1 Field sensor for *in situ* detection of marine bacterial biofilms

Novel sensor concept enables time-resolved detection of bacteria from initial attachment to mature cell clusters

By Matthias Fischer, Martin Wahl and Gernot Friedrichs

1.1 Introduction

In marine environments nearly all surfaces become rapidly covered by microorganisms forming a biofilm. Following a biochemical conditioning phase, the biofilm formation starts within the very first minutes when pioneer bacteria cells adhere to the submerged surface {Wahl, 1989}. Marine biofilms cause serious technical problems by settling on ship hulls and their water conduits, navigational equipment, stationary port structures, industrial pipelines and tidal power plants. They cause severe damage by increasing the drag, roughness and friction resistance of submerged objects and accelerate biocorrosion of metals {Tamachkiarow and Flemming, 2003}. In natural ecosystems bacterial biofilms play more differentiated roles by enabling or preventing further biofouling by micro- and macroorganisms and serve as a unique living habitat {Harder, 2009}. Biofilms on the surfaces of marine organisms may substantially change their ecology and well-being {Wahl et al., 2012}. In order to gain insight into biofilm formation kinetics and dynamics, continuous and *in situ* monitoring of biofilm establishment in the marine habitat is desirable but the required temporal and spatial resolution is difficult to achieve. This article briefly outlines the design, development, and field application of a novel bacterial biofilm sensor {Fischer et al., 2012}.

1.2 Biofilm sensor concept

For marine applications, sensor requirements differ considerably from those for highly sophisticated laboratory instrumentation. The aim was to develop a robust and reliable ready-to-use sensor that allows the detection of biofilm formation dynamics *in situ*, on-line and non-destructively in the marine environment. The sensor concept should allow for

autonomous operation with an operational time over several months as well as selective detection of the biofilm, i.e. distinguishing between organic and inorganic material on the surface. Additionally, a sufficient penetration depth is desired to account for the three-dimensional structure of biofilm that typically constitute highly patchy cell clusters of up to several hundred micrometers in diameter {Dalton et al., 1996}. To ensure a representative sensor signal of the inhomogeneous biofilm, a large detection area on the order of a square centimeter is required while keeping a low detection limit and a wide dynamic range to quantify the entire growth range from initially adsorbed bacteria cells up to complex biofilm community. The fact that all organisms contain natural intracellular fluorophores can be utilized for fluorescence-based detection methods as they provide high sensitivity and selectivity, fast response time, and the capability of monitoring large areas *in situ* without sample contact. The natural protein fluorescence of bacteria, stemming for instance from amino and nucleic acids, has long been known as indicator for biomass and metabolic activity {Arrage et al., 1995; Determann, 1998; Sohn et al., 2009}. At wavelengths in the ultraviolet (UV) range, intrinsic protein fluorescence originates mainly from the three aromatic amino acids tyrosine, phenylalanine and tryptophan. Due to a very low quantum yield of phenylalanine and common quenching mechanisms of the emission of tyrosine, the native fluorescence in proteins is dominated by tryptophan. The indole chromophore of tryptophan can be selectively measured by optical excitation at a wavelength of 280 nanometers and detection of the corresponding peak fluorescence centered around 350 nanometers {Vivian and Callis, 2001}.

1.3 Field sensor layout

The portable fiber optic probe for field application is contained in a waterproof housing (12 centimeters in diameter and 40 centimeters in length) that is deployable down to 50 meters water depth. The sensor head consists of a substrate for biofilm establishment, the light source with an excitation filter on top, the collecting fiber optics and a motor-driven cover plate. A UV transparent quartz window equipped with a gas permeable and low fluorescent foil for optimized bacteria growth is employed as settling substrate. The biofilm is back-illuminated and excited through the substrate using a 280 nanometer UV-light emitting diode (LED) in combination with a narrow bandwidth interference filter. Eighteen bundles of 30 optical fibers each are arranged hemispherically and in two rings around the LED for collection of the emitted fluorescence light. The tilt angle of the fibers is optimized for constraining the detected fluorescence volume to a layer close to the surface and for spatially uniform sensing of an effective area of approximately 0.5 square centimeters. At the end of the combined fiber bundles, the collected fluorescence emission is spectrally separated by a combination of two interference filters centered at 350 nanometers prior detection on a

photomultiplier tube (PMT) operating in single-photon counting mode. The timing of the electronics, the readout of the detector, and the data recording on a 1 Gigabyte secure digital (SD) memory card is accomplished by a programmable microcontroller making the sensor package ready for use as a field data logger. A National Instruments Corp. LabVIEW-based graphical user interface (GUI) allows the user to control the sensor settings including the timing and sampling interval via universal serial bus (USB). Either a universal AC adaptor or seven nickel-metal-hydride rechargeable batteries with charge capacities of 4,500 milliampere hours at 1.2 volts could power the biofilm sensor unit.

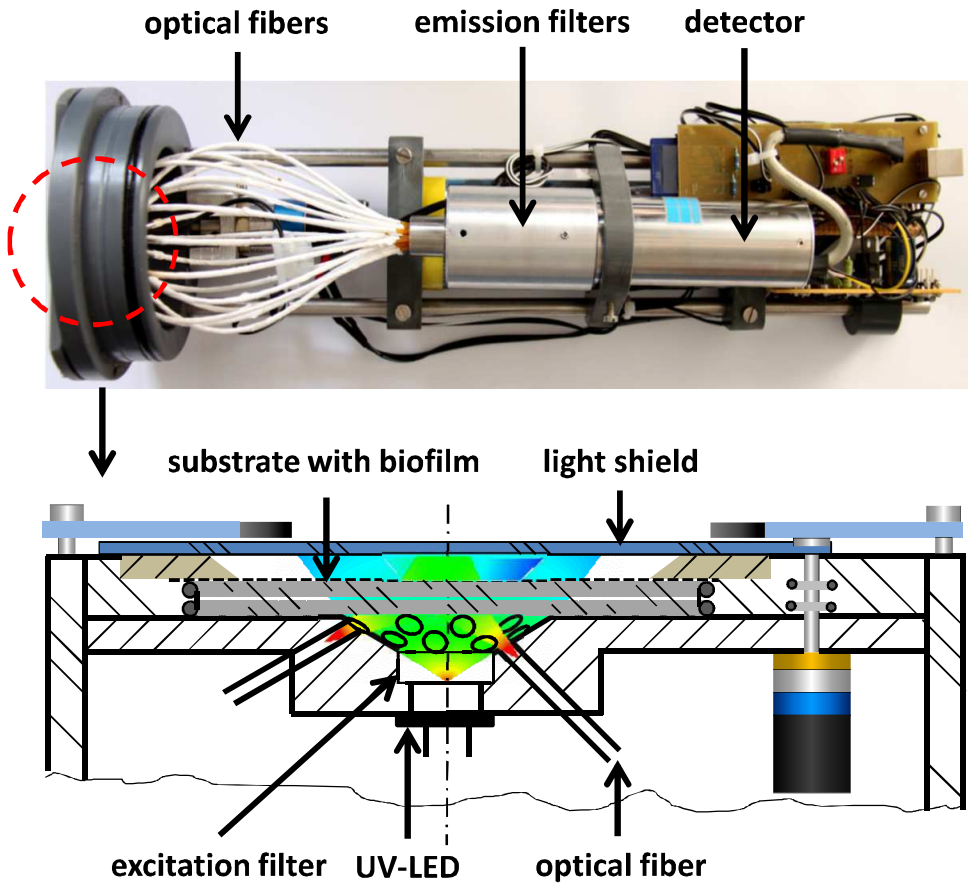

Figure 1.1. Top: A photograph of the developed biofilm sensor without water proof housing. Bottom: Schematic longitudinal cross-sectional view of the cylindrical sensor head with the main components and a color-coded region illustrating the excitation of the UV-LED and fluorescence detection efficiency pattern.

1.4 Biofilm measurement sequence

In order to measure the intrinsic fluorescence intensity of a marine biofilm under field conditions, a light shield in the form of a black anodized cover plate is closed to prevent the incidence of direct sunlight. The remaining background intensity is measured five times and its averaged value is subtracted from the total fluorescence intensity. To ensure a stable light output, the UV-LED is illuminated one second before launching the subsequent biofilm detection sequence. Typically, five fluorescence measurements with integration times of 10 milliseconds each are performed. Supplementary, a high-precision digital thermometer takes a measurement after each detection cycle. The background and total fluorescence intensity values, the setup parameters of the biofilm sensor, the actual time, and the temperature are recorded on the SD memory card. Possible damage to the DNA of the microbial community, which is expected to occur for a prolonged UV exposure {Elasri and Miller, 1999}, is prevented by switching off the UV-LED immediately after the fluorescence measurements. Finally, the light shield opens to allow for biofilm formation under natural light conditions. A complete measurement cycle takes about 12 seconds. Repeated measurements at preset intervals, typically every 15-60 minutes over time periods of several weeks, yield quasi-continuous sampling of the biofilm growth dynamics.

1.5 Detection range

The sensor performance has been tested in the laboratory in April 2010 by placing a UV transparent cell culture dish on the sensor head filled with L-tryptophan solution in artificial seawater. Dilution series in the concentration range of 5×10^{-9} moles per liter $\leq c \leq 1 \times 10^{-4}$ moles per liter yield linear correlations between sensor signal and tryptophan concentration demonstrating the wide dynamic range from the nanomolar to the millimolar range. Further calibration measurements in combination with epifluorescence microscopic counts of bacteria cell numbers with two common marine bacteria strains , namely *Pseudoalteromonas carrageenovora* and *Bacillus subtilis*, and a natural bacterial biofilm community confirmed a low detection limit of about 4000 bacteria cells per square centimeter {Fischer et al., 2012}. Taking into account geometrical factors of the sensor, it can be concluded from these measurements that approximately thirty million tryptophan molecules per cell contribute to the measured signal, which is in good agreement with reported protein content of bacteria {Neidhardt and Umbarger, 1996}.

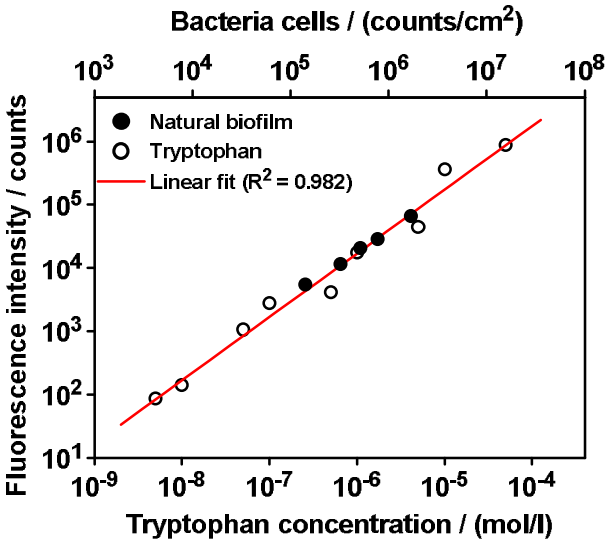

Figure 1.2. Double-logarithmic plots indicating the linear dependence of the fluorescence intensity with tryptophan concentration and bacteria cell number

1.6 Field experiment

The field sensor prototype was applied hourly to quasi-continuously monitor biofilm formation dynamics on a ship cruise in December 2011 about two sea miles south of Hawaii. The sensor unit was installed on deck of the vessel in an outdoor mesocosm with steady exchange of Pacific Ocean water. Over 11 days, the intrinsic biofilm fluorescence intensity was measured together with daily measurements of several physico-chemical water parameters. The average water temperature was 25.2 °C, the salinity was 53.5 millisiemens per centimeter, the pH value was 8.4, and the oxygen content was 5.8 milligrams per liter. Reference settling substrates were placed inside the mesocosm under same hydrodynamic conditions to quantify the corresponding accumulated bacterial cell density of the biofilm. The bacteria were stained by a DNA-binding fluorescent dye, DAPI, and were quantified daily by epifluorescence microscopy. Twenty random images of the substrate were captured, and the bacteria cell numbers were counted by an ImageJ software program {Schneider et al., 2012}. The sensor readouts revealed an exponential trend in marine biofilm growth. Nevertheless, with 2.5 percent, even after 11 days, the area covered by the biofilm remained quite small. Moreover, it has been observed from the microcopy images that the biofilm community of the Pacific Ocean was dominated by bacteria, as the average cell size was only 0.83 micrometers. For a more detailed assessment of the December 2011 experiment, the relative abundance of major bacterial groups was accomplished by fluorescence in-situ hybridization (FISH) {Amann et al., 1990}. Common bacterial oligonucleotide probes revealed that the bacte-

rial biofilm composition was dominated by *Gammaproteobacteria*, which a GAM42a probe {Manz et al., 1996} detected to be about 44 percent of biofilm; *Alphaproteobacteria*, which an ALF968 probe {Glöckner et al., 1999} detected to be about 40 percent; and *Betaproteobacteria*, which a BET42a probe {Manz et al., 1996} detected to be less than 2 percent. Overall, a strong linearity, 0.93, has been found between bacterial cell numbers of the reference subsamples and the biofilm sensor fluorescence data. This also demonstrates that

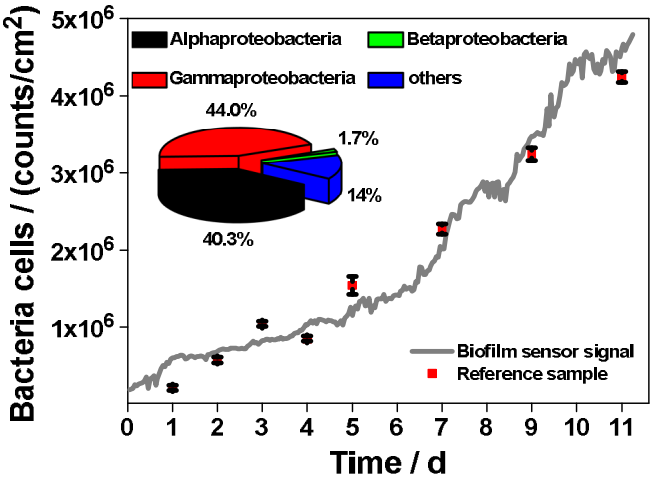

Figure 1.3. Monitoring of bacterial biofilm growth in Pacific Ocean water (solid line). Analysis of bacteria cell density using DAPI-stained subsamples by optical microscopy (squares). Error bars represent standard error means (n = 20) of the bacterial cell density. The inset shows average percentages of dominant bacterial groups.

natural bacterial films in the field, despite their changing and heterogeneous composition, exhibit linear signal response and that tryptophan fluorescence can be used as a universal measure for bacterial abundance. Between 2010 and 2011, other test experiments have been performed in the Baltic Sea with longer settling periods. These experiments yielded a saturation level of bacterial coverage and partly revealed diurnal signal patterns.These results show that interesting new aspects of biofilm formation dynamics wait to be uncovered, and their detailed investigation requires the sensor's quasi-continuous measurement capabilities.

1.7 Conclusion

The developed sensor enables the detection of bacterial biofilms in the marine environment by detecting intrinsic tryptophan fluorescence of bacteria. It has been shown that biofilm fluorescence excited by a UV-LED at a wavelength of 280 nanometers and detected at 350 nanometers reveals a linear relationship between fluorescence intensity and bacteria cell

number. The sensor was calibrated with two marine bacteria strains. The developed sensor enables the detection of bacterial biofilms in the marine environment based on detecting intrinsic tryptophan fluorescence of bacteria. It has been shown that biofilm fluorescence excited by a UV-LED at a wavelength of 280 nanometers and detected at 350 nanometers reveals a linear relationship between fluorescence intensity and bacteria cell number. The sensor was calibrated with two marine bacteria strains. A detection range of approximately four thousand to fifty million cells per square centimeter enables monitoring of biofilms from initial attachment of bacteria cells up to fully developed complex biofilms. The results of the field experiment represent the first quasi-continuous dataset of bacterial biofilm establishment in the Pacific Ocean. Moreover, the flexibility, robustness and sensitivity of the sensor offer a high potential for applications in marine industry, biotechnology, and life sciences. Further upgrades of the sensor will allow simultaneous recording of biomass and metabolic activity of bacteria opening up novel applications.

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