Linking biofilm spatial structure to real-time microscopic oxygen decay imaging

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Abstract

We combined two non-destructive techniques, confocal laser scanning microscopy (CLSM) and planar optode (ViviSens imaging), to relate fine-scale spatial structure of biofilm components to real-time images of oxygen decay in aquatic biofilms. To this aim, both techniques were applied to biofilms grown for 7 days at contrasting conditions of light and temperature (10/20ºC). The geostatistical analyses of the CLSM images indicated that biofilm structures consisted of a combination of small size (~10^0 µm) and middle size (~10^1 µm) irregular aggregates. Cyanobacteria and EPS (extracellular polymeric substances) showed larger aggregate in 20ºC dark-grown biofilms while, for algae, aggregates were larger in light-20ºC conditions. Light-20ºC biofilms were the densest ones, while dark-10ºC

biofilms showed the sparsest structure and lowest respiration rates. There was a positive relationship between number of pixels occupied by biofilm and oxygen concentration decay rate. The combination of optodes and CLMS, taking advantage of a geostatistical analysis, is a promising way to relate biofilm architecture and metabolism at the micrometric scale.

Keywords: confocal laser scanning microscopy, real-time images of oxygen concentration, biofilm growth, planar optodes, biofilm respiration, geostatistics, nested structures

1. Introduction

Biofilms grow on wet surfaces and consist of a combination of autotrophic and heterotrophic microorganisms embedded in self-produced extracellular polymeric substances (EPS) (Costerton et al., 1978). They play a beneficial and critical role in the metabolism of water bodies, being responsible for most of the nutrient cycling, uptake and mineralization processes occurring in aquatic ecosystems, as well as in natural and manmade infiltration devices (Romani et al., 2004; Gette-Bouvarot et al., 2014; Battin et al., 2016). Conversely, biofouling processes (Sala et al., 2013) represent the main adverse effect of biofilms in industrial water systems, including drinking water distribution systems, water treatment applications (such as porous membranes, Nguyen et al., 2012), and marine systems (e.g., aquaculture nets, oil and gas installations, and ship hulls). Both positive and negative macroscopic effects represent the collective result of processes occurring at microbial hotspot scales (µm to cm). Analyzing the fine spatial-scale changes in biofilm structure and metabolism under different environmental conditions is of paramount

42 importance to better understanding the macroscopic effects of biofilm in the environment
43 where it develops.

44 Biofilms present complex and dynamic heterogeneous structures, with sparse and
45 dense zones modulated spatially and temporally by environmental drivers, such us nutrient
46 availability, light, and flow rate (Singer et al., 2010; Proia et al., 2012; Mora-Gómez et al.,
47 2016). Light influences the biofilm thickness, as well as the distribution and relative
48 proportion of autotrophic and heterotrophic microorganisms, responsible for changes in
49 respiration rates (Ylla et al., 2009). Directional flow, shear stress and soil heterogeneity
50 also induce changes in the direction of biofilm growth, leading to anisotropic spatial
51 distributions (e.g., Jung-Woo et al., 2010; Hödl et al., 2011; Bozorg et al., 2012) and the
52 formation of preferential flow paths (Rubol et al. 2014). Another key environmental factor
53 is temperature, which promotes fast biofilm colonization and enhances young biofilm
54 growth (Díaz Villanueva et al., 2011). However, limited information is currently available
55 on whether oxygen consumption distribution in space and time is affected by these
56 environmental conditions and how it is linked to the biofilm architecture (Fenchel and
57 Finlay, 2008).

58 A plethora of techniques are currently available to investigate the spatial distribution of
59 microbial assemblages. These include, among others, confocal laser scanning microscopy
60 (CLSM) and planar optodes. The former is a well stablished technique to study biofilm
61 structure (New and Lawrence, 2016), as it provides simultaneous information about the
62 three-dimensional structure of thin (young) biofilms (Barranguet et al., 2004) and the
63 identification of different components by both auto-fluorescence (for algae and
64 cyanobacteria) and fluorescent dyes (for DNA and EPS). The latter offers bidimensional

oxygen concentration images of surface biofilm based on dynamical quenching of a luminescence indicator by oxygen (O_2). Optodes have been successfully applied in the study of microbial active soils and sediments (Rubol et al., 2016) and biofilms (Borzog et al, 2012; Staal et al, 2011a), with particular focus on the biofilm functioning (Kühl et al, 2007; Kühl and Polerecky, 2008). Both CLSM and optodes are non-destructive techniques capable to highlight differences in spatial distribution that cannot be properly observed by other point measurement devices such as optical fibers or micrometers.

The link between structure and metabolic function of biofilm is under current debate. Staal et al. (2011b) showed that biofilm in tap-water present distinct hotspots of activity modulated by flow rates, with no clear link to structure. Other works, however, suggest that biofilm structure and functionality are connected, but only limited information is available at the very fine scale (e.g., Proia et al, 2012).

Here, we quantify the link between the spatio-temporal concentration distribution of oxygen in the biofilm and the spatial structure of its components (algae, cyanobacteria, bacteria, and EPS) with emphasis on the quantification of aggregate sizes. We used one-week old biofilms grown at four combinations of light and temperature conditions, processed by both CLSM and Visisens planar optode (PreSens Precision Sensing, Germany). The corresponding CLSM 3D and 2D information obtained were then used to determine the spatial statistics of biofilm growth. In parallel, the Visisens plus imaging technology allowed mapping the percentage of air saturation in the biofilm as a function of time and space. The combination of the two high precision techniques allowed studying the correlation of biofilm structure and oxygen consumption for some specific temperature and light conditions.

2. Materials and Methods

2.1 Biofilm growth

Artificial glass tiles (of area 1cm^2) were used as substrata for biofilm growth and were incubated in 8 microcosms with a biofilm extract inoculum obtained from an oligotrophic stream (Fuirosos stream) following the protocol outlined in Ylla et al. (2009). Each microcosm consisted of a sterile glass jar (19 cm in diameter, 9 cm high) with autoclaved glass tiles attached to the bottom. Microcosms were filled with 1.5 L of water of known chemical composition mimicking that of the Fuirosos stream, and obtained by dissolving some pure salts (12 mg/L Na_2SO_4, 20 mg/L Na_2SiO_3, 30 mg/L $CaCl_2$, 1 mg/L KCl, 2 mg/L $MgSO_4$, and 20 mg/L $NaHCO_3$) in MilliQ water. Water was continuously recirculated by means of a submersible pump (Hydor, Pico 300, 230V, 50 Hz, 4.5 W) (one pump for each microcosm).

The microcosms were placed inside an incubator (SCLAB-PGA500) at four different combinations of light and temperature (with two replicates in each case): Light-10ºC (from now on denoted as 10L), Dark-10ºC (10D), Light-20ºC (20L), and Dark-20ºC (20D). Light treatments consisted of a daily cycle of 12 hours of light (160-180 µmol photons m^{-2} s^{-1}) and 12 hours of complete darkness. Dark treatments consisted of 12 hours of very low light (<10 µmol photons m^{-2} s^{-1}) and 12 hours of complete darkness. Two extra microcosms with glass tiles were previously incubated under 20L conditions for 28 days in order to obtain a mature biofilm for testing sensitivity of oxygen consumption (measured by the optode technique) to biofilm age (and correspondingly, thickness). Random samples

were selected from each microcosms, and used with the CLSM and spatial optode techniques.

2.2 Confocal laser scanning microscopy (CLSM): images on biofilm architecture

CLSM (Leica TCS-SP5 AOBS CLSM, Leica Microsystems Heidelberg GmbH, Mannheim, Germany) was performed to obtain images that were relevant to assess the spatial organization of biofilms for the four components visualized: algae, cyanobacteria, EPS, and DNA. After day 7, one replicate per treatment was transferred into an individual flask with enough water to allow the analysis in vivo by CLSM.

Autofluorescence of photosynthetic pigments was viewed in the red channel for phycobiliproteins (570–615 nm emissions) using a 561nm laser diode (to detect cyanobacteria) and in the blue channel (670– 790 nm emissions) for chlorophylls using a 594 nm Helium-Neon laser (to detect algae). EPS was stained with lectin concanavalin A (ConA)-Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) and observed in the green channel (495 to 550 nm emissions) using a 488-nm Argon laser. DNA (as a proxy to visualize bacteria) was stained with Hoechst (Hoechst 33342, Molecular Probes, Invitrogen, H-3570), excited by UV light at 405 nm, and observed in the blue/cyan fluorescence light at 414-458 nm.

For three dimensional analysis, 20 slices were recorded, separated at a fixed distance of 1 micrometer. This is valid for all treatments and for all variables, such that the statistics were computed over samples of the same size (i.e., the same volume was considered for all the treatments and biofilm components), following the same approach used to determine the respiration rates. To decide the depth and number of slices to adopt,

we first measured the maximum depths of the different biofilms in different locations within the biofilm (up, down, left, right). Then, based on this initial inspection, we chose 20 µm to include the maximum volume of biofilm from all biofilm types. Image analyses were performed with the IMARIS code (v. 6.1.0 software, Bitplane, Zürich, Switzerland). Images were taken at different magnification levels. The analysis of the spatial organization of the biofilm structures was performed on the one-week old 10X images that allow the analysis of algae, cyanobacteria and EPS, but not of bacteria which was analyzed only in the 40X images. Most of the results presented in this paper correspond to the 10X images, corresponding to 512x512 pixels, each of size 0.76 x 0.76 μm^2 (representing a subset of the total image equal to a square of side 0.39 mm). The lenses used were PlanApochromatic 40X (NA 1.25, oil) and PlanApochromatic 10X/0.40 CS. The direct comparison of the 10X and 40X images was not possible given that the two set of images were obtained using lenses with different resolution (in the planar direction) and signal elongation (in axial direction). The purpose of using the 40X images, is solely to relate the microbial respiration to the amount of DNA.

2.3 Visisens: image acquisition and sensor foil calibration

The system consisted of a USB-Microscope device (VisiSens; PreSens GmbH, Regensburg, Germany) with an embedded software. The technique is based on the dynamic quenching of a luminescence indicator by oxygen. Using the LEDs incorporated into the USB microscope, the indicator and reference dyes immobilized in the sensor foil (SF-RPSU4, Presens GmbH, Regensburg, Germany) are simultaneously excited with the same blue light source. The red light emitted by the reference dye is not affected by oxygen changes. On the contrary, the green light emitted by the indicator dye is quenched by O_2.

Since the emission wavelengths of the red and green lights match the red and green channel sensitivity of a RGB chip, both signals are recorded within a single image. Quantification was obtained by the ratio of the red and green channels of the RGB image applying a calibration function derived from the sensor output. The calibration function was determined from exposure to known oxygen concentration values, which were 0% (obtained covering the sensor foil with sodium sulfite solution) and 100% (obtained exposing the sensor foil to air). We refer the reader to Holst and Grunwald (2001) and Gansert and Blossfeld (2008) for a detailed description of the method.

The oxygen-sensitive foils were cut into 1cm^2 squares and glued with silicone to the inner bottom of 6 well-plates (polystyrene, Nunc). The sensitive side of the foil was in direct contact with the biofilm grown glass tiles. Superficial oxygen concentration distribution was recorded with the Visisens unit detector. Measurements were performed on the one-week biofilms grown at the four conditions of temperature and light described, as well as in four-week old biofilms grown at 20°C in light conditions. The image accounted for an area of 1280x1024 pixels (1mm=115pixel). Images were recorded every 30 seconds over 30 minutes (a total of 60 images per treatment). Spatial averaging and hotspot evaluations were performed by using the z-function in the Vivisens software. A schematic overview of the setup can be found in the supplementary material.

2.4 Statistical analysis

CLSM images were analyzed for spatial distribution description, based on the 2D projections along the vertical axis of the different color bands for the 10X visualization fields. Intensity values (for each channel) were measured by an *ad-hoc* script in Matlab. The intensity values for each channel (chlorophyll, phycobilin, concanavaline, DNA)

varying in a 0-255 scale, were converted to binary sets, after selecting a threshold light intensity, so that at each pixel the variable I(x, y), indicated presence (I=1) or no presence (I=0), based on whether the actual measured light intensity at each pixel exceeded the threshold value. For all channels, the threshold value chosen was set to 10% of the maximum potential intensity (i.e., corresponding to an intensity value of 25). This threshold was determined by visual inspection between the resulting binary map and that of the original images with intensity color plots. The sensitivity of the choice of threshold was tested in the red channel, using also a value of 50, and then comparing the results of the statistical analysis. Finally, we extended the work to the green and blue channels, and found no reason to use a threshold different from 25 (again, from visual inspection of the resulting binary maps).

The resulting binary (indicator) fields were analyzed for the number of non-void pixels, and then the omnidirectional sampled variograms were obtained (horizontal isotropy in the xy plots was adopted based on preliminary tests). The variograms were then fitted to standard existing models.

To account for the heterogeneity of the biofilm samples, we used two stacks of images for each set of environmental conditions. For each stack, we used a sampling window large enough to guarantee that the ergodicity of the sample was ensured (i.e. we checked in preliminary analysis that sampled windows were at least 25 times larger than the largest integral scale for all components and all treatments). For all treatments and channels, the ranges obtained in the two stacks were quite similar (see Table 1 in the SI). Note that ergodicity could not be assessed properly in the 40X images (due to the smaller sampled domain size). Therefore, the ranges determined for the bacteria may not be representative

201 of the whole biofilm. However, we included these data, since they were collected at the
202 center of the sample where the respiration rates were measured and, as stated before, the
203 purpose of using the 40X images is solely to relate the microbial respiration to the amount
204 of DNA.

205 Note that, in our study, the small proportion of non-void pixels close to the
206 substraum and the comparison of the 3D and 2D images indicated that the statistics for the
207 one-week old biofilm were preserved in the 2D projections (see Appendix A). Thus, the 2D
208 projections allowed obtaining the statistics of the aggregates sizes and the planar distance
209 between aggregates. However, the use of 2D projections in older/denser biofilm, may result
210 in artifacts and requires further investigations.

211 The combination of univariate (histograms) and bivariate statistics (indicator
212 variograms) in the 2d projected images provided simple but very useful information about
213 the way the different components of the biofilm are spatially organized under different

214 conditions of light and temperature.

215 The rates of oxygen consumption were measured using oxygen concentration values
216 taken every 30 sec in the interval 0 to 30 min for each individual sample. Non-linear
217 regression analyses using Sigmaplot 11.0 (Systatsoftware, Inc CA. USA) were performed
218 in each data set in order to calculate the slope of the oxygen concentration versus time plot,
219 equivalent to the consumption rate.

220 **3. Results**

221 **3.1 Geostatistical analysis of confocal images.**

Figure 1 represents the 3D images (at 10X magnification) of the biofilms grown at the four environmental conditions (10L, 20L, 10D, and 20D) and for all four biofilm compartments analyzed in this work (algae, cyanobacteria, EPS, and bacteria). These images revealed the presence of highly structured microbial aggregations, whose sizes and distribution varied depending on both temperature and light conditions. More precisely, the 20L biofilm CLSM image exhibited the largest number of total non-void pixels of all the compartments, with algae, cyanobacteria, and EPS unevenly distributed within the biofilm. On the contrary, the 10D image was characterized by largest amounts of empty pixels.

Figure 1 goes here

The amount of area occupied by cyanobacteria, EPS and algae (approximated by the proportion of non-void pixels) ranged from 0.12 for EPS in one of the 10D stack, to 15.4% for algae in the 20L biofilm in the 2d projections, as shown in Figure 1b. The 10D treatment presented always the lowest values for all the three components. In the 10D and 20D, the largest amounts of non-void pixels corresponded to cyanobacteria while in the 10L and 20L, algae were the most abundant component (see Figure 2 and Table 1).

Figure 2 goes here

The CLSM images were interpreted by variography of the binary images to determine selected spatial characteristic sizes and statistical parameters of the biofilm compartments analyzed. The variogram associated to a stochastic process is a powerful tool to describe how the variable of interest correlates as a function of distance. The sample variogram is then fitted by one of the standard stationary models. In our analyses, we studied the number of structures required to model the experimental variograms of the 10X images for algae,

244 cyanobacteria, and EPS. For each one of them, we obtained a fitted range (i.e., the distance
245 beyond which data are not spatially correlated), equivalent to the average size of the
246 microbial aggregates for each component; also, each structure displays a sill, that represents
247 the fraction of the total variance explained.

248 The statistical analysis for the 3D images revealed that microbes were mostly
249 concentrated in the bottom slices (results not shown). The vertical dimension displayed a
250 slightly larger range than the horizontal one, but the actual estimate of range is very
251 uncertain due to the limited number of slices available. As a consequence, the approach
252 presented in this work based on 2D projections was devised only for thin biofilms, with
253 sizes of hundreds of microns in the x-y directions and only a few micrometers in the z
254 direction, and would not be useful for thick (mature) biofilms. While the statistical
255 descriptors obtained for the 2D and 3D cases were not equal, the overall behavior was
256 similar, and thus the 2D projected data was considered suitable to study the spatial structure
257 of the different biofilm compartments. In any case, an example of the comparison between
258 the 2D and the 3D images for the 10D red channel is included in Appendix A. In the
259 following, we report only the 2D results.

260 The variables explored for the 2D projections included the three channels observed in
261 the 10X images: cyanobacteria (red - R), EPS (green - G), and algae (blue - B). The
262 resulting sample variograms were then fitted by two superimposed (nested) stationary
263 models with no nugget, providing two sills (their sum corresponds approximately to the
264 total variance of the indicator parameter, indicative of a stationary stochastic process) and
265 two ranges (Table 1). Stationarity implies that the aggregates are placed throughout all the
266 domain, without any spatial trend, or in other words, that aggregates grow following a

267 similar pattern in different areas. The first variogram model displayed a short range being
268 an indication of the size of the smallest aggregates, and accounted for most of the
269 variability (on the average, it explains 72% of the total variance); the second one showed a
270 larger range, indicating the characteristic distance between aggregates, and it completes the
271 full variance description. In the following, we refer to them as small-scale and large-scale
272 aggregates respectively. The different sample variograms could be fitted by means of
273 exponential models (thus, displaying no continuity at the origin). This is an indication that
274 the shapes of the aggregates are not smooth, but irregular.

275 **Table 1 goes here**

276 Overall, the average size of both the small- and large-scale aggregates of biofilms
277 grown at 20ºC were larger than those grown at 10ºC. In contrast, aggregate sizes associated
278 to light variations (dark vs. light) were just slightly different depending on the channel
279 analyzed. For cyanobacteria (R - channel), the short-range variogram structure (small-scale
280 aggregates) explained 59% to 81% of the corresponding total variance, and provided ranges
281 (i.e. average sizes) of 2-3.9 pixels (the average is equivalent to 2.2 μm), just slightly larger
282 under 20ºC dark conditions (Table 1). The second structure (large-scale aggregates) showed
283 ranges of 10-42 μm (13-55 pixels) and 22-33 μm (29-41.5 pixels) for biofilms incubated
284 under dark and light conditions, respectively. The combination of variogram ranges and
285 percentage of occupied pixels for cyanobacteria indicated that small-scale aggregates are
286 quite similar regardless of channels or environmental conditions; on the other hand, the
287 number of pixels occupied is largest under light conditions, indicating that in such a case
288 there was a larger number of cyanobacteria distributed throughout the domain forming
289 average size aggregates.

290 For EPS, the small-scale aggregates ranged between 1.6 μm (10D) and 3 μm (20D),
291 describing 53%-78% of the total variance, while for algae the average size was between 2.1
292 μm (10D) to 2.3 μm (20D and 10L) and accounted for 64%-80% of the total variance. The
293 range of the second structure (large-scale aggregates) was quite variable, being of 4.6-27.8
294 μm for EPS and 9.1-32.7 μm for algae, with a clear increase with temperature and light.
295 Again, the combination of ranges and non-void pixel percentage, indicates that for the 10D
296 treatment, biofilm occupied just a very reduced area that tended to be localized in very few
297 clusters; on the contrary, for the 20L case, aggregates were distributed all along the area,
298 with a large number of clusters covering the full space.

299 Bacteria (DNA stain) could only be assessed in the images with a 40X magnification
300 and therefore are treated separately. The results from the variographic analysis are reported
301 in Table 2. In the 10D biofilm the area occupied by bacteria was the lowest of all treatments
302 (see also the inset in Figure 2), and their location displayed no apparent spatial correlation,
303 characteristic of an uncorrelated random function. The remaining three experimental
304 variograms could be studied using second order stationary models. Due to the high-
305 resolution images it was not possible to obtain large ranges (anything above 100 pixels;
306 here 1 pixel = 0.19 μm). In all three cases, the sampled variogram could be fitted by a
307 single model with a nugget effect indicating a combination of variability at a very low
308 range (uncorrelated random process), representing 60-65% of the total variability, and a
309 correlated process that accounted for the remaining 35-40% of the variance, with ranges of
310 2.3 μm, 6.5 μm and 7.8 μm for treatments 10L, 20D, and 20L, respectively. This indicates
311 that clustering of bacteria is more sensitive to temperature than to light conditions.

312 **Table 2 goes here**

3. 2 Oxygen respiration patterns

Metabolic processes in biofilms are the result of autotrophic and heterotrophic organisms happening at the μm to mm scale. Oxygen is produced by photosynthetic microorganisms and consumed by aerobic respiration. To study the resulting spatial distribution of oxic/anoxic zones in the biofilms at the micro-scale, we monitored the changes of oxygen concentration on the surface of the 10D, 10L, 20D, 20L and mature biofilms over thirty minutes at a temporal frequency of 30 seconds. Figure 3 shows the spatially averaged oxygen concentration for the one-week-old biofilms grown at 10D, 10L, 20D and 20L and for a (four-week-old) mature biofilm grown at 20º C under light conditions. The mature biofilm had an initial oxygen concentration of 160% (expressed as % of air saturation) due to the high colonization of algae and cyanobacteria. This value was comparable to the initial oxygen concentration for the 20D and 20L biofilms, which ranged 140-160%. Oxygen concentration initial values were lower (60-80%) for 10D and 10L.

The associated oxygen concentration consumption rates were obtained by fitting an exponential decay curve, $S = a\exp(-bt)$, on top of the oxygen saturation (S) versus time (t) data displayed in Figure 3. The values of the coefficients a (in percentage saturation) and b (in min^{-1}) are listed in Table 3. The slope of the oxygen decay curve (b) increased with temperature, with the largest value measured for the mature biofilm.

Figure 3 goes here

Table 3 goes here

The real-time images of oxygen concentration at selected times is illustrated in Figure 4 for all 7-day old biofilms, as well as for a 28-day old biofilm grown at 20L (an example

of the whole time series for the 20L biofilm is included in the Supplementary Material, see Video S1). Anaerobic hotspots, defined as zones with disproportionally high reaction rates with respect to the surrounding matrix (McClain et al., 2003) developed under all environmental conditions, but were most intense for the biofilms grown under light.

Figure 4 goes here

An example of the fine-scale spatial variability (i.e., heterogeneity) in oxygen decay is presented in Figure 5, corresponding to the one-week 20L biofilm. Five subzones (of 12 mm^2 surface area) were cropped from the original plot. The evolution of the spatially averaged oxygen saturation is plotted, for each subzone, as a function of time. The oxygen decay rates presented a very large variability of the a and b coefficients, with values ranging from $b = 0.03$ - 0.10 min^{-1}.

Figure 5 goes here

Histograms of the spatial oxygen concentration distribution are presented in Figure 6 for two selected times, 3 minutes and 30 minutes. Figure 6 displays a large variability in space among the oxygen concentration histograms studied. Overall, the largest reduction in oxygen concentration is observed for the mature biofilm. For young biofilms, the largest reduction is observed in the 20L treatment. In almost all the young biofilms the initial oxygen concentration distribution is positively skewed, with a mean ranging 87-95% air saturation, and a median close to 100% air saturation. The only exception is the 10L case that presented a negatively skewed distribution with a low initial value (58% air saturation).

All the 30 minute oxygen concentration histograms were positively skewed, with mean air saturation values ranging between 53% and 32%. Contrarily, the oxygen concentration

357 histograms of the mature biofilm were very different, with initial distribution mostly
358 symmetrical and an average value of 167% air saturation, that decreased very rapidly to
359 0.6% at t=30 minutes.

360 **Figure 6 goes here**

361 **3.3 Linking biofilm structure to oxygen respiration rates**

362 The heterogeneous distribution of oxygen concentration in space and time (highlighted
363 in Figure 4 to Figure 6) can be understood from a combination of statistical and biological
364 considerations. The oxygen concentration maps have a pixel size of about 8.7 μm, therefore
365 always larger than the range of the smallest biofilm structure determined by the variogram
366 analysis (i.e., characteristic length scale of the small-scale aggregates). On the other hand,
367 8.7 μm is smaller in general (comparable in the 10D case) than the ranges obtained for the
368 second structure This suggests that all pixels in the oxygen concentration maps have some
369 amount of biofilm (non-void pixels) that can produce/consume oxygen. In addition, the
370 variographic analysis performed (at the 10X resolution) indicates only presence/no-
371 presence of biofilm, but does not provide information about activity. Therefore, it is
372 expected that the oxygen dynamics is variable both in space and time. Finally, biofilm
373 respiration rates have been traditionally described by a lognormal model (e.g., del Giorgio
374 and Williams, 2005; Forney and Rothman, 2012), thus it can be expected that the statistical
375 distribution of oxygen concentration values displays a positively skewed, describable by a
376 lognormal distribution, as shown in Figure 6.

377 Figure 7 explores the potential correlations between the biofilm structure and the
378 respiration rates for the one-week old biofilms analyzed in this study. To this aim, the

379 panels in Figure 7 displays the values of the respiration rates, *b* (see Table 3), *versus* the
380 amount of non-void pixel (i.e., the amount of biofilm for each of its components) and
381 *versus* the corresponding ranges of both the small and large scale aggregates. Additionally,
382 in Figure 7b we show the amount of non-void pixel of DNA *versus* the respiration rates,
383 stressing that these values were obtained with the 40X images. Results show that in all
384 cases there is positive correlation between oxygen decay and the proportion of non-void
385 pixels. This is regardless of the biofilm component analyzed (phicobilins, EPS, or Chl-a).

386 **Figure 7 goes here**

387

388 **4. Discussion and conclusions**

389 The 3D spatial structure of the biofilm analyzed in this study showed the presence of
390 microbial structures tightly associated, whose size and spatial distribution were strongly
391 modulated by different environmental conditions. Previous studies have already shown the
392 effect of light and temperature on biofilm structure by CLSM observations (e.g., Díaz
393 Villanueva et al., 2011). Our work represents a step further in the analysis of biofilm
394 structures, by quantifying the spatial distribution and characteristic scales of the microbial
395 assemblages of algae, EPS, cyanobacteria and bacteria. To this aim, the geostatistical
396 analysis of the CLSM images represents a powerful tool to determine the most significant
397 features of the microbial spatial distribution at the micro-scale.

398 The 3D information based on 20 slices separated 1 micrometer, indicated that most of
399 the biological structures were located at the bottom slices, impeding to assess the statistical
400 stationarity along the vertical. Therefore, the 2D projection of the 3D data was used to

401 study the one-week-old biofilms. The sampled variograms of the 2D projection displayed a
402 well-marked sill, characteristic of a stationary variable (no observed spatial trends), and the
403 best fit was obtained by a nested model of two exponential components. The range of the
404 first structure provided the characteristic length of small aggregates of 1.5-3 μm, quite
405 constant regardless environmental conditions. More, the small-scale aggregates were not
406 located following an independent (Poisson-type) distribution, but rather, part of the total
407 variance (an average of 28%) was explained by a second structure with somewhat larger
408 ranges, indicating the presence of middle-size aggregates whose size is highly dependent on
409 temperature and light conditions. Additionally, the lack of continuity at the origin in the
410 variogram models, indicated that the aggregates presented an irregular shape (far from an
411 spherical shape).

412 In the case of cyanobacteria, the variographic analysis indicated that the largest range
413 is observed for the 20D case, suggesting that smaller aggregates could be observed under
414 light conditions than under dark ones, and in the dark such larger aggregates, are more
415 scattered in space. As expected, the number of pixels occupied by cyanobacteria was larger
416 under light than dark conditions for a given temperature. The differences in ranges between
417 dark/light treatments could be related to the capacity of cyanobacteria to adapt their
418 morphology based on light condition (Iijima et al. 2015). For instance, cyanobacteria have
419 been found to exhibit elongated shapes when grown under low light conditions (de Marsac
420 et al 1993), and in our study this is visible for the 20 ºC.

421 On the contrary, in the case of bacteria, clustering was more sensitive to temperature
422 than to light conditions, suggesting that bacteria clusters included a majority of
423 heterotrophs, whose growth is favored by temperature and non-affected by the light

424 conditions. For algae, main differences were found on the size of large-scale aggregates,
425 which increased with light and temperature, and could be related to a change in species
426 composition such as greater abundance of filamentous green algae (Pillsbury and Lowe,
427 1999). The ranges for EPS were quite similar to those of cyanobacteria (around 20%
428 smaller in the latter), as EPS are expected to be placed surrounding the cells. The relative
429 area occupied by EPS under 10L conditions was unexpectedly larger than in 20D and 20L;
430 however, Varin et al. (2012) found that cyanobacteria may produce large amount of EPS in
431 cold environments to enable optimal growth at low temperatures.

432 The characteristic spatial scales of the aggregates were then compared to the
433 respiration rates measured in the biofilms grown at different conditions of light and
434 temperature to investigate the link between biofilm function and structure. Variations in
435 respiration rates were observed between the one-week (young) and the four-week (mature)
436 biofilms. In addition, respiration rates increased in biofilms grown at 20°C, as compared to
437 those grown at 10°C, and no differences were observed for light conditions. The fact that
438 the 10L biofilm presented similar respiration rates than the 10D, although in the former a
439 larger area was occupied by microbes, may suggest that microorganisms of biofilm grown
440 under dark conditions are more efficient (higher respiration activity per cell) than the ones
441 of biofilms grown under light conditions. The latter may contain a largest presence of
442 inactive cells, as observed for natural grown biofilms (Romaní et al., 2004).

443 The temporal evolution of the 2D oxygen concentration distribution highlighted the
444 development of micro-environments with steep oxygen gradients in all the samples
445 analyzed. In statistical terms, the distribution of oxygen concentration was positively
446 skewed and resembled lognormal distributions both after 3 minutes and 30 minutes of the

447 respiration monitoring period. Note that the average size of the pixels in the oxygen maps
448 (8.7 μm) was comparable to the spatial scale of the large aggregate, being about 11 times
449 greater than the size of the pixel in the confocal images (0.76 μm). Thus, most of the pixels
450 in the oxygen maps contained some non-void cells of biofilms, and respiration occurred in
451 mostly all pixels. That is, for all environmental conditions there was a fast transition during
452 the first minutes of respiration, with a significant reduction for all pixels and a positively
453 skewed distribution, which has often been found to adequately describe the distribution of
454 the microbial populations (e.g., Hirano et al., 1982; Hosoda et al., 2011). Overall, we found
455 that higher values of respiration rates were associated to larger amount of non-void pixels
456 of bacteria, suggesting that microbial respiration was responsible for the oxygen decay
457 depicted at the fine (mm) scale. We conclude that: (i) using geostatistical analyses is a
458 valuable tool to quantify the size and spatial pattern of microbial aggregates, and (ii) the
459 combination of optodes and CLMS is a promising way to relate the oxidative metabolic
460 activity to the biofilm architecture at the micrometric scale.

461

462 **Appendix A: 3D vs. 2D characterization**

463 This appendix aims to compare the statistical analysis for the 3D and 2D projection of the
464 10D condition for cyanobacteria (red channel). A total of 512x512x18 pixels were
465 analyzed, with only 0.03% being considered positive, i.e., occupied by biofilm. Positive
466 pixels were mostly concentrated in the bottom slices. For the 3D case, the sample
467 variogram in the horizontal direction was fitted by the superposition of two models, both
468 spherical. The first one accounted for 90% of the total variance and had a range of 2 pixels
469 (=1.5 μm). The range of the second one was of 15 pixels (about 11 μm). Similar to the 3D

field, the sample variogram of the 2D projection was fitted by the superposition of a short- and a medium-range structures, both exponential. The first one accounts for 91% of the total variance and has a range of 3.6 pixels (=2.6 μm). The second one accounts for the remaining 9% variance, with a 10 pixel (=7.3 μm) range. The total amount of occupied pixels is 0.033%.

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List of Tables

Table 1. Statistical (variographic) analysis of the CLSM 2D projections from the 10X magnification images for cyanobacteria (red channel; R), EPS (green channel; G) and algae

(blue channel; B). The table includes the proportion of non-void pixels (p), and the variance (p(1-p) in a binary field), as well as the parameters representing a two-nested statistical structure, each range indicative of the size of aggregates and each sill their relative contribution to the total variance. Values are means for the two analyzed stacks.

Table 2. Statistical analysis of the different models indicating the fraction of pixels occupied by bacteria (DNA stain, grey channel), and the parameters corresponding to the variographic study. Analysis was performed on the 40X magnification images. In this case, only the range of the smallest aggregate could be assessed.

Table 3. Coefficients *a* and *b* (with standard deviations in parenthesis) representing the parameters of exponential regression for mature (28 days old) and young (7-day) biofilms growing at different conditions of light and temperature. The regression coefficients R^2 are also reported.

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Figure 1. Top: 3D CLSM images (corresponding to 10X magnification) of biofilm grown over 7 days for the four combinations of environmental conditions of light and temperature: 10D, 20D, 10L and 20L. The different biofilm compartments are shown in red (phycobilins autofluorescence, cyanobacteria), green (concanavalin stain, EPS), blue (chlorophyll autofluorescence, algae), and light grey (DNA stain, bacteria). Bottom: the corresponding two-dimensional projections.

595 **Figure 2.** Percentage of non-void pixels of cyanobacteria, algae and EPS corresponding to
596 the 2D projections of the 3D CLSM 10X magnification images for the one-week-old
597 biofilms. In the case of bacteria (shown in the inset), 40X images were used for the
598 analysis. Values are the average from the two stacks analyzed per biofilm.
599
600 **Figure 3.** Spatially averaged oxygen values (expressed as % air saturation) for the one-
601 week-old biofilms grown under different conditions of light and temperature and for a
602 mature (four-week-old) biofilm grown at 20L conditions. Bars represent the standard
603 deviation. The specific rates calculated for each decay curve are summarized in Table 3.
604
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608 mature biofilm (28-day-old).
609
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611 oxygen concentration decay rates for the 20L biofilm. The oxygen map corresponds to t=30
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613 function of time is shown for the each subzones.
614
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Figure 7. Relationship between respiration rate (coefficient *b* in Table 3) and different statistics related to the CLSM (proportion of non-void pixels and ranges of the nested variograms).