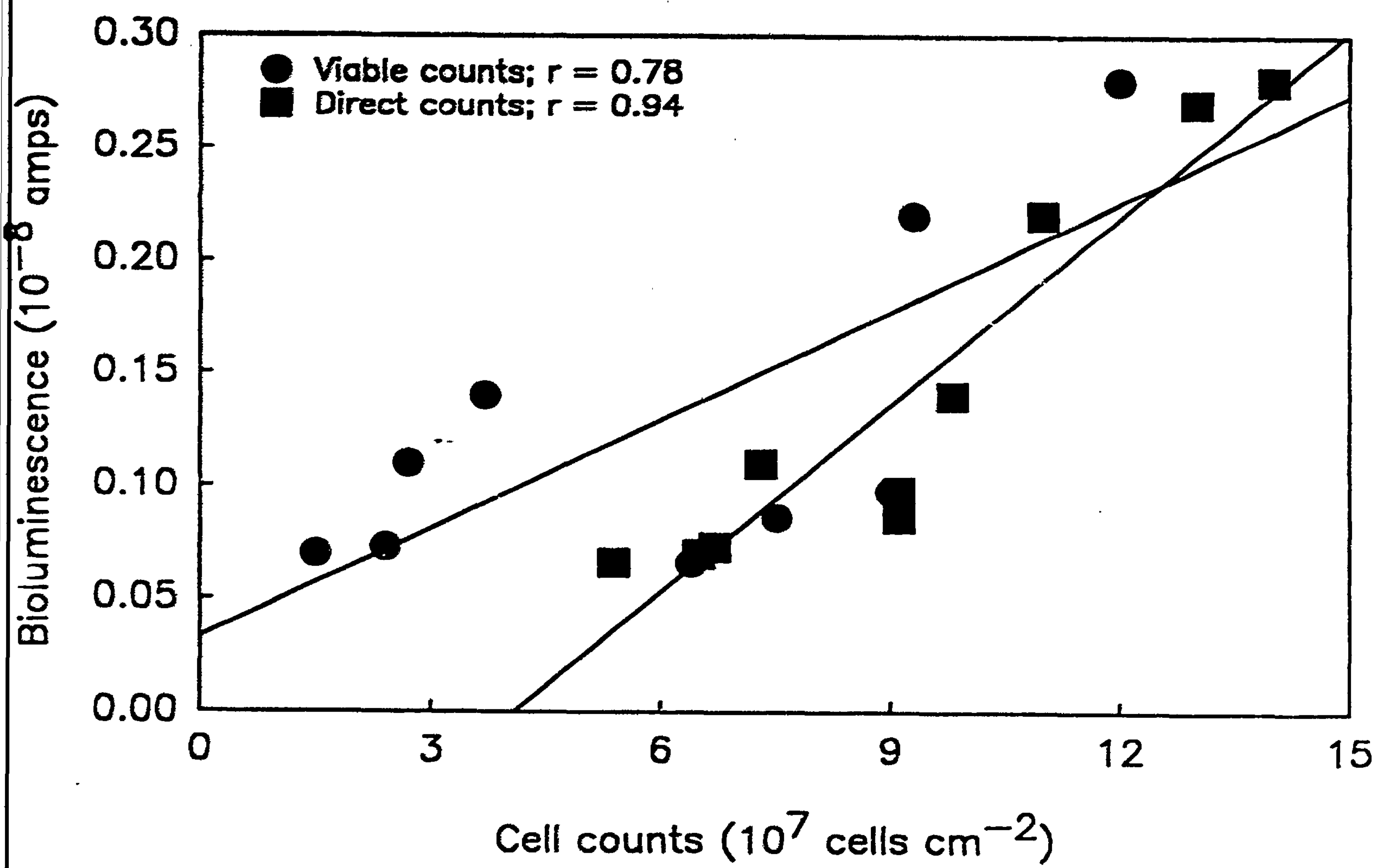
On-Line Bioluminescence Measurement System

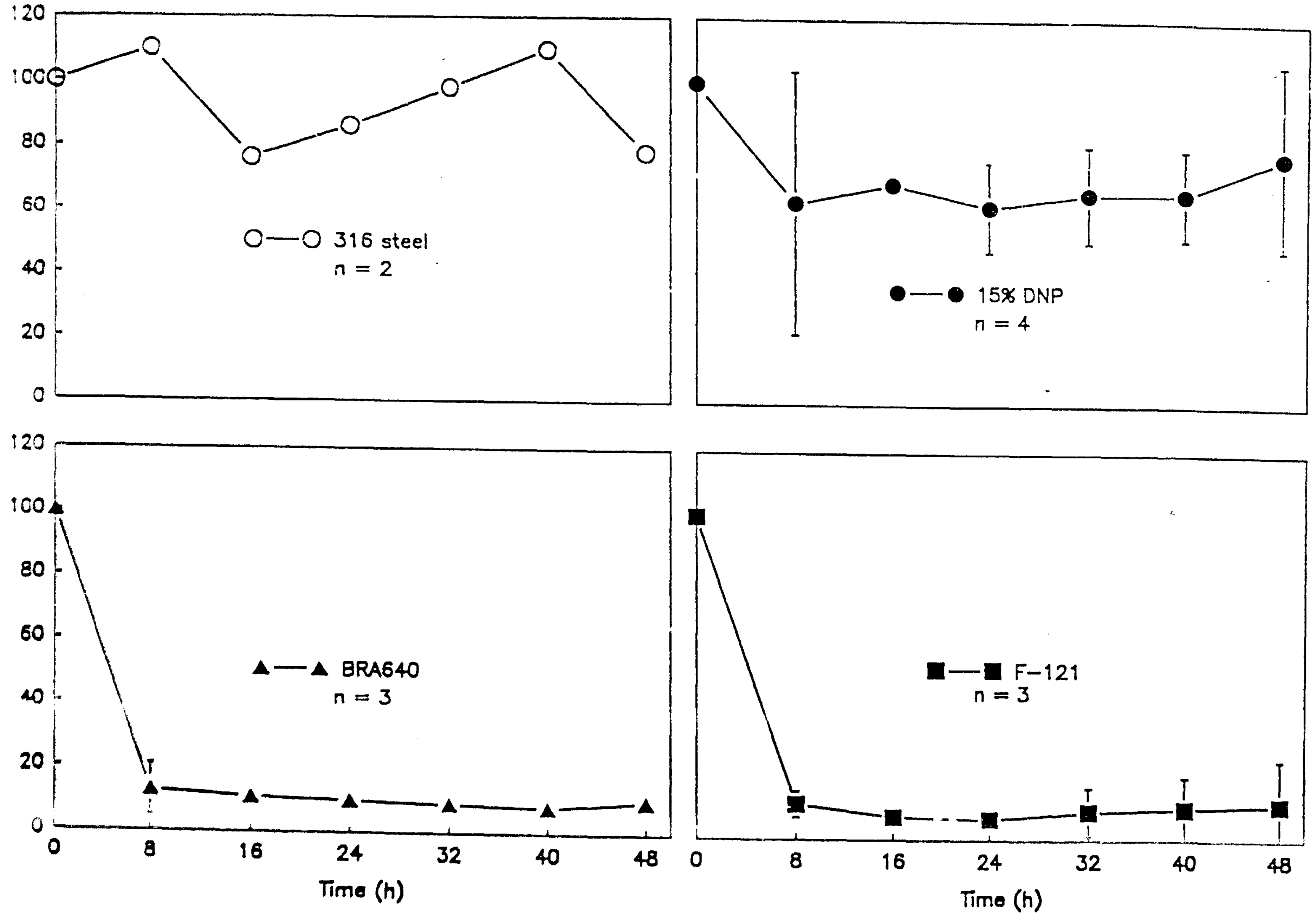


316 Stainless Steel Coupons



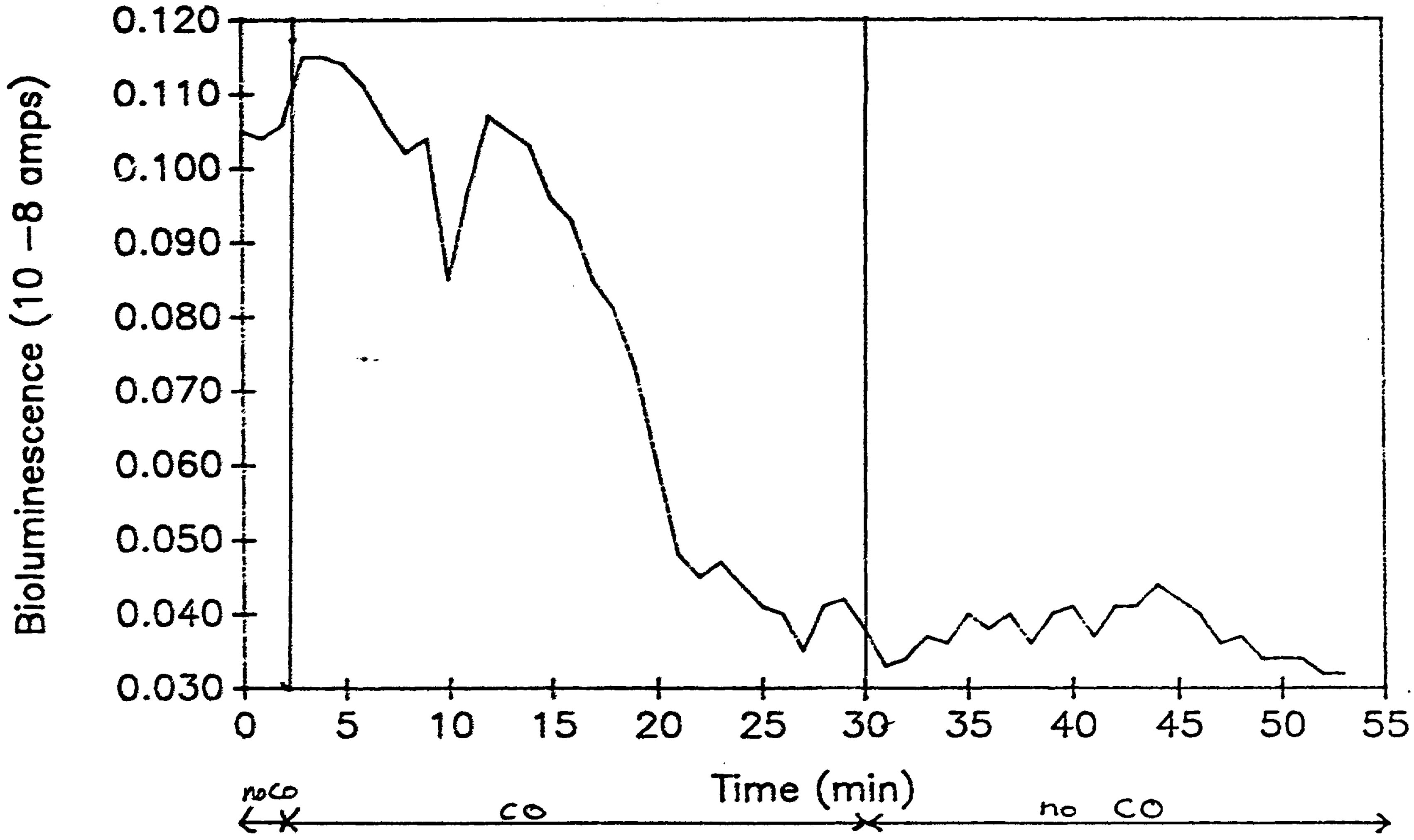
Correlation of Bioluminescence to Viable and Total Cell Counts on 316 Stainless Steel Coupons



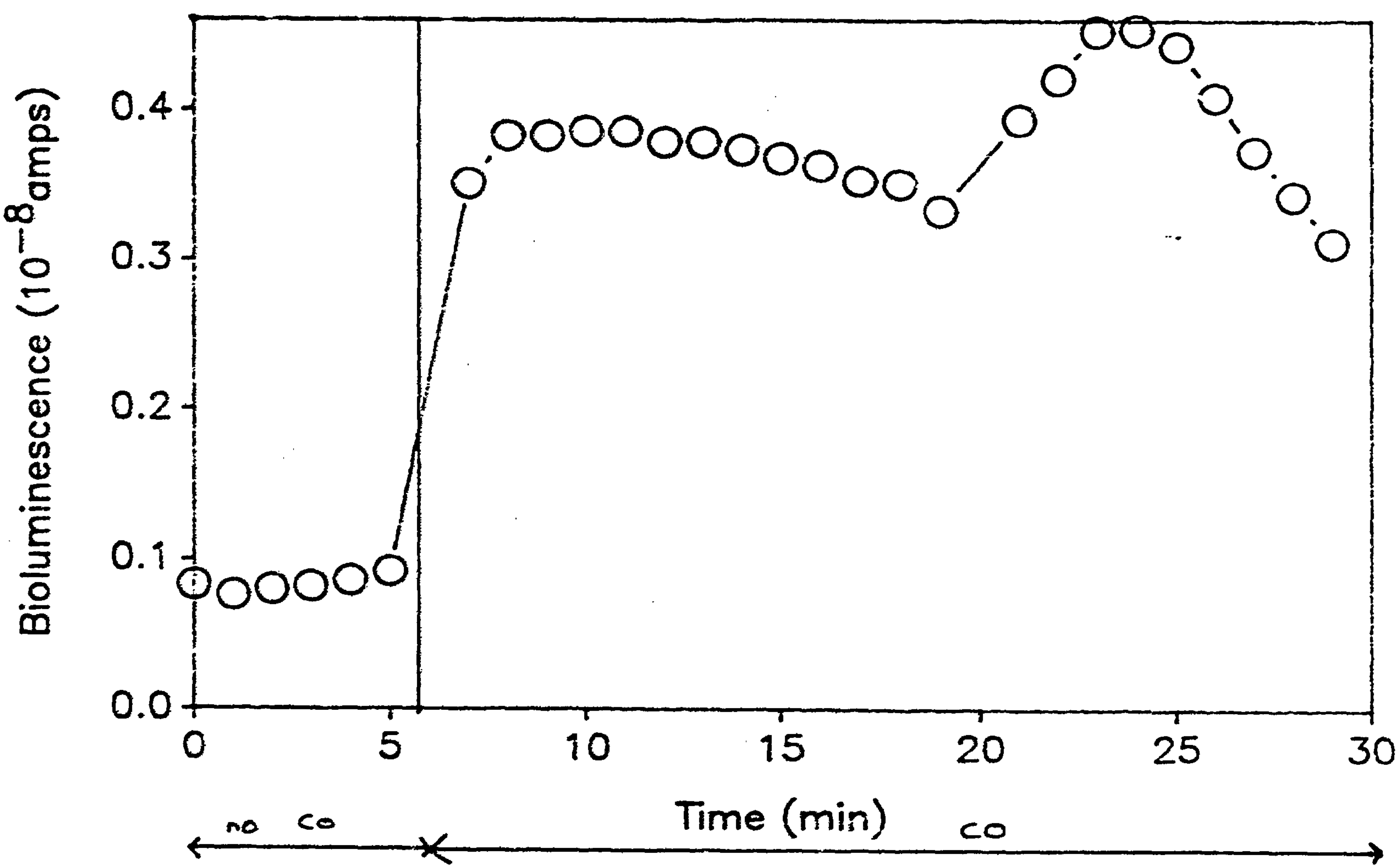

120
100
80
60
40
20
0
316 steel
n = 2
15% DNP
n = 4
BRA640
n = 3
F-121
n = 3
0
8
16
24
32
40
48
Time (h)
Time (h)

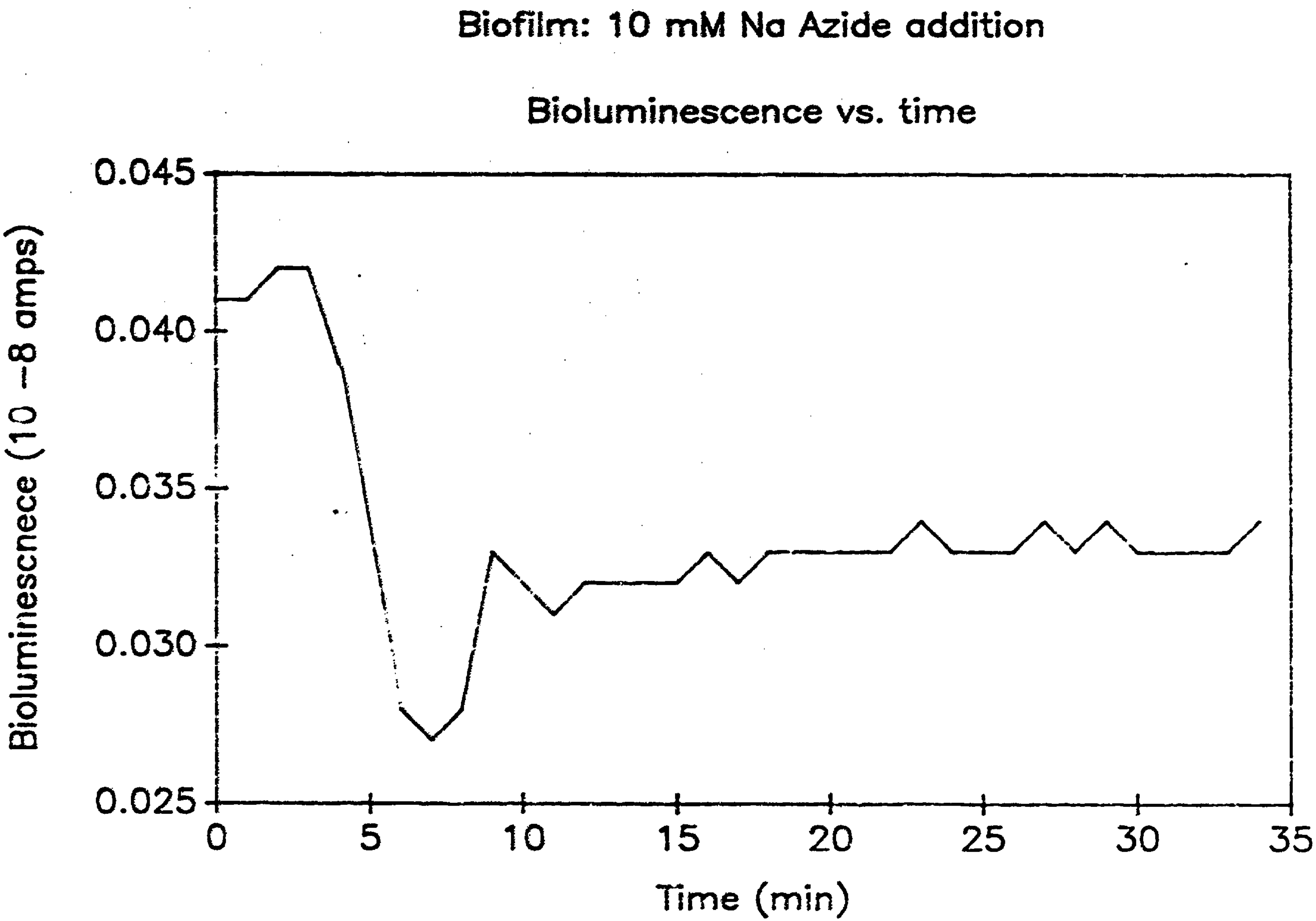
5

2 day old biofilm with carbon monoxide
Bioluminescence vs. time



2 day old bulk phase with carbon monoxide
Bioluminescence vs. time

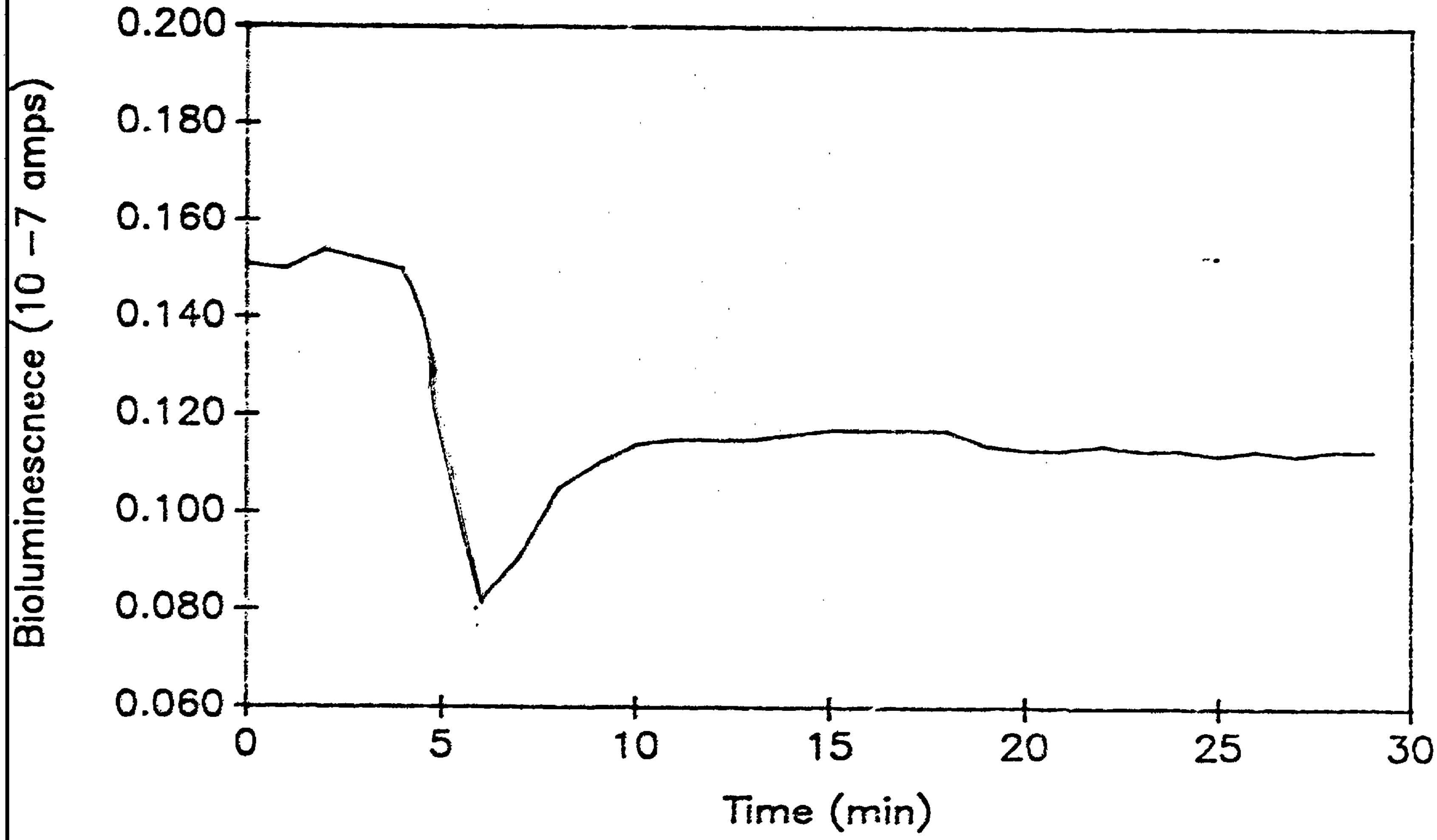




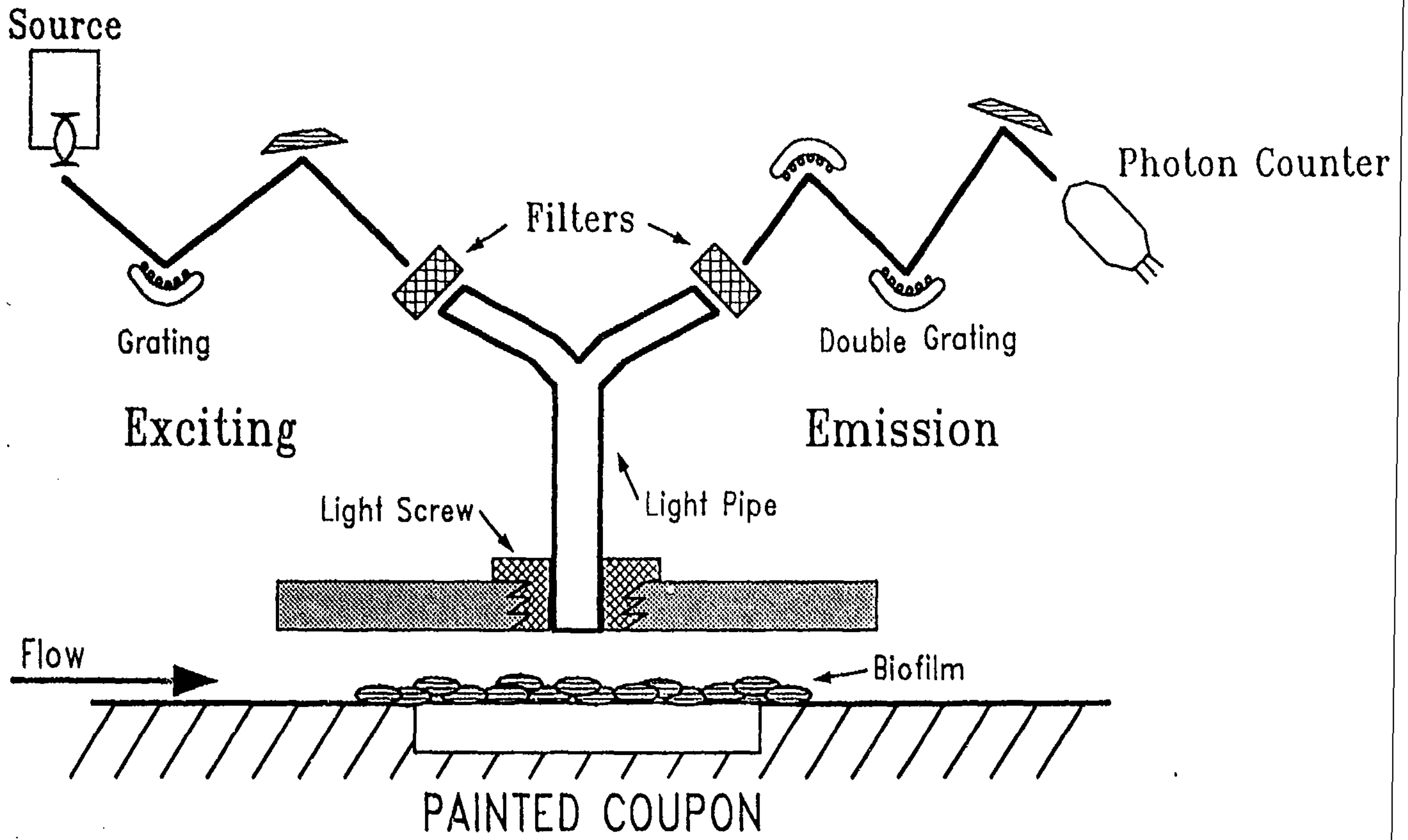
Biofilm: 10 mM Na Azide addition
Bioluminescence vs. time
0.045
0.040
0.035
0.030
0.025
0
5
10
15
20
25
30
35
Time (min)
Bioluminescnece (10 −8 amps)

Glowing bulkphase: 10 mM Na Azide addition

Bioluminescence vs. time



Fluorometer Schematic



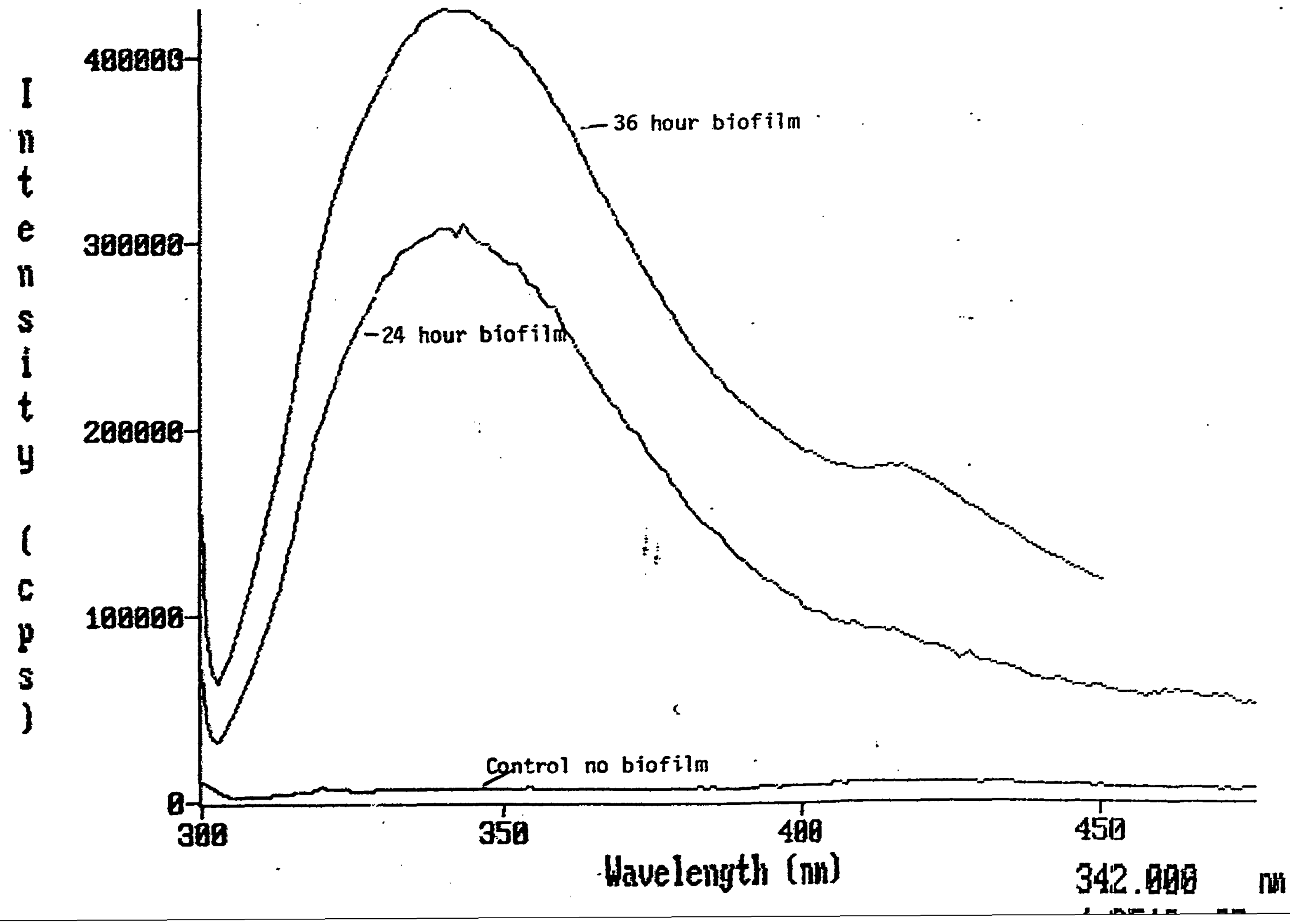
Bioluminescence (Activity)
Fluorescence (Biomass)
Tryptophan
Chlorophyll

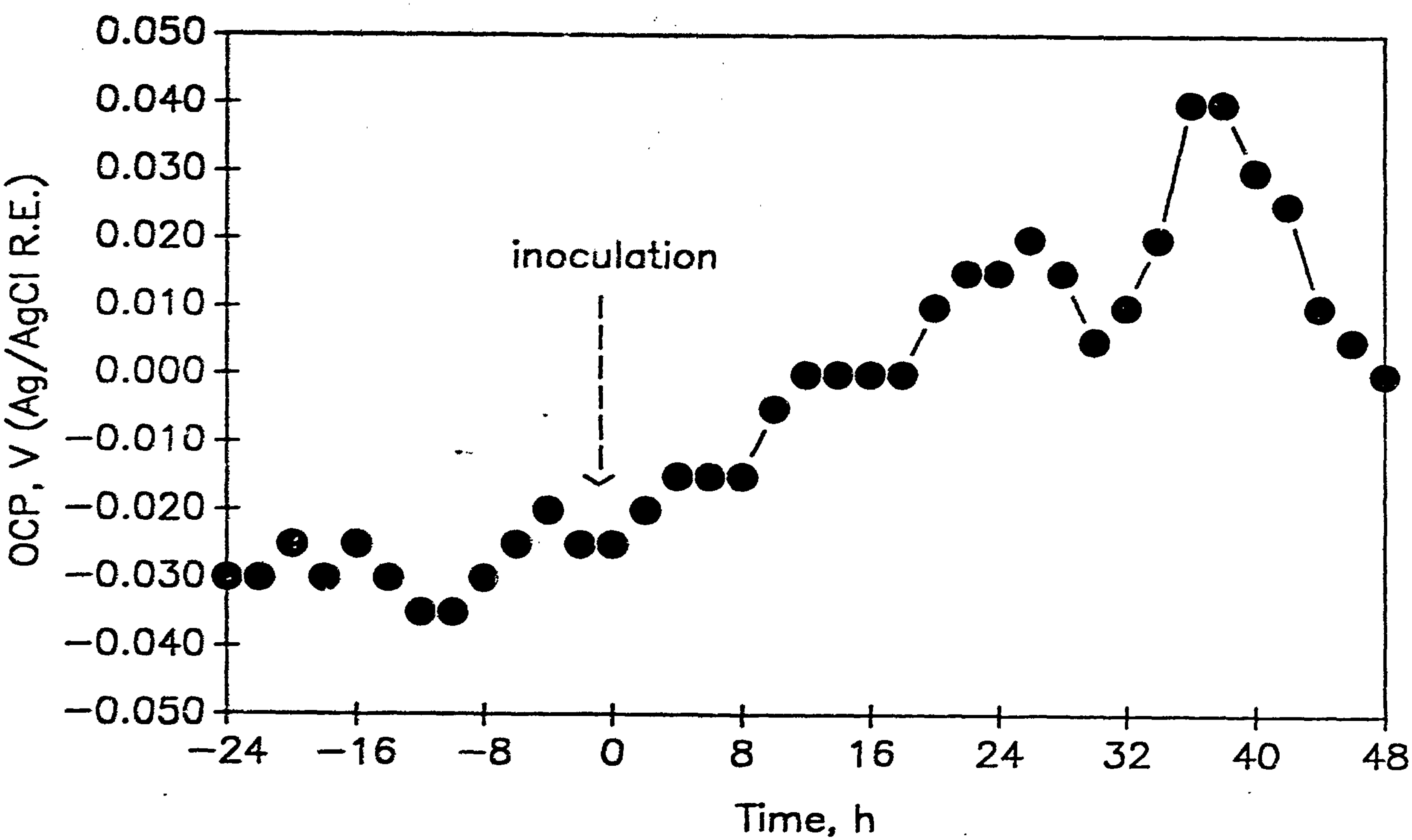
Controlled Shear
Fouling Release

Adherence
Colonization
Succession
Desquamation

Sublethal Toxicity = ↓ Activity/Biomass

Biofilm Ecology


Intensity (cps)
400000
300000
200000
100000
0
300
350
400
450
Wavelength (nm)
36 hour biofilm
24 hour biofilm
Control no biofilm
342.000 nm



inoculation
OCP, V (Ag/AgCl R.E.)
0.050
0.040
0.030
0.020
0.010
0.000
−0.010
−0.020
−0.030
−0.040
−0.050
−24
−16
−8
0
8
16
24
32
40
48
Time, h