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High-throughput method development for *in-situ* quantification of aquatic phototrophic biofilms

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ABSTRACT

In the maritime field where biofouling has both economic and environmental impacts, *in situ* quantification methods of biofilm development are of outstanding importance. Indeed, it is challenging to temporally monitor biofilm formation due to the complexity of the marine ecosystem, common inaccessibility of sampling location and lack of standardized techniques. Here, an artificial polymeric surface was tested *in situ* and *in vitro* against natural phototrophic biofilms and monoculture biofilms using plate reader fluorescence. The suitability of the developed method was verified using fluorescence microscopy coupled with image analysis - a common quantification technique - demonstrating a strong correlation between the tested methods. The results indicated the efficiency of inherent chlorophyll fluorescence in quantifying undisturbed phototrophic biofilms in field and laboratory conditions using microplate reader. This work demonstrated that the suggested approach is promising for biofilm high-throughput testing, and therefore has the potential to be used in several research and industrial sectors for monitoring phototrophic biofilm development.

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Phototrophic biofilm; biofouling; microalgae; diatoms; chlorophyll fluorescence; quantification bioassay; fouling control


Introduction

Aquatic phototrophic biofilms are mixed microbial conglomerations formed and attached to submerged solid surfaces, typically composed by light-driven autotrophs and heterotrophs that are surrounded and stabilized by self-producing extracellular polymeric substances (EPS) (Hoagland et al. 1993; Cooksey and Wigglesworth-Cooksey 1995; Landoulsi et al. 2011). In a nutshell, at the top biofilm phototrophic layer, oxygenic photoautotrophs are prevailing, whilst the internal part of the biofilms consists of heterotrophs (bacteria, protozoa, fungi) and anoxygenic phototrophs (Roeselers et al. 2007; Bharti et al. 2017). Oxygenic photoautotrophs (primary producers) primarily consist of diatoms, green algae, and cyanobacteria that possess photosynthesizing components enabling them to use light energy and reduce carbon dioxide, thus producing oxygen and organic substrates (Roeselers et al. 2007, 2008).

In industries where the investigation of biofilm development is of great interest, high-throughput

quantification tools are fundamental for assessing the performance of novel fouling control material. Phototrophic biofilm quantification is important in a wide range of research and industrial applications, such as wastewater treatment (Schumacher et al. 2003), aquaculture (Bender and Phillips 2004), conservation of cultural heritage (Sasso et al. 2016), antifouling industry (Salta et al. 2013), etc. Marine phototrophs constitute the first surface colonisers along with bacteria, leading to the marine biofouling problem (Characklis 1981; Callow and Callow 2002; Anil et al. 2006). The uncontrolled biofilm growth on surfaces can interfere with their performance (Luo et al. 2018), for example biofouling greatly impacts the efficiency level of antifouling coatings (Molino and Wetherbee 2008; Papadatou et al. 2021). Moreover, phototrophic biofilms commonly possess specific features, such as active metabolites (Bhadury and Wright 2004) that could significantly affect marine biofouling, and consequently constitute attractive candidates for further exploration in many biotechnological applications. Hence, in order to assess the

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biofilm development on biofouling-impacted artificial surfaces, such as fouling control coatings, it is essential to monitor *in situ* biofilm formation over time.

Biofilm quantification can be employed with a variety of techniques depending on the research objectives, budget, and instrument availability. Among the biofilm monitoring techniques, the non-invasive approaches are of great importance for the reflection of the spatial and heterogenous undisturbed environmental biofilm structure consisting of phototrophic and heterotrophic microorganisms (Barranguet et al. 2004). Additionally, *in situ* methods are of principal importance as they generate real-time information and set the basis for effective control (Wolf et al. 2002), however, field studies are greatly lacking (Briand 2009). Phototrophic biofilms have the advantage of possessing chlorophyll α (chl- α), a green pigment essential for photosynthesis (Woodward et al. 1960; Jeffrey and Humphrey 1975) that has been widely used as a biomass biomarker (Cattò and Cappitelli 2019) for label-free and non-destructive detection (Serodio et al. 1997; Cerovic et al. 2002). The estimation of intrinsic chlorophyll fluorescence (ChlF) provides a direct implication of the photosynthetic activity in chlorophyll-containing cells and has been used as a proxy for cell number estimation (Best et al. 2016). Intrinsic natural auto-fluorescence could be examined by both spectrophotometric and microscopic measurements (Cattò and Cappitelli 2019).

The conventionally used fluorescence-based microscopy methods in biofilm studies including confocal laser scanning microscopy (CLSM) and epifluorescence, provide quantitative and qualitative information (Mosleh et al. 2012), while allowing the non-disruptive visualisation of young aquatic phototrophic biofilms (Lawrence et al. 1998; Eggert et al. 2006). CLSM is an optical high-resolution microscope coupled with a laser beam that provides time-resolved three-dimensional images of the biofilm structure (Lawrence and Neu 1999; Mueller et al. 2006) and the capacity to simultaneously explore different biofilms components, using fluorescent dyes to target bacterial DNA or EPS, and the intrinsic autofluorescent pigments for phototrophs (Barranguet et al. 2004). However, CLSM is specified but not commonly available equipment (Salta et al. 2018), that requires considerable sample preparation (Fabbri et al. 2018) and often expensive generation of results. Epifluorescence microscopy is a method for *in situ* microbial quantification with the application of fluorescent tags (Nadeau et al. 2008) or exploitation of autofluorescent pigments, that has been extensively used to study

different biofilm structures (Amann et al. 1992; Surman et al. 1996; Clarke et al. 2010). Another traditional method to manually determine planktonic microalgal concentration is cell counting using light microscopy, although this technique could be laborious, susceptible to misuse of the counting chamber, dependent on the experience of the operator, and impractical for dense aggregates (Takahashi 2019). Plate readers constitute standard high-throughput infrastructure equipment in research laboratories, applied to facilitate spectrophotometric and fluorescence measurements inexpensive (Warren 2008; Salta et al. 2018), often with faster and more reliable results compared to direct microscopic counting (Camps et al. 2011).

A simple, nonintrusive, fast technique was developed by Carreira and colleagues (Carreira et al. 2015) to explore the distribution of several benthic phototrophic groups based on different autofluorescence excitation spectra. The designed custom-made method was successfully applied for laboratory cultures (diatom; *Nitzschia capitellata*, cyanobacterium; *Geitlerinema* sp.) and natural microbial mats using an imaging system (excitation for diatoms; blue 470 nm, cyanobacteria; amber 600 nm) that enabled discrimination between the two phototrophic groups. Best et al. (2016) reported a novel ChlF-based method for screening microalgae and cyanobacteria using a fabricated microdroplet (microfluidic) platform. Two cyanobacteria (genus *Synechocystis*, *Synechococcus*) and a green alga (genus *Chlamydomonas*) were monitored through ChlF measurements encapsulated in droplets. The recommended label-free detection approach was directly associated to the physiological properties of each single-cell and displayed applicability of ChlF in picolitre microdroplets for different cyanobacteria and microalgae strains. Warren (2008) established a robust, rapid approach to assess chlorophyll using microplate reader. Chlorophyll obtained from eight different plant families (Acacia, Allium, Araucaria, Avicennia, Eucalyptus, Gossypium, Marsilea, Schleggera) was extracted with methanol, following measurement of chlorophyll with microplate and spectrophotometer. The results indicated a strong linear relationship between the two methods for chlorophyll concentration estimation.

The utilisation of plate readers has been successfully adapted for laboratory assessment of bacterial (e.g. Stafslie et al. 2006, 2007) and microalgal (e.g. Cassé et al. 2007a, 2007b) biofilm attachment. Stafslie et al. (2006) developed a fabricated temperature-controlled, circulated water bath incubator tank.

The novel high-throughput method based on crystal violet staining and plate reader absorbance measurements, enabled the assessment of marine bacterial biofilm growth. Effectiveness and versatility of the bioassay was then tested for assessment of bacterial biofilm retention (*Pseudoalteromonas atlantica*, *Cobetia marina*, *Halomonas pacifica*, *Cytophaga lytica*) on antifouling surfaces, and demonstrated to be successful in the primary screening of antifouling coatings for identification of promising candidates (Stafslien et al. 2007). Cassé et al. (2007a) constructed a water-controlled spinjet apparatus with variable pressure (application of hydrodynamic forces) to facilitate biomass quantification of *Navicula perminuta* diatom and *Ulva linza* macroalga sporelings using fluorescence plate reader, and cell adhesion strength using epifluorescence microscope. The laboratory bioassay facilitated the screening of the adhesion strength of algae that allowed evaluation of novel fouling-release coatings. Cassé et al. (2007b) established an algorithm to quantify the algal (*Ulva linza* zoospores) biomass coverage from coatings using digital image analysis for visual assessment, which proved to be reproducible semi-high-throughput for characterising coatings performance.

Salta et al. (2018) developed a combinative bioassay to assess *in situ* the performance of natural product- and biocide-containing coatings against bacterial biofilm formation (*Cobetia marina*) using microplate reader and CLSM with nucleic acid staining coupled with COMSTAT analysis. The developed method reported comparable and repeatable results between the two methods and demonstrated the natural product (NP)-based coatings to exhibit good antifouling efficacy and inhibition of initial biofilm attachment and growth. Fabbri et al. (2018) designed a novel flow cell to assess marine biofilms on biocidal antifouling coatings under shear flow using optical coherence tomography (OCT). The combination of biofilm flow cell and OCT successfully allowed screening for coating-specific differences in the tested biofilms. Le Norcy et al. (2019) developed a method to characterise microalgal biofilms using CLSM combined with flow cells, and multi-well plate assays by microplate reader. The results displayed the ability of model microalgae (*Cylindrotheca closterium*, *Porphyridium purpureum*) to form biofilms in the tested flow-cells and showed that the reference microplate reader method confirmed the flow-cell (CLSM) findings.

Taken together, a powerful biofilm quantification method should be reproducible, rapid, accurate, cost-effective, non-destructive, and simple (Donlan 2001;

Olwal et al. 2017). However, there is a lack of consensus for a standard phototrophic biofilm quantification technique, which might impede the comparison and lead to discrepancies between different biofilm studies (Olwal et al. 2017; Corte et al. 2019). In light of these considerations, the principal aim of the present study is to deliver a rapid method for *in situ* quantification of non-destructive marine biofilm-forming phototrophs. Phototrophic biofilms were *in vitro* and *in situ* monitored through ChlF estimation using fluorescence microscopy and microplate reader. The purpose of the developed low-cost and high-throughput bioassay is to support applications with primary interest in the initial phototrophic biofilm colonization of artificial surfaces, ranging from laboratory assays using model diatom strains to field tests of mixed photosynthetic communities.

Materials and methods

In vitro laboratory assays

Phototrophic biofilm-forming microalgae strains

The marine microalgal strains *Cylindrotheca closterium* (AC-170) and *Nitzschia thermaloides* (AC-712) were obtained from Algalbank Caen, Microalgal Culture Collection of Caen Normandie University. *Cylindrotheca* is a well-known microfouling candidate (Briand 2009; Briand et al. 2012; Le Norcy et al. 2017), regardless the hydrodynamic treatment, i.e. static/dynamic immersion (Zargiel and Swain 2014) with the capacity of strong adhesion and thick biofilm formation on artificial surfaces (Le Norcy et al. 2019). *Nitzschia* was identified as one of the major fouling diatoms during several studies (Beveridge et al. 1998; Briand 2009; Zargiel et al. 2011; Muthukrishnan et al. 2017), with some cases only being observed under static conditions (Zargiel and Swain 2014). Both diatom strains belong to the family Bacillariophyceae, commonly involved in colonisation of marine surfaces. The selected model strains constitute rapid-growing organisms that allow experimental design of replicated treatments within reasonable timescales.

Culturing conditions and planktonic growth rates

Microalgal strains were cultivated in sterile filtered artificial seawater (35 g/L Sea Salts, Instant Ocean, Aquarium Systems, St. Blacksburg, VA, USA), supplemented with 2% Guillard's F/2 Marine Water Enrichment Solution (Sigma-Aldrich, UK) at 17°C and 28 ppt salinity. Fresh culture medium was stored at 17°C prior to subculturing. Cultures were maintained aerobically in 500 mL Erlenmeyer flasks

(VWR) under illumination from white light-emitting diode (LED) lamps with an irradiance of $32.2 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (2300 LUX) on a 14:10 h light:dark cycle (photoperiod). Stock cultures were subcultured every 20–30 days. The subculture formula was 1:7 from previous culture in sterile filtered medium (e.g. 50 mL culture in 350 mL medium).

Microalgal cells were monitored every 2–3 days over a 32-day period, using three replicates of 1 mL sample in Eppendorf tubes for cell counting. Manual cell counting was performed using a Neubauer counting chamber (Fisher Scientific) to evaluate cell density of suspended cells. Concentration estimations were determined using the formula:

$$\text{Concentration (cells/mL)} = \frac{\text{Number of cells counted} \times 10,000 \times \text{Dilution factor}}{\text{Number of squares}}$$

Flask cultures set-up for *in vitro* attachment assays

Thermanox coupons (VWR UK, diameter 15 mm), i.e. round plastic coverslips, were used as artificial surfaces to monitor *in vivo* the fluorescence of chlorophyll-containing *C. closterium* and *N. thermaloides* biofilms under static conditions in the laboratory. Thermanox polyester material was selected as it exhibits optimal cell attachment and growth. Each monoculture treatment was tested in 6 replicate flasks, where a total of 50 mL of the precultured strain at the early logarithmic phase was used as an inoculum (5 h after onset of the light cycle). Inoculum was added into a 500 mL Erlenmeyer flask enclosing 350 mL Guillard's F/2 medium and a sterile fishing line with 8 Thermanox coupons attached (Figure S1A). Control flasks were prepared with the addition of 400 mL sterile filtered medium (subculture medium) and Thermanox coupons attached to a fishing line (16 control coupons in total).

Diatom sample collection

Monitoring of monoculture phototrophic biofilms was performed over a 9-day period under controlled laboratory conditions. Prior to sampling, flasks were shaken gently manually for 1 min. Under sterile conditions two coupons from different control flasks and six coupons from each culture flask were randomly collected using sterile forceps. Treatment coupons were rinsed with sterile filtered artificial seawater (35 g SS, 1 L dH₂O). In total, 6 treatment coupons and 2 controls were collected at each sampling point

(time = 1, 3, 4, 5, 6, 7, 8, 9 days) for downstream fluorescence measurements.

In situ field assays

Rosette preparation for *in situ* attachment assays

Thermanox coupons were used as artificial surfaces to monitor *in situ* the fluorescence of marine chlorophyll-containing biofilms. Coupons were attached to an experimental rig termed rosette, designed with 8 round holes at the top and bottom part supported by a metallic material in the middle (Figure S1B). A sterile fishing line with two Thermanox coupons attached and supported by swivel, was tied on two parallel holes. The maximum number of coupons on a single rosette was limited to 16 to avoid entanglement and overlapping. Rope was passed through two diametrically opposed holes at the top side of the rosette to be fasten for deployment. The rosette was externally covered with plastic net of $1.5 \times 2.2 \text{ cm}^2$ aperture (hole) size, to hold in place the samples and eliminate access to marine organisms, such as fish, that often tend to feed/graze on biofilms.

Natural biofilm sample collection

Rosettes were immersed vertically to the seawater surface at 0.5–1 m depth. Phototrophic biofilms were monitored in the marine environment at Southsea Marina UK ($50^{\circ}47'27.5''\text{N } 1^{\circ}02'04.7''\text{W}$) during three sampling campaigns in 2017. The first field experiment was conducted for 19 days in Spring (May 5–24), the second experiment for 28 days in Summer (June 15–July 13) and the third for 30 days in Autumn (September 12–October 12). The experiments were designed for the collection of samples with 2–3 days frequency, which was the case for Spring (8 sampling points) and Autumn (11 sampling points) experiments. For the purpose of other experiments, access to the sampling location at preselected dates was feasible during Summer, therefore additional samples were deployed and collected in Summer (3 sampling points) with 7 days frequency (1 point missing due to technical issues). Every coupon was collected using sterile forceps and scissors and placed into a sterile glass tube containing 5 mL of artificial seawater. Six coupons were collected at each sampling point for downstream fluorescence measurements.

Fluorescence measurements

Following collection of monoculture or mixed biofilms, coupons were placed into a sterile 24-well plate that

contained 1 mL sterile artificial seawater and a black cover internally walled around each well to avoid light scattering during measurements. Phototrophic biofilm quantification was based on the intrinsic autofluorescent capacity of the chlorophyll-containing cells, with no staining. Chlorophyll fluorescence (ChlF) was recorded with two different methods including microplate reader fluorescence and epifluorescence microscopy coupled with image analysis.

Microplate reader fluorescence

Fluorescence excitation spectra was measured on a Hidex Sense microplate reader (425-301, Tukru, Finland) using the following assay settings: Fluorescence (Assays by technology), Well scan 7×7 mode, $\lambda_{\text{excitation}} = 436 \text{ nm}$, $\lambda_{\text{emission}} = 680 \text{ nm}$. At the end of each measurement, a Microsoft Excel file with fluorescence estimates in Relative Fluorescence Unit (RFU) value was exported from the reader.

Epifluorescence microscopy and image processing

In order to maintain the samples hydrated during measurements, sterile artificial sea water was placed between slide and the coverslip if deemed necessary. *In vitro* laboratory assays of monoculture biofilms were examined using a fluorescence microscope (Carl Zeiss Axio Scope.A1, Jena, Germany) with equipped filters for $\lambda_{\text{excitation}} = 469 \text{ nm}$ and $\lambda_{\text{emission}} = 592$. *In situ* field assays of mixed biofilms were investigated using a fluorescence microscope (Leica DFC310FX, Wetzlar, Germany) with equipped filters for $\lambda_{\text{excitation}} = 515 \text{ nm}$ and $\lambda_{\text{emission}} = 590 \text{ nm}$.

At least five randomly selected biofilm areas were captured from each coupon. Fluorescence images were collected and then processed using the image analysis software *ImageJ* V. 1.51j8 (MacBiophotonics, ImageJ, Canada) for percentage area coverage estimation. Surface area was estimated by converting a sample image (all replicates of the same sample) to stack, manual adjustment of thresholds for biofilms cells against the background (contrast compared with the light microscopy image), transform to binary, and measurement of area fraction (%). The results were exported and analysed in Microsoft Excel, where ChlF was presented in percentage surface area (% Area).

Statistical analysis

Measurements of ChlF from the *in vitro* and *in situ* experiments were processed in Microsoft Excel and scatter plots were generated merging fluorescence microscopy and plate reader fluorescence results. For

each experiment, a Pearson correlation t-test (Paired Two Sample for Means) was conducted in Excel, where $\alpha = 0.05$ and two-tail *P* value, to determine significant differences between the two tested ChlF methods.

To test the correlation coefficient for the relationship between ChlF recorded with plate reader and microscopy, Pearson's product-moment correlation method was applied for all experiments using *R* (version 4.0.2) (R Core Team 2020). Regression analysis was performed to determine significant differences among the different treatments and scatterplot was generated using *ggplot2* *R* library (Wickham 2016). Confidence interval was set at 95% on the fitted values (around the regression line).

Results

Five experiments for phototrophic biofilm quantification were implemented using two fluorescence techniques: microscopy and plate reader. The developed approach was designed to test the outcomes and evaluate the performance between the two tested techniques under various conditions and did not focus on biofilm kinetics.

Monitoring phototrophic growth in the laboratory

Growth rate of model microalgal planktonic strain

Growth rate of the pennate diatom *C. closterium* was estimated with manual cell counting over a 32-day period. The planktonic strain exhibited a decrease in cell concentration during the initial lag phase (0-5 days) (Figure S2). Following the logarithmic phase where cells increased exponentially, concentration of *C. closterium* cells exhibited a decline after 32 days of incubation (Figure S2). This indicated a potential nutrient depletion or increase of metabolic by-products. Therefore, the subculturing strategy was determined to take place every 20 days up to maximum 30 days.

Testing monoculture Cylindrotheca closterium biofilms

In vitro quantification of the chlorophyll-containing *C. closterium* biofilms was implemented using fluorescence microscopy (Figure 1A) and plate reader fluorescence (Figure 1A and 1B). Fluorescence measurements were performed during the initial lag until the start of the log phase, where growth fluctuations have been previously confirmed for planktonic cells of *C. closterium* (Figure S2). Here, it was observed biofilm cell increase until day-3 of

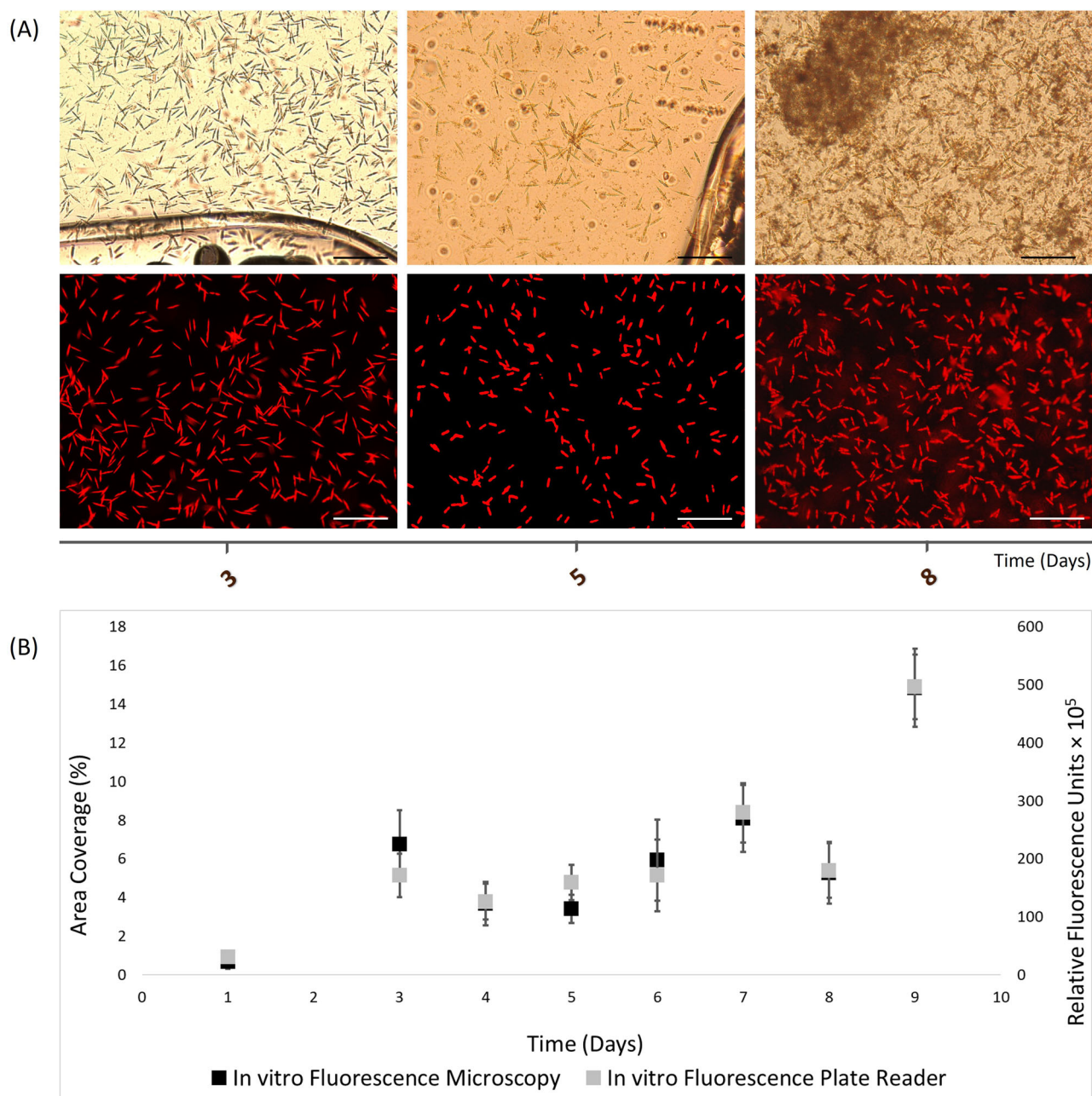


Figure 1. *In vitro* autofluorescence measurements of 9-day *Cylindrotheca closterium* biofilms, as recorded with (A) light (top) and fluorescence microscopy (bottom) images (scale bars = 20 μm), and (B) merged fluorescence microscopy and plate reader fluorescence records, where bars represent standard error.

incubation, which was then stationary until day-5, followed by cell growth until day-7, decrease in day-8 and then exponential increase at day-9.

The two-tail *p*-value (*p*-value = 0.004) was smaller than the significance level ($\alpha = 0.05$), therefore there is statistical proof of close relationship for the ChlF values obtained with microscopy and plate reader (Pearson coefficient (r) = 0.99, t Stat = -4.16, t Critical = 2.37, $df = 7$). As illustrated in Figure 1B, *C. closterium* biofilms exhibited comparable ChlF measurements between the two fluorescence methods during the 9-days monitoring.

Testing monoculture *Nitzschia thermaloides* biofilms

In vitro autofluorescence assessment of the biofilm-forming pennate diatom *N. thermaloides*, exhibited cell growth up to day-3, with subsequent decrease until day-5, followed by cell increase up to day-7, then slight decrease at day-8 and again cell increase at day-9.

The two-tail *p*-value (*p*-value = 0.0008) was significantly less than alpha value, and t statistic (t Stat = -5.60) did fall in the left rejection area (t Critical two tail = [-2.36, +2.36]), hence there was strong correlation between microscopy and plate reader measurements ($r = 0.98$, $df = 7$). As shown in Figure 2 the two

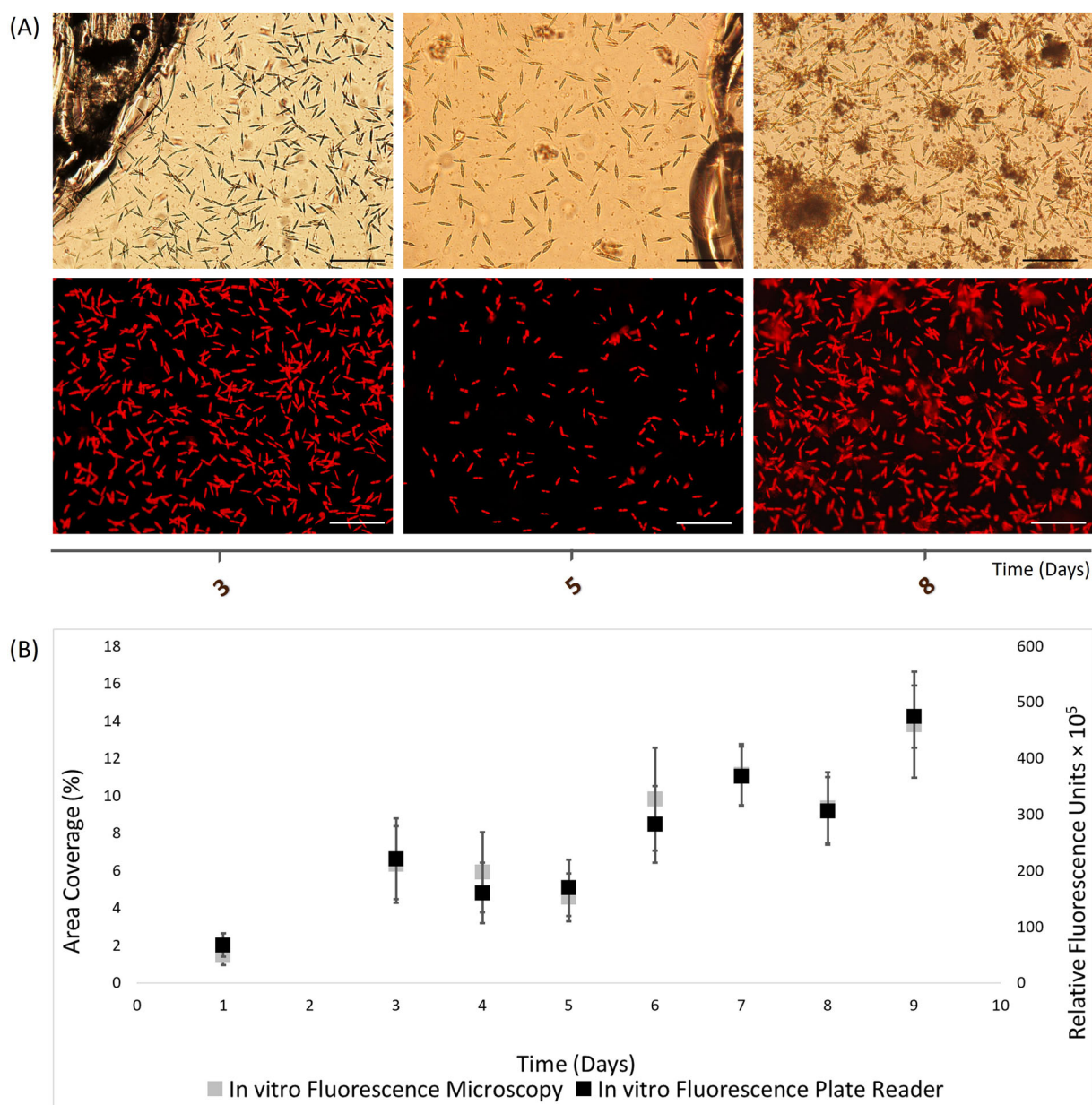


Figure 2. *In vitro* autofluorescence measurements of 9-day *Nitzschia thermaloides* biofilms, as recorded with (A) light (top) and fluorescence (bottom) microscopy images (scale bars = 20 μ m), and (B) merged fluorescence microscopy and plate reader fluorescence records, where error bars represent standard error.

fluorescence techniques indicated similar records for all data points. Overall, the ChlF results displayed the utility of microplate reader for *in vivo* quantification of monoculture phototrophic biofilms.

Monitoring phototrophic growth in the marine environment

Testing marine phototrophic biofilms in spring

In situ assessment of chlorophyll-containing natural biofilms was performed for 19 days in Spring 2017. The recorded natural biofilms exhibited a gradual increase until day-12, followed by stationary phase

until day-14 and then exponential increase until day-19. Both techniques showed similar ChlF measurements for almost all time points (Figure 3A and 3B), except at day-17 where values varied slightly (Figure 3B).

A strong correlation ($r=0.97$) was evident for ChlF measurements between microscopy and plate reader methods during the Spring field experiment (p -value = 0.047, t Stat = -2.61, t Critical = 2.57, df = 5). Overall, it was shown that both fluorescence microscopy and plate reader provided similar ChlF values for *in situ* monitoring of marine phototrophic biofilms.

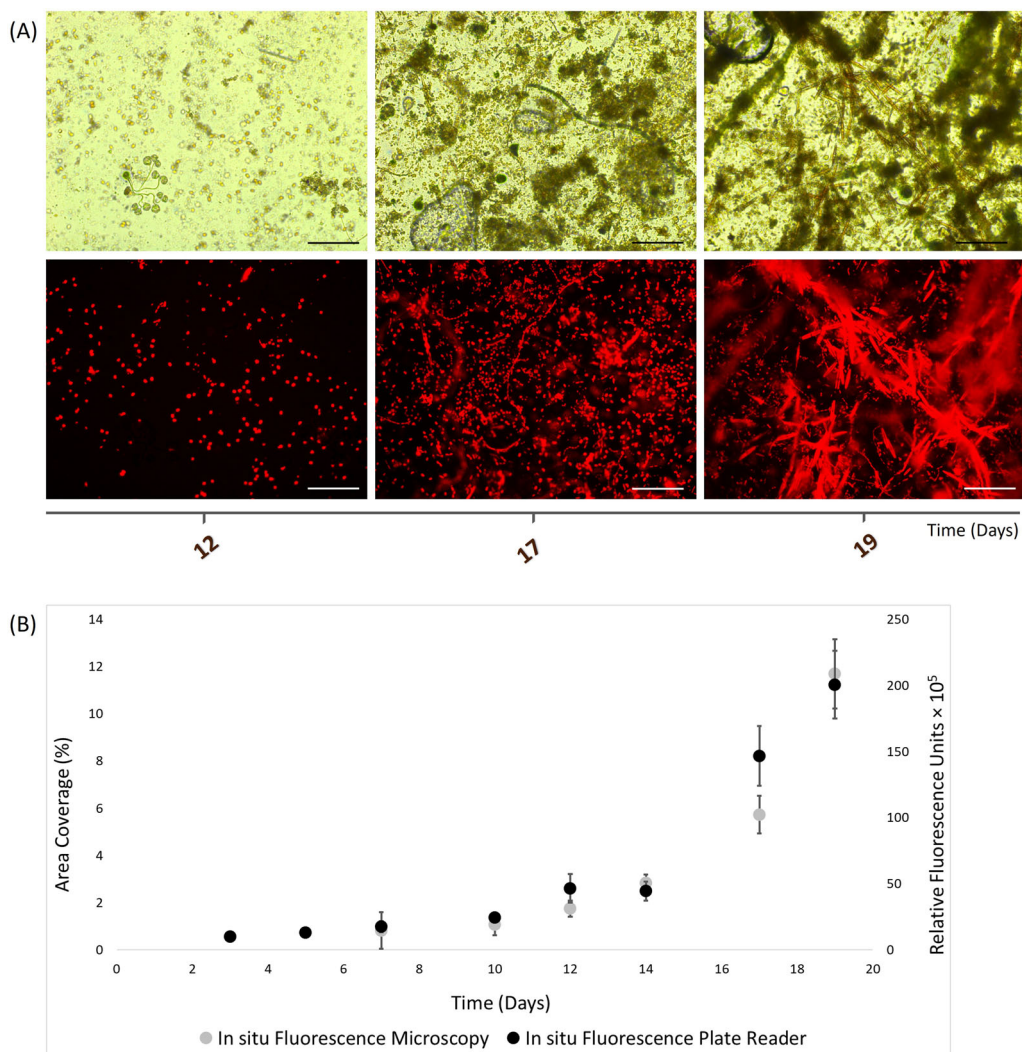


Figure 3. *In situ* autofluorescence measurements of 19-day natural phototrophic biofilms in Southsea Marina UK during Spring 2017, as recorded with (A) light (top) and fluorescence microscopy (bottom) images (scale bars = 20 μ m), and (B) merged fluorescence microscopy and plate reader fluorescence records, where error bars represent standard error.

Testing marine phototrophic biofilms in summer

In situ intrinsic autofluorescence of marine biofilms was recorded for 28 days in Summer 2017. Biofilm cells were increased from day-7 to day-21, and then potentially displayed stationary phase until day-28.

Statistical test revealed close correlation ($r=0.98$) but not significant (p -value = 0.09) between the ChlF values obtained with microscopy and plate reader during the Summer field experiment (t Stat = -2.98 , t Critical = 4.30, $df = 2$). As shown below (Figure 4B), measurements of ChlF with microscopy (Figure 4A and 4B) and plate reader presented comparable results for the *in situ* assessment of phototrophic biofilms.

Testing marine phototrophic biofilms in autumn

In situ quantification of chlorophyll-containing marine biofilms was performed for 30 days in Autumn 2017. Here, biofilms were recorded with increasing cells until

day-14, followed by cell decrease until day-20, then cell growth at day-22 and stationary phase up to day-30.

Quantification of *in situ* ChlF exhibited comparable estimates between fluorescence microscopy (Figure 5A) and plate reader (Figure 5B) for about 2-week biofilms. Nevertheless, small deviations between microscopy and plate reader measurements were shown at day-20 and day-30 (Figure 5B). Any potential discrepancy was statistically examined. A good correlation ($r=0.93$) was shown for ChlF measurement between microscopy and plate reader during the Autumn field experiment (p -value = 0.00059, t Stat = -4.93 , t Critical = 2.23, $df = 10$).

Correlation of fluorescence techniques

The developed method was examined using a general linear model to test the correlation of ChlF values

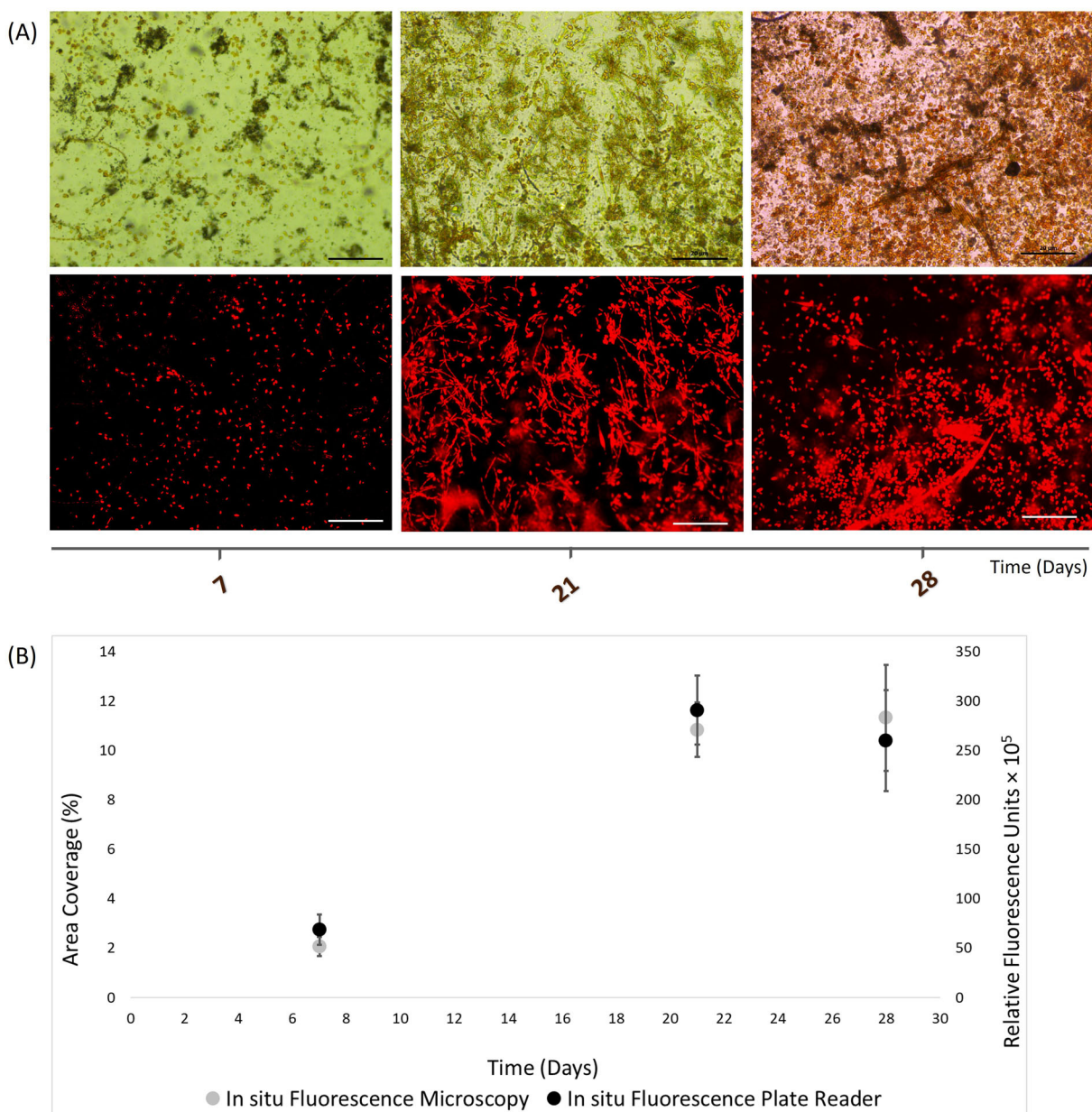


Figure 4. *In situ* autofluorescence measurements of 28-day natural phototrophic biofilms in Southsea Marina UK during Summer 2017, as recorded with (A) light (top) and fluorescence microscopy (bottom) images (scale bars = 20 μ m), and (B) merged fluorescence microscopy and plate reader fluorescence records, where error bars represent standard error.

between the plate reader (Relative Fluorescence Units) and fluorescence microscopy (% Area Coverage) techniques considering all experiments. A strong correlation coefficient $\rho=0.84$ (p -value = $1.081e-10$, $t=9.05$, $df=35$, $conf.int=[0.7, 0.9]$) indicated positive association between the two fluorescence techniques considering all data points.

Scatterplot of ChlF measurements for the *in vitro* assay illustrated a very strong linear relationship as indicated by the coefficient of determination ($R^2 = 0.96$) (Figure 6a) and the established close clustering of all data points to the regression line. The scatterplot for the *in situ* assay also exhibited an

evident strong linear relationship ($R^2 = 0.77$) (Figure 6b). The linear regression model ($R^2 = 0.70$, $df = 37$, p -value = $1.081e-10$, F -statistic = 81.9) used to define the relationship between all variables, demonstrated that fluorescence microscopy (Area Coverage %) explains 70% of the variance in plate reader measurements (Relative Fluorescence Units) (Figure 6c). Estimates of fluorescence microscopy (x axis) could be predicted almost perfect ($\rho=0.84$) from plate reader fluorescence (y axis). The result of correlation coefficient close to 1, confirms that these techniques could generate comparable ChlF results.

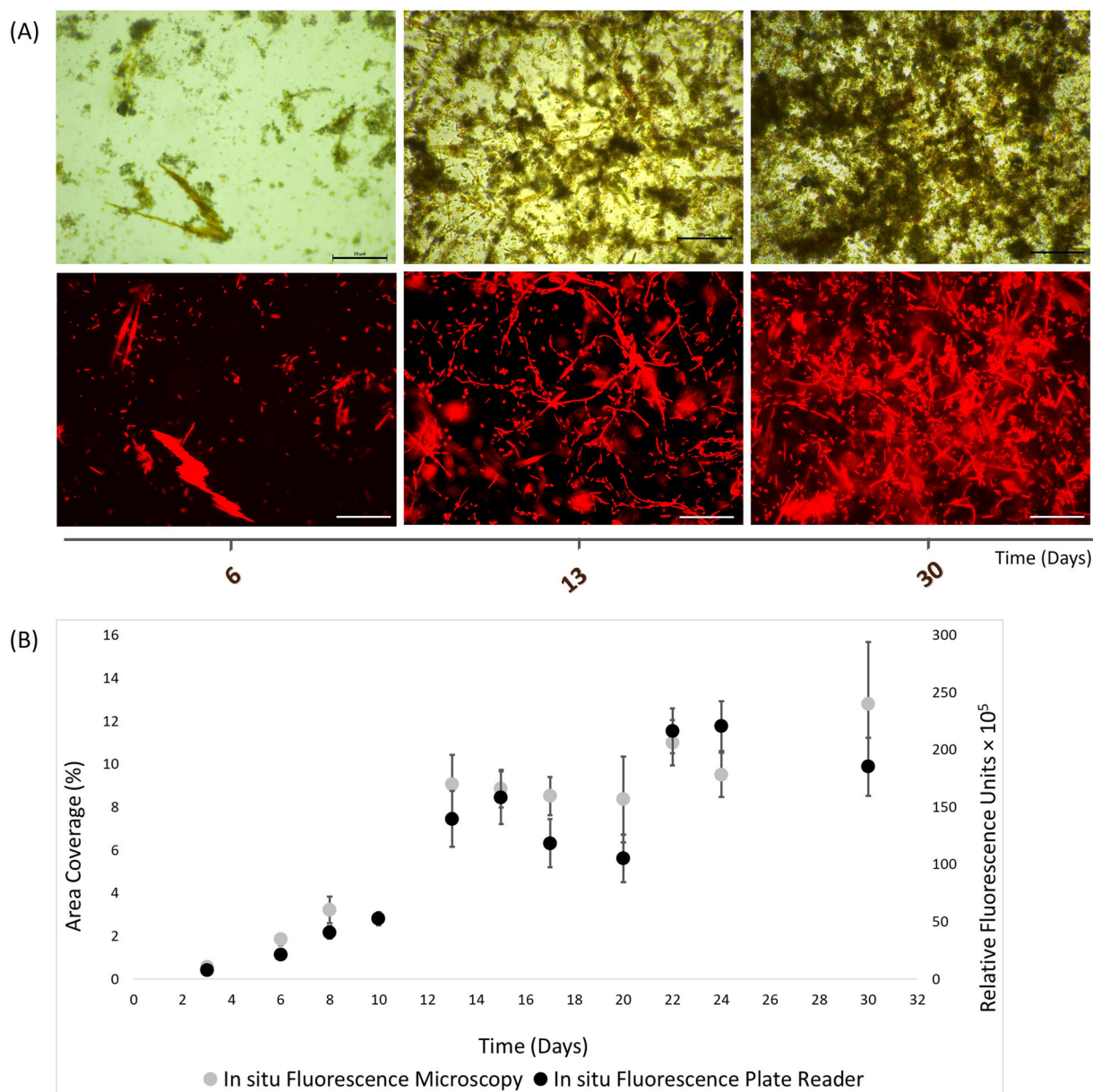


Figure 5. *In situ* autofluorescence measurements of 30-day natural phototrophic biofilms in Southsea Marina UK during Autumn 2017, as recorded with (A) light (top) and fluorescence microscopy (bottom) images (scale bars = 20 μm), and (B) merged fluorescence microscopy and plate reader fluorescence records, where error bars represent standard error.

Discussion

Until recent years, the phototrophic biofilm activity has been underestimated since the main research focus of biofilm studies was centred around bacterial biofilms (Hall-Stoodley et al. 2004; Flemming et al. 2016; de Carvalho 2018), whilst phycological studies were focused mainly on the planktonic mode of life (Roeselers et al. 2008). It is accepted that due to the complexity of characterising biofilm systems, there are no standardized methods for their enumeration (Pantarella et al. 2013), which probably explains the scanty information yet generated for characterising phototrophic biofilms (Bharti et al. 2017). The

development of simple, reliable, and straightforward methods will eliminate the labour-intensive sample preparation, collection and examination of phototrophic biofilms, and next to this, enhance the consistency between different biofilm studies.

The current paper reports a non-invasive method for rapid *in situ* phototrophic biofilm quantification based on the use of plate reader fluorescence. Epifluorescence microscopy is used as a reference technique for biofilm quantification. The high-throughput assay assessed two sets of experimental conditions: (1) *in vitro* monoculture biofilms in a controlled medium, and (2) *in situ* mixed biofilms in

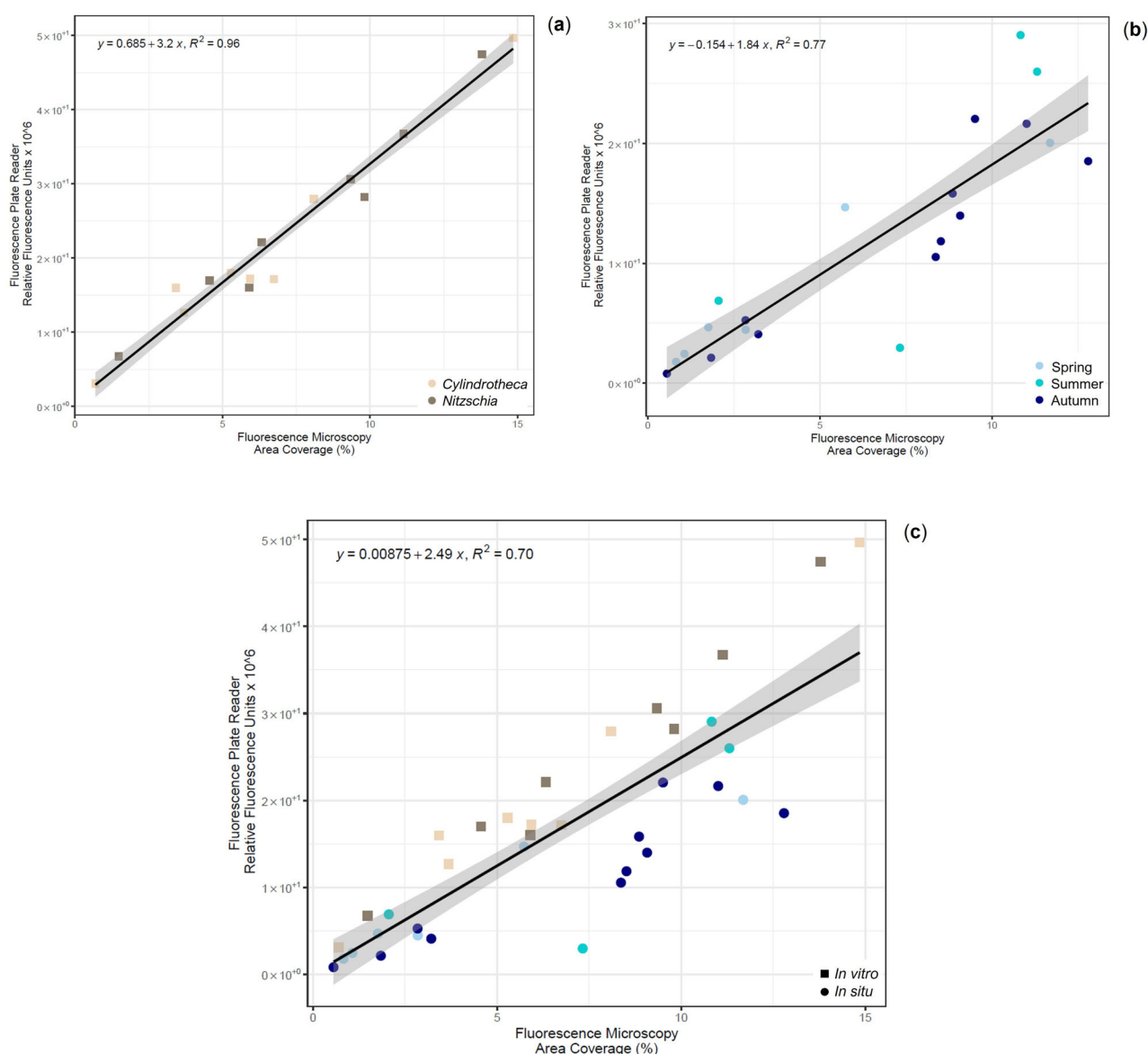


Figure 6. Scatterplot of general linear model for fluorescence measurements between plate reader and microscopy, testing (a) *in vitro* monoculture biofilms ($R^2 = 0.96$), (b) *in situ* marine phototrophic biofilms ($R^2 = 0.77$), and (c) both *in vitro* and *in situ* biofilms ($R^2 = 0.70$).

natural seawater. Ultimately, this work demonstrated the utility of chlorophyll fluorescence measurements under varying natural and laboratory conditions.

Bioassay for *in vitro* assessment of monoculture biofilms

Our results showed that *in vitro* monoculture biofilms in artificial seawater could be efficiently monitored using plate reader. An earlier study by Warren (2008) has established accuracy and precision in chlorophyll measurements using microplate reader combined with chemical chlorophyll extraction. This paper tested plant species from eight different families in freeze dried form, whilst our proposed approach presented

both laboratory and field experiments of model and mixed biofilm samples. Even though chlorophyll extraction with an organic solvent is a widely applied method for measuring chlorophyll, Prieto et al. (2004) have reported inefficient total extraction of chlorophyll from mixed culture of cyanobacteria biofilms (*Nostoc*, *Oscillatoria*, *Scytonema*), and suggested further experiments for robust biofilm quantification. Taking this outcome into account and aiming at designing a method for avoiding cell disruption, the present study selected to compare outcomes of microplate reader with a method of minimum biofilm disruption (i.e. epifluorescence microscopy) compared to chlorophyll extraction. A non-destructive method based on the organism's colour was suggested by

Prieto and colleagues (Prieto et al. 2002) where they demonstrated phototrophic quantification of mixed model cyanobacteria; *Nostoc* and *Scytonema*, under laboratory conditions using chroma meter to monitor colour shifts. This cost-effective approach did not compare outcomes with a reference method, and it is limited to laboratory set up since further *in situ* experiments were required for method validation of environmental monitoring. Another investigation applied CLSM, digital image analysis and *in vitro* chlorophyll extraction to test natural biocides efficiency against multi-species phototrophic biofilms (Chlorophyta; *Chlorella*, *Stichococcus*, Cyanobacteria; *Leptolyngbya*, *Pleurocapsa*) (Sasso et al. 2016). The laboratory-based enrichment experiment indicated good direct correlation of the results obtained, however monitoring phototrophic colonization under environmental conditions was not shown. Our study introduces the *in situ* experiment of natural biofilms, in addition to a rapid method, i.e. plate reader - contrary to the time consuming digital image analysis and CLSM, or destructive chlorophyll extraction that were applied by Sasso et al. 2016. A recent *in vitro* bioassay explored microalgal growth of the model microalgal biofilm-forming *Cylindrotheca closterium* and *Porphyridium purpureum* using microplate reader and CLSM- microfluidic flow-cell (Le Norcy et al. 2019). This work established the tested methods to be complementary for biofilm characterisation, although it is limited to laboratory monitoring of monoculture phototrophic biofilms. Moreover, CLSM constitutes a very delicate, expensive to use and time consuming (depending on the parameters tested) method compared to our suggested reference method of epifluorescence microscopy.

Overall, the proposed high-throughput bioassay was shown to adequately facilitate *in vitro* assessment of phototrophic biofilm growth through the use of plate reader alongside fluorescence microscopy. Plate reader scan results exhibited a similar performance to the fluorescence microscopy data as illustrated from the initial growth of the model fouling diatoms; *Cylindrotheca* (Figure 1) and *Nitzschia* (Figure 2) biofilms, and the linear regression model ($R^2 = 0.96$) (Figure 6a). During growth rate estimation of *Cylindrotheca closterium* diatoms, a decrease in cell concentration was evident during the first five days (Figure S2), which could be a result of slow acclimation (adaptation) (Takahashi 2019) and/or stress after inoculation (subculturing). The *in vitro* bioassay confirmed that the developed non-destructive and rapid quantification method could be successfully applied

for laboratory studies of monoculture phototrophic biofilms, as previously suggested (Le Norcy et al. 2019), taking advantage of the intrinsic properties from chlorophyll-containing cells, with no need for destructive chemical extraction. Considering that mono-species systems are less reflective of the real environment (Cattò and Cappitelli 2019), while *in vitro* assays can partially predict the *in situ* conditions, it was essential to further validate our designed bioassay in the marine environment.

Bioassay for *in situ* assessment of natural mixed-species biofilms

Our study demonstrated that *in situ* mixed biofilms (up to 1-month old) in natural seawater could be effectively examined using plate reader. An *in situ* study implemented by Carreira et al. (2015) quantified and discriminated benthic photosynthetic microbial mats (i.e. cyanobacteria and diatoms), in addition to mixed planktonic diatom *Nitzschia capitellata* and cyanobacterium *Geitlerinema* sp. cultures, in the laboratory using autofluorescence imaging (charge coupled device with filters) that was compared to cell counts of the laboratory culture samples. The results of this nonintrusive and simple technique reported close correlation of the two tested methods for the first 8-days, although measurements were not consistent until day-15. Carreira et al. (2015) compared imaging with cell counting only for the *in vitro* experiment of planktonic cells, whilst our approach applied two techniques feasible to be tested for both natural and culture biofilm samples, therefore comparisons were performed between the methods for the *in vitro* and *in situ* experiments (Figure 6). Another study for *in situ* assessment of marine biofilm growth introduced a method applied a novel designed flow cell coupled with optical coherence tomography (Fabbri et al. 2018). They successfully confirmed biofilm assessment (in terms of thickness, roughness, area coverage) in a dynamic biofilm culturing facility of filtered natural seawater with access to natural sunlight. Advantage of this method is the biofilm testing under controlled flow, yet the development of the specific flow cell does not encourage the wider application of the method, compared to the use of plate reader.

The suggested high-throughput method for *in situ* phototrophic biofilm quantification was successfully confirmed based on plate reader and fluorescence microscopy measurements. Chlorophyll fluorescence measurements were performed during three field

experiments that demonstrated successful quantification of mixed marine biofilms. More specifically, plate reader measurements were shown to be complementary to fluorescence microscopy during the 19-day old Spring biofilms (Figure 3), the 28-day old Summer biofilms (Figure 4), and the 30-day old Autumn biofilms (Figure 5). Some inconsistencies were observed between the two methods - after 17-days in Spring and 20-days in Autumn - which could potentially be attributed to the complex and uneven structure of environmental biofilms, especially with time as aggregates mature. Another reason that could explain this variation is the fact that the plate reader measured the entire surface area (surface scan mode) of the coupon samples, whilst only five fields of view from the coupon samples were randomly selected for epifluorescence microscopy measurements (selective number of optical areas). During the spring sampling campaign, diatoms belonging to the *Cocconeis*, *Navicula* and *Amphora* genera were identified, with *Amphora* sp. prevailing with time (data not shown).

The intrinsic biological variation in our data was to be expected, however the results statistically showed that intrinsic fluorescence from phototrophic biofilms could be predicted *in situ* with great confidence with both fluorescence techniques (Figure 6b). Overall, these findings confirmed the efficiency of the plate reader bioassay for *in situ* quantification of 30-day biofilms in the marine environment. Even though there is an existing methodological gap concerning phototrophic biofilms due to the inherent complexity of natural biofilm assemblages, the current work illustrates a promising method that can be applied with confidence on these natural systems.

Conclusion

In this proof-of-concept study, chlorophyll fluorescence measurements of phototrophic biofilms were successfully conducted under different conditions i) *in vitro* monocultures, ii) *in situ* mixed natural biofilms, and iii) during three different seasons. To the best of our knowledge, this is the first *in situ* bioassay for high-throughput rapid, and non-destructive quantification of up to 1-month natural phototrophic marine biofilms utilizing plate reader - a universally available equipment - complemented by epifluorescence microscopy. The presented biofilm method aimed to set a comprehensive baseline for natural phototrophic biofilm assessment without the need for chlorophyll extraction from the microalgal biofilms, but rather by measuring the intact cells directly from

the artificial surface while still being immersed in water. The developed microplate reader-based method is anticipated to serve as a reliable tool for *in situ* monitoring of phototrophic biofilm formation on several artificial surfaces with commercial interest and contribute towards further development of phototrophic biofilm applications and antibiofilm testing.

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