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Title: Early biofouling detection using fluorescence-based extracellular enzyme activity

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Graphical Abstract:

Highlights:

- Biosensor for early membrane biofouling detection based on *in situ* fluorescence measurements
- Extracellular enzyme activity validated with OCT to track biomass accumulation
- *In vitro* platform for fluorogen and fluorogen-substrate characterization
- Robust assay for range of pH and temperature testing

Abstract:

Membrane-based filtration technologies have seen rapid inclusion in a variety of industrial processes, especially production of drinking water by desalination. Biological fouling of membranes is a challenge that leads to increased costs from efficiency reductions, membrane damage and ultimately, membrane replacement over time. Such costs can be mitigated by monitoring and optimizing cleaning processes for better prognosis. Monitoring bacterial accumulation *in situ* can therefore advance understanding of cleaning efficiency. A fluorescence-based sensor for early biofouling detection capable of measuring extracellular enzyme activity was developed and tested in a lab-scale seawater reverse osmosis (SWRO) biofouling model for use in monitoring bacterial accumulation proximal to the surface of a membrane. We tracked bacterial biomass accumulation rapidly and non-invasively using exogenously applied fluorogen-substrates and corroborated with optical coherence tomography imaging of the membrane surface in real-time. The selected fluorogen and fluorogen-substrate were characterized and down selected by high throughput screening *in vitro* for compatibility in seawater and profiled over relevant Red Sea desalination parameters (pH and temperature). This approach demonstrates the practicality of prototyping an early-detection biofouling sensor in membrane

based processes, such as seawater desalination, using extracellular enzyme activity as a measure of bacterial abundance.

Keywords: Biofouling; Biotechnology; Desalination; Enzymes; Extracellular enzyme activity; Fluorogen-substrate

1 Introduction

The use of membrane filtration technologies for water purification has gained popularity with the ever-increasing demand for clean water among industries as well as the general population [1]. As a specification-driven process, membrane filtration technology is now considered as mainstream industrial practice due to reduced footprint and energy requirement while increasing high quality product water. Many water-stressed or arid countries are augmenting their water supply with Reverse Osmosis (RO) membrane filtration for primary desalination in nearly all recently built plants [2]. Desalination is a primary water resource for domestic and industrial supplies in counties such as Qatar, Kuwait, Saudi Arabia, and parts of the USA. Present global desalination capacity is around 100 million m³/d with ~64% of the total capacity produced by membrane processes [3]. A critical cost driver to operation of membrane based desalination is biological fouling, commonly referred to as biofouling. Biofouling is growth on the membrane surface (by bacteria) that leads to reducing the effective membrane area, and therefore decreased flux of permeate [4].

Figure 1 Schematic of bacterial biofilm formation in which bacteria are present in an aqueous medium (Stage 1); initiation of adherence to the surface (Stage 2). Bacteria continue to replicate and grow, forming a protective layer around the immature biofilm (Stage 3). At maturation, the biofilm is recalcitrant to removal and has a propensity to cause fouling while actively releasing colonies to replicate the process (Stage 4). Current detection methods to guide cleaning processes rely on measuring physical parameters (flow impediment, pressure drops) that are indicative of Stage 4 biofilm formation. Developing early detection techniques based on molecular techniques shift the focus closer to Stage 2, resulting in both favorable prognosis of the industrial application and improved cleaning regimens.

Biofilm (bacterial communities) formation is a natural process whereby groups of bacteria adhere to a membrane surface and mature via an encapsulation process as schematically shown in Figure 1. Mature biofilms exhibit anti-bactericidal properties and are often resistant to detachment [5]. When exacerbated, biofilm formation leads to biofouling, which in desalination systems is evidenced by decreased efficiency of water production and/or degradation of the membrane, resulting in increased costs associated with cleaning regiment and shortened membrane lifespan [6]. In current practice, biofouling is mainly detected through physical

measurements such as increased transmembrane pressure or reduced permeate flux through the membrane [7]. Since these parameters are manifest during the mature biofilm stage, biofouling is difficult to remedy and cleaning efficiency is limited. Detection of bacterial activity prior to mature biofilm formation can thus help alleviate recurring costs associated with biofouling and increase productivity of the desalination process.

There are a number of methods capable of detecting bacterial abundance both qualitatively and quantitatively that can be adapted for use in non-invasive and non-destructive biofilm characterization [8]. Briefly, current techniques can be divided into two categories depending on the mode of operation, physical and reporter based detection.

Physical detection relies on observing the surface and monitoring changes therein for initiation of biofilm formation. This approach is embodied mainly in a variety of imaging based systems, such as different light and epifluorescence microscopy techniques, electron microscopy, confocal laser scanning microscopy [9], magnetic resonance imaging (MRI) [10], and more recently optical coherence tomography (OCT) which has gained popularity in visualizing biomass accumulation at the membrane surface [11,12]. The OCT has been both employed to study biofouling development in membrane filtration systems [13] and has been used to evaluate the efficiency of two-phases flow cleaning in a spacer filled channel [14]. Physical detection is limited by the requirement to isolate the area under investigation to a pre-defined field of view, making it difficult to apply in full-scale membrane installations, but is a powerful tool in studying biofouling fundamentals.

Reporter based systems can rely on cellular-level processes such as enzyme based cleavage or receptor-specific binding to generate a measurable signal. This approach has been

leveraged for indirect measurements of bacterial abundance with varying degrees of sensitivity. Flow cytometry, fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR) are highly sensitive methods that can be used to enumerate bacterial numbers but require technical expertise, complicated sample preparation, and relatively long incubation periods (4-6 hours) [15–17] limiting its application in early biofouling detection. Similarly, immuno-based assays and similar receptor based technologies are fast and sensitive, but are limited in application to specific bacterial receptors that the assay is designed to detect with the need to troubleshoot background binding that dampens signal to noise [18].

Enzyme based cleavage of reporter molecules balance between specificity and ease of use while generating meaningful bacterial enumeration in a rapid and sensitive manner [19]. Previously measurements of extracellular enzyme activity (EEA) have been utilized in bacterial enumeration in drinking water [20–22], nutrient turnover at an ecosystems level [23–25], freshwater [26], seawater [27], soil samples [28] and field monitoring of coastal waters [29,30]. The concept of bacterial enumeration via reporter-based methods can therefore be explored as a technique for simple, rapid and scalable monitoring of biofouling in seawater reverse osmosis membrane modules.

The extracellular enzyme activity approach measured fluorescently using exogenously applied fluorogen-substrates may be the most robust technique available today for biofouling monitoring. Sensitivity of this method was further improved by the exchange of chromatographic indicators with fluorophores. These fluorophores when bound to bacterial substrates [19], referred to as fluorogen-substrates, can be applied exogenously to measure bacterial extracellular enzyme activity [31]. By design, the fluorogen-substrate complex contains a quenched fluorogen moiety, which when catalyzed by specific enzymes is liberated and can fluorescence up to 500x

of the quenched signal [32]. Figure 2 shows a graphical representation of the enzymatic process. Both the substrate molecule and fluorogen moiety can in theory be chemically engineered to interact with extracellular enzymes released by pertinent bacteria. This has resulted in the synthesis of a growing library of fluorogen substrates over the past decade [33]. Therefore, a gap exists in rapidly profiling novel fluorogen-substrates in varying operating conditions for quantification of microbes in seawater.

Figure 2 Graphical representation of a fluorogenic substrate cleavage event by extracellular enzyme activity resulting in the release of the fluorescently active fluorogen moiety. While bound to the substrate, the fluorogen moiety is quenched. As the fluorogen-substrate bond is catalyzed by specific enzymes, the fluorogen molecule is liberated and fluoresces. The rate of catalysis is dependent on microbial enzyme concentration, therefore extracellular enzyme activity could be an indirect measure of bacterial abundance.

Fluorogen substrate libraries have not been thoroughly characterized in seawater for requisite incubation period, pH and temperature mainly due to the lack of a fast and reliable assay. The need to characterize fluorogen and fluorogen-substrate is especially important in industrial processes, where non-physiological conditions can exist (extreme temperature or pH). We propose an *in vitro* assay that is sensitive, inexpensive and easy to deploy for screening and characterizing fluorogen and fluorogen-substrates over a range of parameters. This approach was developed using Red Sea water, but can be adapted to other aqueous samples.

The main objective of this study was to identify fluorogen-substrates compatible with Red Sea water and test the feasibility of using fluorescence-based extracellular enzyme activity to develop a sensor capable of monitoring biomass accumulation in a flow cell.

2 Materials and Methods

- 2.1 *Absorbance and fluorescence measurements*- A spectrophotometer, SpectraMax M5e (Molecular Devices, USA), was used to measure absorbance (600 nm) in 96-well polystyrene flat-bottom microplates (Model #3358, Corning, USA) and fluorescence (excitation 355 nm; emission 465 nm) in 96-well flat black microplates (Model #655096, Greiner Bio-One, Austria). Kinetic measurements, data collected at set intervals over a period of time, were used to measure each well of the plate temporally. Data was recorded using SoftMax Pro Data Acquisition Software (Molecular Devices, USA). Graphs and statistical analysis were generated in GraphPad Prism (GraphPad Software Inc., USA).
- 2.2 *Water samples and reagents*- Unfiltered Red Sea water was collected locally to the KAUST Thuwal Campus. All water samples were collected and used same day. Sterile yeast extract was added as nutrient supplement (62.5 mg/L) and incubated for 24 hours 30°C without agitation. Flow cytometry was used to estimate bacterial cell counts and correlated with absorbance measurements using the BD Accuri™ C6 (BD Biosciences, USA) as described previously [34]. For all samples, an absorbance of 0.08 ± 0.02 correlated to an average of 1.6×10^5 bacterial cells per 50 μ L. Dilution matrices of both seawater and drinking water were prepared in which samples were boiled for 15 minutes, acclimated to room temperature and filtered through a 500 mL 0.22 μ M Vacuum filter unit (model #431097, Corning, USA). Two fluorogen standards, 4-methylumbelliferone (MUF) and β -Naphthylamine (β -NA), and two fluorogen-substrates, 4-Methylumbelliferyl Phosphate (MUF-Phosphate) and 4-Methylumbelliferyl N-acetyl- β -D-glucosaminide (MUF-nabdG) were purchased from Sigma-Aldrich as lyophilized powder, reconstituted as

per manufacturer instructions and aliquoted into sterile black micro centrifuge tubes. Fluorogen standards were stored at 4°C while fluorogen-substrates were stored at -20°C.

- 2.3 *Fluorogen stability testing under light conditions*- Photoreactivity testing of fluorogens diluted in the seawater dilution matrix were performed in polystyrene plates at ambient temperature over a period of 24 hours. Freshly prepared fluorogen standards were added to 4 mL of seawater dilution matrix, at different time points, into wells of 6-well plate (Model #3506, Corning, USA) and covered with the polystyrene plate lid. Plates were subsequently placed inside a chamber with and without exposure to light. All samples were collected and measured for fluorescence in triplicates.
- 2.4 *Titration of bacterial inoculum using constant fluorogen-substrate*- Titrations of bacterial inoculum using constant concentrations of fluorogen-substrate were setup in 96-well black flat-bottom microplates (Supplementary Figure 1). Briefly, 100 µL of dilution matrix were added to all wells of rows B - H. Water samples were added (200 µL/well) starting at A1 to A10. In this manner, 5 samples were tested in duplicate for each titration. 200 µL of fluorogen standard were added to wells A11 and A12. Using a multi-channel pipette, 100 µL from row A were serially diluted two-fold to row B, repeating until row G and discarding the final 100 µL. Wells H1 - H10 were negative controls containing no bacteria. For assay calibration, columns 11 and 12 were used to generate fluorogen standard curves. Wells of H11 and H12 control for background fluorescence in the dilution matrix. Prior to microplate read, 10 µL of fluorogen substrate were added to columns 1 - 10.
- 2.5 *Titration of fluorogen-substrates with constant inoculum*- Two 96-well microtiter plates, Sample Read Plate and Fluorogen-Substrate Dilution Plate, were setup as follows:

(A) Sample Read Plate were setup bacterial titration plate described previously, with the following modification. Aliquots of standardized water inoculum (100 μ L per well) instead of dilution matrix were added to Columns 1 – 10 and stored at room temperature until use.

(B) Fluorogen-Substrate Dilution Plate were setup by filling 25 μ L of dilution matrix in all wells of Columns 1 – 10, except Row A. Fluorogen-substrate at 10X concentration, prepared in dilution matrix, were aliquoted (50 μ L / well) to Wells A1 – A10. Fluorogen-substrates were serially diluted two-fold 25 μ L from Row A to Row G, discarding the last transfer. Final volume of each well is 25 μ L.

Once prepared, 10 μ L from each well of the Fluorogen-Substrate Dilution Plate was transferred to the corresponding well of the Sample Read Plate. Fluorescence measurements were started immediately after completion of the transfer.

2.6 *Profiling pH and temperature effect on fluorogen signal intensity and extracellular enzyme activity*- For pH profiling, aliquots of water samples (dilution matrix for fluorogen standard and standardized inoculum for fluorogen-substrate testing) were adjusted to pH (7 – 10) using 1M CaCO₃. The pH-adjusted samples were transferred to wells of a 96-well plate, at which point either fluorogen standard or fluorogen-substrates was added and fluorescence measurements recorded. For temperature profiling, dilution matrix or standardized inoculum were aliquoted into micro centrifuge tubes and acclimated to the desired temperature using dry baths (ThermoFisher Scientific, USA). As appropriate, fluorogen or fluorogen-substrate of the corresponding temperature were added to samples. Samples were removed after the incubation period, transferred to wells of a 96-well plate and measured for fluorescence signal.

2.7 *Configuration of the lab scale biofouling study*- A lab scale setup was operated using a membrane founding simulator (MFS) using recirculated Red Sea water as described by Fortunato and Leiknes [35] with the following modifications. Three-way valves were fitted immediately upstream and downstream of the MFS for fluorogen-substrate injection and subsequent sample collection as shown in Figure 3. 5 mL aliquots of MUF-Phosphate were prepared in luer lock syringes, stored at -20°C and used after thawing to room temperature. At each sampling time point, the inlet and outlet for the MFS was closed, MUF-Phosphate (5 μ M final concentration in system) was injected into the MFS and incubated for 15 minutes. After incubation, ~2 mL was removed via the sampling port syringe for fluorescence testing. At this point the system was flushed with Red Sea water through the injection and sampling ports before reopening the inlet and outlet valves and restarting the system.

Figure 3 Schematic representation of the extracellular enzyme activity sensor, consisting of a modified fouling simulator with injection and sample port fitted with a three-way valve. A probe integrated with video camera housing the optical coherence tomography unit was used to collaborate biofilm development in time. At each extracellular enzyme activity sampling point, the flow from the inlet and outlet is halted while fluorogen-substrate is injected via the injection port and incubated inside the fouling simulator. After incubation, the sample is removed via the sample port for fluorescence measurements. The system is flushed and restarted. Total sampling time is 20 minutes.

2.8 Optical Coherence Tomography (OCT) Imaging- Simultaneously, a spectral domain OCT system device Ganymede II from Thorlabs, GmbH (Germany) armed with the scan lens (LSM 03BB) was used to assess the fouling development non-invasively in the flow cell. The OCT probe was mounted on the MFS for continuous monitoring in a confined area.

Scans were acquired periodically for the whole period of observation (84 hours). The cross-sectional scans (1557 x 613 pixel) corresponded to 6.23 mm x 1.80 mm (width x height). The OCT scans were processed with Fiji software. The images were filtered to reduce the noise. Then the contrast and brightness were adjusted. A false color lookup table was applied for visualization purposes.

2.9

3 Results

Early biofouling detection can be a key parameter in reducing the cost associated with membrane based desalination by guiding membrane cleaning processes. In this study, fluorogen-substrate cleavage was explored as a method to enumerate bacterial abundance in seawater and tested in a lab scale membrane biofouling model. Determination of the effect of matrix on fluorogen molecules was assessed in drinking water and Red Sea water. Fluorogen, fluorogen-substrates and standardized Red Sea water inoculums were characterized *in vitro* to assess:

- Concentration dependence and speed (rate) of catalysis of fluorogen-substrates by Red Sea water bacterial inoculums;
- Effect of pH and temperature on fluorogen signal intensity and fluorogen-substrate cleavage;
- Correlation of fluorescence signal intensity to concentration of bacterial inoculum based on MUF-phosphate cleavage.

Lab scale experiments were performed with MUF-Phosphate to track bacterial abundance in a MFS. Fluorescence signal resulting from enzymatic cleavage of the fluorogen-substrate was monitored every 6 hours over a period of 84 hours. Simultaneously, visualization of the membrane surface by OCT was conducted every 12 hours to confirm progression of biofouling.

3.1 Fluorogen stability dependence on water type

Fluorogen stability and signal intensity vary based on water type and are thus important to characterize before screening fluorogen-substrate libraries. Photoreactivity of two fluorogens,

MUF and β -NA were observed in seawater dilution matrix over a period of 24 hours (Figure 4A). Differences between fluorogen signal intensity with and without light exposure were either negligible (β -NA) or slightly higher in light protected samples (MUF). To assess inhibitors and quenching in seawater, MUF (0 – 240 μ M) was characterized in seawater every five minutes for one hour as shown in Figure 4B. Signal intensities for similar concentrations were higher in seawater than in drinking water, demonstrating the impact of matrix on fluorogen intensity. Although signal intensity varied with matrix composition, stability of the signal remained constant over the monitoring period, pointing to no inhibition or quenching over time in the seawater matrix.

Figure 4 Fluorogen fluorescent signal profile with and without exposure to light for two fluorogen standards, 4-Methumbelliferone (MUF) and β -Naphthylamine (β -NA), over 24 hours (A). MUF was assessed over a range of concentrations for signal inhibition in seawater (B). Mean was plotted with error bars representing range.

3.2 *Fluorescence intensity impact of concentration, pH and temperature for two fluorogen-substrates in seawater inoculums*

Two fluorogen-substrate molecules, MUF-Phosphate (0 – 200 μM) and MUF-nabdG (0 – 80 μM), were titrated two-fold in seawater standardized inoculums over 60 minutes (Figure 5). Concentrations of MUF-Phosphate ≥ 25 μM are rapidly cleaved to liberate the fluorogen product, exceeding detection limit within 30 minutes. MUF-nabdG signal intensity increased linearly over the hour-long measurement across the concentrations measured. For both fluorogen substrates tested, signal intensity correlated to concentration of fluorogen-substrate whereby increased concentration led to higher signal intensity within a shorter time frame. This result is important for practical reasons as a balance between decreasing sampling time and reducing cost associated with increased fluorogen-substrate required need to be maintained.

Figure 5 Titrations of two fluorogen-substrates, MUF-Phosphate (0 – 200 μ M) (A) and MUF-nabdg (0 – 80 μ M) (B) in drinking water (left) and seawater (right) using standardized bacterial inoculums. MUF-n-a-b-d-glucosaminide exhibited almost no signal in drinking water as opposed to seawater, indicating the necessity to screen in the water of interest. Mean was plotted with error bars representing range. Note scale of (B) Drinking Water

Florescence properties of the fluorogen moiety and enzyme catalytic rates are impacted by pH and temperature shifts. Fluorogen signal intensity was tracked for MUF, MUF-Phosphate and MUF-nabdG to assess the effect of pH and temperature (Figure 6).

Figure 6 MUF-Standard, MUF-Phosphate and MUF-nabdG were profiled over a range of pH (A) and temperature (B) in seawater standardized samples at a single concentration. Both pH and temperature have an effect on signal intensity. Fluorogen intensity for the standard generally increased across the tested pH and temperature ranges.

Fluorogen-substrates, MUF-phosphate and MUF-nabdG, exhibited different optimums for both variables. All measurements were taken following 30 minutes of incubation. Mean was plotted with error bars representing range.

Fluorescence signal measurements were conducted for a pH range of 7-10 and temperature range of 25-55°C. Although the range of pH and temperature tested are not seen in reverse osmosis desalination, it is possible for ambient parameters to reach these levels in other industrial applications. Fluorescence signal intensity of MUF doubled over both pH and temperature ranges tested. Measurements of the two fluorogen substrates demonstrated pH dependence in line with pH optimums previously reported of enzymes [36–38]. Seawater standardized inoculums were used to measure MUF-Phosphate and MUF-nabdG, showing signal optimums at pH 9 and 8 respectively. MUF-nabdG signal significantly dropped after pH 9, indicating either the enzyme responsible for MUF-nabdG cleavage or the active site of the fluorogen-substrate was denatured by the pH shift. Increase of temperature from 25 to 55°C correlated to an increase of fluorescence signal for MUF-Phosphate from 1800 to 7500 RFU (+400%). Meanwhile, the fluorescence signal from MUF-nabdG increased from 1800 to ~4500 RFU (+250%) as temperature increased from 25 to 45°C. A drop of 50% was measured when temperature increased from 45 to 55°C. Temperature dependence is a known factor of enzyme catalytic rates, typically increasing as temperature rises to an optimum [39,40]. Increases in temperature past the optimum result in molecular destabilization, leading to decreased enzymatic activity. The aim was to demonstrate the versatility of the assay to rapidly assess pH and temperature impact over a range of values.

3.3 Extracellular enzyme activity; an indirect measure of bacterial numbers tested *in vitro* and in a lab scale biofouling model

Fluorogen liberation as a result of enzymatic activity provides an indicator for the determination of relative concentration of bacteria. Bacterial concentration and fluorescence signal measured *in vitro* at 15 and 30 minutes are shown in Figure 7. A standardized seawater inoculum was titrated two-fold from 100 to 1.5%, with 0 as seawater without bacteria. These samples were tested with MUF-Phosphate at 5 μM . At each time point, the fluorogen signal correlated to percent bacterial inoculum, indicating a linear relationship between bacteria present and signal generated. As bacterial inoculum titrates down, fluorescence signal also decreases due to the reduction in concentration of bacterial enzymes.

Figure 7 Seawater bacteria inoculum fluorescently measured at 15 and 30 minutes with MUF-Phosphate (5 μM).

Incubation time impacts the fluorescence signal intensity and hence can be modulated to fit a range of application requirements. Mean was plotted with error bars representing range.

A lab scale biofouling model was operated while measuring EEA and capturing 2D cross-sectional OCT scans of the monitored area over time (Figure 8). Fluorogen-substrate were sampled every 6 hours and OCT images were captured every 12 hours to determine progression of biomass accumulation. Initiation of biomass deposition was noted from 12 hours onward by both increasing EEA-based fluorescence signal and representative OCT scans of the flow cell. As biomass deposits, fluorescence signal resulting from fluorogen-substrate cleavage increases from baseline linearly over 36 hours and then exponentially after 36 hours, indicative of rapid fluorogen liberation. Extracellular enzyme activity fluorescence measurements at 60 hours (~20,000 RFU) and 84 hours (~30,000 RFU) correlate to OCT 2D cross-sectional scans at 60 and 84 hours depicting enhanced biomass accumulation, specifically on the feed spacer.

Figure 8 Results of biofouling tracking using Extracellular enzyme activity (EEA) (A) and Optical Coherence Tomography (OCT) imaging (B). EEA was measured using fluorescence based cleavage of MUF-Phosphate at discrete sampling points. The fluorescence signal increased as bacterial biomass accumulates inside of the fouling

simulator. Representative OCT images from the fouling simulator surface are shown on the right. White arrows are representative and indicate biomass accumulation on the membrane feed-spacer.

4 Discussion

4.1 *In vitro* profiling of fluorogen and fluorogen-substrates

Selecting fluorogen-substrate for use in seawater biofouling, or other applications, requires the use of a rapid screening platform. Characterization of the fluorogen moiety and screening suitable fluorogen-substrate target entails standardizing an inoculum that is consistent and replicable in a short period of time. Inoculum standardization is a difficult step in assay development as sample source, collection time and composition directly affect bacterial communities present. This requires measuring samples multiple times longitudinally to minimize artificial results and warrant appropriate comparison. A clear advantage of screening in microtiter plates is multiple samples can be measured in a single experimental plate, complete with controls for sample and microenvironment consistency. This feature is critical for deriving insights for the variables being tested. Sample and microenvironment consistency are more so important when working with environmental samples, as small variability inevitably arise due to sampling time, source and environmental heterogeneity [41]. Fluorogen-substrates were tested in standardized bacterial inoculums to measure signal over a range of incubation periods, pH and temperature. During the process, we refined a simple assay to test the seawater matrix, characterize fluorogens and fluorogen-substrates and measure extracellular enzyme catalysis of fluorogen-substrates in 96-well microtiter plates.

Fluorogen stability in seawater was also assessed for signal inhibitors or quenchers, as well as potential degradation during manipulations in ambient lighting. Within the methodological context of the microtiter plate assay, there were no significant bleaching or deterioration in seawater or by exposure to light over 24 hours. Although the literature suggests rapid fluorogen degradation should occur in the presence of light [42], this phenomenon was not observed while assessing the fluorogens MUF and β -NA in polystyrene plates covered by the polystyrene lid. We believe that the polystyrene protected the fluorogen moiety from degradation, therefore it is important to assess photodegradation if other material types are employed.

MUF-Phosphate and MUF-nabdG signal in seawater was measured over time using a standardized bacterial inoculum to gauge enzyme functionality and catalytic speed. MUF-Phosphate was cleaved rapidly (< 15 minutes) as compared to MUF-nabdG (> 15 minutes), evidenced by fluorogen liberation and subsequent fluorescence signal intensity. Concentrations of MUF-Phosphate greater than 25 μ M exceeded machine detection limits within 30 minutes. This is an important consideration when determining two requirements for the final application; time availability and fluorogen-substrate required. Time constricted measurements, such as in monitoring of real-time processes, benefit from fluorogen-substrates with fast catalytic activity to minimize or negate off-line time associated with sampling. Understanding concentration of fluorogen-substrate to signal strength permits utilizing fluorogen-substrate concentrations to optimize the “measurement window”. The “measurement window” can be considered the entire time duration required to sample the system (pre-sampling preparation, sampling and post-sampling). If lengthy or advanced sample handling or manipulation is required, lowering the concentration of fluorogen-substrate prolongs the time in which measurements are within detection limits. Conversely, raising fluorogen-substrate concentrations shrink the measurement

window resulting in faster signal generation. Modulating the fluorogen-substrate concentration impacts the measurement window and can best be determined by calibration and assessing application requirements.

Since pH and temperature are critical parameters that are application-specific, a robust method to test fluorogen and fluorogen-substrate cleavage in different pH and temperature conditions to gauge impact on signal intensity and enzyme kinetic rates was established. Signal intensity of the MUF fluorogen moiety increased with basification two-fold as pH increased from 7 to 10, in line with previous reports for similar compounds [43]. The fluorogen moiety is affected independently of the fluorogen-substrate cleavage, which depends on the pH and temperature optimums of the enzyme responsible and is therefore required to be assessed independently. Temperature increases resulted in similar trends for the fluorogen MUF. This trend was not observed for MUF-Phosphate and MUF-nabdG signal intensities. Enzyme and substrate are proteins and hence stability is contingent on pH and temperature, which cause conformational changes at the active sites – promoting or restricting substrate binding or denature the enzyme, diminishing catalysis and thereby leading to decreases in fluorescence signal [36]. Variability between optimums for pH and temperature are also expected as different enzymes are responsible for cleavage of the two fluorogen-substrates tested. MUF-Phosphate signal intensity peaked at pH 9 and 55°C and MUF-nabdG signal intensity peaked at pH 8 and 45°C across the tested range. Characterization of both the fluorogen and fluorogen-substrate profile is important in determining suitable candidates for downstream application. Understanding the ambient conditions of the final application is therefore important to designing the assay for assessing both fluorogen intensity and enzyme catalysis. Although not within the scope of our work, the generality of the method permits testing additional variables, such as ionic

concentration or metallic ion effect, on fluorogen intensity and enzyme activity in the matrix of interest. Industrial processes occur in both natural and artificial conditions and therefore a quick method to test ambient parameters for fluorogen stability and enzyme activity is essential.

4.2 *Biofouling study using fluorogen-substrate cleavage in a modified membrane fouling simulator*

Previous studies have demonstrated the feasibility of using EEA measurements for assessing biomass abundance in drinking water systems [20]. In this study, *in vitro* assay results indicate that bacterial titrations can be measured within 15 minutes using MUF-Phosphate in seawater. We therefore adapted this method for a lab scale monitoring of biofouling in a MFS. As described previously, the MFS is representative of industry standard spiral-wound membrane modules used in the desalination of saline water [44]. Our modifications included the introduction of an injection port upstream and a sampling port downstream of the MFS. Due to the diffusive nature of both bacterial enzymes and the fluorogen-substrate molecule, it is possible to assess the state of the entire membrane module. We operated this system using Red Sea water to assess biofouling over time.

To corroborate our method of tracking biofouling using EEA, OCT was chosen from amongst other imaging techniques since it is the most robust technique for monitoring biofilm growth on-line, *in situ*, and non-invasively in a membrane based flow cell. Although Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) have higher resolution and have been classically used, both techniques have limitations when used *in situ*, often necessitating destructive imaging *ex situ*. Moreover, both SEM and CLSM require the use

of staining or contrast agent, which risk altering the biofilm morphology [45]. We therefore utilized OCT to acquire 2D cross sectional scans of the membrane surface to track biofouling and validate the EEA signal.

Our results indicate that EEA based on fluorescence is comparable to OCT in tracking biomass accumulation in the MFS. Fluorescence signal intensity rose linearly at the early bacterial attachment phases, rising rapidly as the biomass accumulated. This can be associated with the increase in bacterial enzyme production as bacterial abundance increases. In Figure 8, we observe early biomass deposition on the feed spacer using the OCT, which is in agreement with previous studies [46]. Theoretically, fluorescence-based EEA signal increases are not dependent on where bacteria deposit, demonstrating the practicality of using EEA to monitor biomass accumulation across the membrane module in seawater processes.

4.3 Challenges and future direction

Limitations of this method must be considered before implementation in pilot scale setups. At a fundamental level, further studies on bacterial regulation of extracellular enzymes are required as enzyme production can be regulated by a variety of factors such as bacterial growth phase, metabolic and physiological conditions [47]. Bacterial populations under stress might also produce proportionally more or less enzymes of a specific type than other populations [48–50], leading to potential over- or underestimation of bacterial activity.

The goal of this technique is to transition to practice, where it can be used as a guide to improving cleaning processes. Extracellular enzyme activity as a qualitative measure of bacterial

abundance has application in a number of industrial processes where monitoring of deviations from steady-state levels of bacteria are important. The seawater desalination process is a prime example in which background levels of bacteria require monitoring to prevent growth leading to operational disruption. With further lab scale testing, we postulate fluorogen-substrate cleavage can be aptly tailored as a supplementary tool to qualitatively assess bacterial load in industrial practices where relative monitoring, but not exact quantification, can reduce the overall cost associated with biofouling.

Conclusion

In this study, we developed a biosensor for biofouling detection based on assessing enzymatic cleavage of a fluorogen-substrate molecule. In addition, an *in vitro* assay for profiling fluorogens and fluorogen-substrates interactions in water and over a range of pH and temperature was elucidated. We conclude that:

- A biosensor capable of detecting extracellular enzyme activity measured by fluorogen-substrate cleavage can monitor membrane based biofouling;
- Fluorogen stability of the MUF was not affected by Red Sea water or exposure to ambient light within the context of the assay, making the screening approach practical for bench scale experimentation;
- Fluorescence signal intensity for both fluorogen and enzymes responsible for fluorogen-substrate cleavage were impacted by pH and temperature.

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