A new method for evaluation of antifouling activity of molecules against microalgal biofilms using confocal laser scanning microscopy-microfluidic flow-cells

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A new method for evaluation of antifouling activity of molecules against microalgal biofilms using Confocal Laser Scanning Microscopy-Microfluidic Flow-cells.

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Abstract

Regulatory developments regarding antifouling molecules encourage the search for non-toxic substances. Evaluation tools must evolve to highlight anti-adhesion effects rather than growth inhibition. The work presented here aimed at developing a method based on confocal laser scanning microscopy-microfluidic flow-cells in order to characterize microalgal biofilms. The first part of the work was dedicated to the setting-up of experimental parameters allowing the production of microalgal biofilms. The results obtained showed that it was indeed possible to produce reproducibly biofilms. The size of microalgal strains appeared to be a key-parameter in the adhesion rate and cells adhesion strength. *Cylindrotheca closterium* cells adhered in lower amount but formed denser biofilms than *Porphyridium purpureum*. The second part of the work focused on the evaluation of a known antifouling molecule, dibromohemibastadin-1 (DBHB). A comparison with the conventionally used method, multi-well plates experiments, was established. The multi-well plates experiments allowed the determination of minimum inhibitory concentration (MIC) for growth and adhesion inhibition (around 80 µM). The flow-cells combined with confocal laser scanning microscopy (CLSM) enabled the observation of biofilm, the determination of kinetics parameters of adhesion and an estimation of the adhesion strength.

Keywords: Microalgae, flow-cell, adhesion, biofilm, CLSM, multi-well plate, bioassay, antifouling, methodology, *Cylindrotheca closterium*, *Porphyridium purpureum*.

1. Introduction

Biofilms consists of agglomerates of microorganisms surrounded by a self-produced extracellular matrix (ECM) composed primarily of extracellular polymeric substances (EPS). In seawater, biofilms on submerged surfaces are mainly composed of bacteria and microalgae (Wahl 1989, Cooksey and Wigglesworth-Cooksey1995, Dang and Lowell 2016). These complex structures have been observed by sampling biofilms from various substrates collected in the marine environment (Arrhenius *et al.* 2014, Maso *et al.* 2016, Faÿ *et al.* 2018, Balqadi *et al.* 2018) or by using laboratory methods combining culture and imaging of biofilms (Doiron *et al.* 2012, Reddy *et al.* 2017, He et *al.* 2016, Di Peppo and Congestri, 2017). Indeed, the characterization of biofilms in terms of composition and structure has been made possible by the development of 3-D mapping techniques associated with confocal laser scanning microscopy (CLSM). This method has been extensively used in the studies of bacterial biofilm formation (Azerdo *et al.* 2017, Pamp 2009, Arai *et al*. 2015, Smaldone *et al*. 2014, Haagensen *et al*. 2015), cyanobacteria (David *et al*. 2015) and natural communities (Larson and Passy 2005, Proia *et al*. 2012, Mueller *et al*. 2006, Risse-Buhl *et al.* 2017). The use of microfluidics associated with CLSM is particularly interesting to study microbial adhesion and detachment, biofilm integrity and structural parameters (biomass, thickness and cell viability), as well as biofilm susceptibility to antibiotics (Macia and al. 2014, Pousti et al. 2019). Direct real-time non-destructive visualization of biofilms developed on substrata informs on spatial relationships between organisms as well as with substrata. Nevertheless, the use of flow-cell systems associated with CLSM to observe mono-species microalgal biofilms is so far poorly described (Irving and Allen 2011, Ozkan 2013a). Most of the studies concerning microalgae focus on the design of devices dedicated to biomass and lipid productions, and, treatment of wastewaters (Berner *et al.* 2014, Katarzyna *et al.* 2015, Bruno *et al.* 2012, Boelee *et al.* 2011, Gismondi et al. 2016, Chaudhary *et al.* 2017). Hence, the first objective of this paper was to determine experimental parameters suitable to obtain *in vitro* microalgal biofilms in flow cell by using a system adapted from the protocol of Sternberg and co-workers (Sternberg *et al.* 2006, Tolker-Nielson and Steinberg 2011, Pamp *et al.* 2009). For this, two model microalgae strains were used: C*ylindrotheca closterium* (Bacillariophyceae) and *Porphyridium purpureum* (Porphyridiophyceae). Both strains were chosen because they are involved in colonization of

marine substrates (Jellali et al. 2013) and are commonly studied in colonization assessment (Briand *et al*., 2012, Zargiel and Swain 2014). They are associated with strong adhesion on surfaces and play an important role as exopolysaccharides producers within biofilms (Staats et al 1999, Zaouk et al. 2018). Thus, inhibition of the adhesion of these strains is a real challenge for the maritime industries, especially because they can be as well involved in biocorrosion (Landousli et al., 2011).

In addition, in the context of antifouling coatings and ever more pressing regulatory constraints (for example, EU Biocidal Product Regulation EU 528/2012) the development of tools for assessing anti-bioadhesion molecules is essential. The second part of this study consisted of using the method previously developed to evaluate a known antifouling molecule dibromohemibastadin-1 (DBHB) (Bayer *et al.* 2011, Niemann *et al.* 2015, Ortlepp *et al.* 2007, Le Norcy *et al.* 2017a) in comparison with the reference method (multi-well plates). Generally, microplates or classical microbiological techniques are used to determine minimum inhibitory concentrations (MIC) of compounds against bacteria and microalgae (Plouguerne *et al.* 2010, Iyapparaj and al. 2014, Prakash *et al.* 2015, Gao *et al.* 2014, Bressy et al 2014). However, with these technics, only the impact of compounds on the growth or toxicity can be evaluated and only few papers have studied the impact of compounds on adhesion (Jin *et al.* 2014, Xin *et al.* 2017, Abed *et al.* 2013). In this paper, the impact of a reference compound on cell adhesion, adhesion strength and cell growth rate on surfaces was studied *via* the CLSM-Microfluidic Flow-cells method.

2. Materials and Methods

2.1 Strains

Microalgal strains were obtained from Algobank (Biological Resource Center of the University of Caen Normandie, France): *Cylindrotheca closterium* (AC-170) (Bacillariophyceae, (Ehrenberg) Reimann & Lewin) and *Porphyridium purpureum* (AC-122) (Porphyridiophyceae (Bory) Drew & Ross).

2.2 Growth

Before use, strain axenisation was realized by a treatment with a mixture of three antibiotics: chloramphenicol (100 µg/mL), penicillin (1000 µg/mL) and streptomycin (500 µg/mL) during 24 hours (Druehl and Hsiao, 1969). Both strains were grown in sterile artificial seawater (ASW, 30

g/L, Sigma Aldrich) supplemented with Guillard's F/2 medium at 2% (Guillard and Ryther 1962, Sigma Aldrich) at 20°C. Guillard's F/2 was added after sterilisation and culture medium was stored at 4°C before use. Erlenmeyer flasks were maintained under controlled illumination of 250 µmol.photons.m^{-2}.s^{-1} white fluorescent lamps (Philips Master TL5 HO 54W/840 1SL/20) at 20°C with a 12h:12h light:dark cycle in a Hélios 600 phytotron (Cryotec, Saint-Gély-du-Fesc, France). This procedure favored the cellular growth (Forjan et al. 2005). Cultures of *C. closterium* and *P. purpureum* were initially started with about 10^3 cell mL^{-1}. *C. closterium* reached the exponential phase after 3 days, whereas *P. purpureum* needed more time (6 days). After 15 days, both strains were in stationary phase. *C. closterium* showed longitudinal shape with a length of 65±10 µm, whereas *P. purpureum* showed a round shape (5±0.5µm) (Figure S1).

2.3 Culture in flow-cells

Microalgae were grown under hydrodynamic conditions in flow-cells. The flow-cell system was a tool originally used for the *in vitro* observation of bacterial biofilm (Tolker-Nielsen and Sternberg, 2011) and has been adapted for microalgal biofilm observation. Microalgal biofilm was grown in a three channels flow-cell (1x4x44 mm; 1.76 mL; Biocentrum, DTU, Denmark) prepared with microscope glass coverslip (24x50 mm) (Knittel Glasser, Braunschweig, Germany) (Figure 1). Before the experiment began, the flow-cells were sterilized by flowing a solution of sodium hypochlorite (1.5%, 150 µL/min) for 1 hour. They were then rinsed by ASW supplemented with Guillard's F/2 medium at 2% for 2 hours. All experiments were performed in a thermostatic stove at 20°C under 12:12 h light-dark cycle at 250 µmol.m^{-2}.s^{-1}. Distance between the flow cell and light source (2 fluorescent tubex, T4 TL2004A, 12W, Intertek, France) was 13.5 cm.

2.3.1 Adhesion of planktonic cells

250 µL of microalgal culture (10^5 cells/mL) diluted in ASW supplemented with Guillard's F/2 medium (2%) were inoculated in each channel by using a sterile 1mL syringe. The medium flow was not activated, thus allowing attachment of cells to the glass surface. Hence, the adhesion step was realized in static condition. Four incubation times were studied: 8, 24, 48 and 72 hours. The number of adhered cells per cm^2 after the incubation time was evaluated by CLSM observation (Zeiss, LSM710) equipped with helium-neon laser source and X20 air objective. The acquisition

software was Zen Software (Zeiss). Cells were observed by the natural auto-fluorescence of chlorophyll ($\lambda_{excitation}$ = 633 nm, $\lambda_{emission}$ = 638–720 nm). Experiments were realized with three independent cultures. The three channels of each flow cell were inoculated and three randomly observations were realized by channel. Hence, 27 randomly fields were considered during quantification.

2.3.2 Growth of adhered cells

Biofilm maturation was evaluated after the adhesion step (24 hours without any flow of medium to allow the microalgae adhesion). For this purpose, a flow (90 or 15 µL/min) was activated with ASW supplemented with Guillard's F/2 medium (2%) using a Watson Marlow 205U peristaltic pump (Watson Marlow, Falmouth, UK). Biofilm was observed after 24, 48, 72, 96 and 120 hours. Images were taken every micrometer throughout the whole biofilm thickness. The Zen software (Zeiss) was used to process data of 3D image with maximal intensity projection. The average thickness of the biofilm (µm) and the biovolume ($\mu m^3/\mu m^2$) were measured using COMSTAT software (Heydorn *et al* 2000). Results were representative of 27 randomly fields.

2.4 Evaluation of reference compound by multi-well plate assay

Reference compound was dibromohemibastadin-1 (DBHB). It was furnished by Prof. Proksch (Institute of Pharmaceutical Biology and Biotechnology, University of Düsseldorf, Germany). It was evaluated at 0.016, 1.6, 8, 16, 32 and 80 µM in 12 replicates in 96-wells microplate (black with transparent base, Fisher-Scientific, Waltham, MA, USA). Methanol was used as a carrier solvent to add DBHB in the microplate, and was evaporated prior to the bioassay. A well without DBHB was used as control. Then, one hundred microliters microalgal cultures of 2 weeks at 10^3 cells/mL were added in each well and microplates were incubated at 20°C during 5 days under controlled illumination (250.µmol.photon.m^{-2}.s^{-1}) with a 12:12 light-dark cycle. The fluorescence was measured (λexcitation = 633 nm, λemission = 638-720 nm) by using a TECAN microplate reader (Magellan, France) in order to quantify the total cells density in each well. Then, the microplate was emptied by flipping and washed twice with ASW. The fluorescence was again measured in order to quantify adhered microalgae. The difference between the total cells number and the adhered cells number was calculated to obtain the number of planktonic cells. MIC was defined as the lowest concentration that produced a significant reduction in growth or adhesion. Experiments were repeated three times.

2.5 Evaluation of reference compound by flow-cell assay

2.5.1 Impact on adhesion from planktonic cells

The experimental procedure was the same that described above (2.3.1). The impact of compound was evaluated by adding DBHB in the flow-cell at 16 or 80 µM in the microalgae inoculum immediately prior to the inoculation in flow cell channels. After 24h of adhesion, the flow (120 µL/min) was activated during 30 minutes to remove free diatoms. The number of adhered cells was compared to the control without DBHB.

2.5.2 Impact on adhesion strengths

The impact of DBHB on adhesion strength of microalgae was determined after the adhesion stage. Experiment was realized in the presence of DBHB (16 µM) during adhesion step (72h) as described above (2.5.1), then the application of a high shear stress was realized to quantify the removal percentage. The flow was activated at 330 µL/min (8.10^{-3} Pa) during 30 minutes. The cells number adhered on the surface were counted again and the removal percent was determined as following:

$$\frac{cells.cm^{-2}_{before\ flow} - cells.cm^{-2}_{after\ flow}}{cells.cm^{-2}_{before\ flow}} x100.$$

The removal percentage determined for the experiment in the presence of DBHB was compared to the control without DBHB.

2.5.3 Impact on growth of adhered cells

The impact of DBHB on biofilm maturation was evaluated by adding DBHB (16 µM) in the growth medium flow. After 72 hours of adhesion without DBHB, the growth medium containing DBHB was "activated" as described above (2.3.2) at 150 µL/min during 24, 48, 72, 96 or 120 hours. The biovolume and the average thickness were determined with the COMSTAT program. Experiments without DBHB in the growth medium were used as control. All experiments were realized three times.

2.6 Statistical analysis

The one-factor analysis variance (ANOVA) was used to determine the effect of studied conditions. The level of significance was set to $p < 0.01$. Values were means ± standard deviation (sd).

3 Results and discussion

3.1 Culture of microalgal biofilm in flow-cell system.

3.1.1 Adhesion from planktonic cells

Figures 2 and S2 show the adhesion kinetics of *C. closterium* and *P. purpureum* in flow-cells over 72 hours. The count represents the number of cells irreversibly adhered. Single cells were observed on the totality of the cover glass, which indicated that initial attachment of cells was homogeneous. However, the adhered cells number for both strains was significantly different. The number of adhered cells increased with the time: a significant ($p < 0.01$) difference was observed between each observation. For *C. closterium*, the results indicated that the rate of adhesion to glass can be divided in 3 parts: 1) during the first 8 hours, the density increased with a rate of 1280 cells/cm^2/h, 2) the rate decreased to 330 cells/cm^2/h during the subsequent 48 hours ($r^2 = 0.9998$), and 3) finally in the last 24 hours the rate decreased further to 65 cells/cm^2/h. This profile of cells multiplication on glass slide has been already shown for *Chlorella vulgaris* (Ozkan *et al.* 2013a). For *P. purpureum*, the rate adhesion rate showed only 2 parts: after a high adhesion rate during the first 8 hours (2145 cells/cm^2/h), a linear rate ($r^2 = 0.9989$) of 555 cells/cm^2/h was observed to the end of the experiment.

Cells densities ($2.5.10^4$ and $5.1.10^4$ cells/cm^2 respectively at 72h) were significantly different ($p < 0.01$) for *C. closterium* and *P. purpureum*. This observation could be explained by the morphology of microalgae. *C. closterium* is elongated (65 µm) whereas *P. purpureum* is spherical and smaller (5µm diameter). For an equal space and for reasons of steric constraints, more *P. purpureum* cells are likely to adhere than *C. closterium* cells. These results were consistent with the observation of Sekar *et al*. (2004), who indicated variability in the microalgal adhesion levels and an increase of adhesion with exposure time.

3.1.2 Growth of adhered cells

Biofilm formation of *C. closterium* and *P. purpureum* was evaluated under constant flow conditions, after the step of adhesion (24h). In the case of bacteria, it has been established that two factors influenced the structure of biofilms and needed to be controlled: the flow velocity and the nutrient status. Indeed, the flow velocity determined the hydrodynamic shear and the mass transfer characteristics of a system (Stoodley *et al*. 1999). Hence, microalgal biofilms were grown during application of a continuous flow (90 or 150 µL/min) in the presence of medium culture (ASW supplemented with Guillard's F/2). Microalgal biovolume was quantified during 120 hours and the growth kinetics was determined (Figure 3). The cellular density increased with time and the medium flow velocity. As for bacteria, an increase of fluid flow velocity in the flow-cells resulted in faster development of microorganisms due to higher nutrients transport (Bussher and van der Mei 2006). For both strains, the biovolume increased linearly with time after 48 hours of medium flow which indicated a cellular multiplication at a constant rate during the time of experiment. In the case of *C. closterium* a time lag was observed. Then, a growth rate could be determined. Results showed a significant higher ($p<0.01$) growth rate at a flow velocity of 150 µL/min (0.1102 $\mu m^3/\mu m^2/h$) compared to the flow velocity at 90 µL/µmin (0.0612 $\mu m^3/\mu m^2/h$). The biomass reached after 120 hours a value significantly higher (9.1 $\mu m^3/\mu m^2$ versus 5.5 $\mu m^3/\mu m^2$ respectively). For *P. purpureum*, no time lag was observed. The cellular multiplication took place mainly in the first 48 hours, then a decrease of cellular multiplication was observed indicating the end of the maturation process: the increase in the cells number between two times was not significant ($p>0.05$). Moreover, the impact of flow velocity was not significant ($p>0.05$): similar growth rates (0.0083 $\mu m^3/\mu m^2/h$) were determined. On the other hand, the biovolume was significantly ($p<0.01$) higher for *C. closterium*: the denser biofilm was obtained for a flow velocity of 150 µL/min. This result could be explained by the size of cells and their respective growth rates (10 times greater on average for *C. closterium*).

The microscopic observations of biofilms developed during 48, 72, 96 and 120 hours at a flow velocity of 150 µL/min are shown in Figure 4A. The qualitative results described the spatial organization of microalgae in biofilm: cells agglomerated to each other and at the glass surface. Results showed the capability of *C. closterium* and *P. purpureum* to form a biofilm in flow-cell experiment after 120 hours. Nevertheless, the structure of biofilm was dependent on the strain studied. In the case of *C. closterium* biofilms, cells formed dense aggregates, whereas in *P. purpureum* biofilms cells were more scattered. Based on the biofilm image analysis, biovolumes

were quantified for each strain and results are shown Figure 4B. Biofilm formation was highly repeatable between independent experiments (standard deviation lower than 1 $\mu m^3/\mu m^2$ and 0.2 $\mu m^3/\mu m^2$ for *C. closterium* and *P. purpureum* biovolume respectively). However, the observed biofilms indicated average thicknesses relatively low (< 40 µm) (Figure 4C) compared to those of millimeters or more observed in natural environment (Irvin *et al*. 2011). The biofilm observed for *C. closterium* was denser (9.1 $\mu m^3/\mu m^2$) and thicker (38 µm) than for *P. purpureum* which indicated only 1.5 $\mu m^3/\mu m^2$ and 10.2 µm for biovolume and average thickness respectively. *C. closterium* covered more quickly the surface, then cells adhered to each other to form a 3D structure (from 72 h). Another species of the genus *Cylindrotheca* (*C. fusiformis*) was identified as excellent candidates to form biofilms due to their strong attractiveness to glass but also cell to cell interactions (Ozkan *et al.* 2013b). On the contrary, *P. purpureum* developed longitudinally on the surface. A relatively homogeneous surface pattern interspersed with a few macro-colonies was observed after 120 hours. These differences could be explained in particular by the size of cells.

3.2 Use of culture in flow-cells to evaluate antibiofilm/ anti-bioadhesion compounds

The use of bioassays to evaluate anti-biofilms compounds is essential to screen potential substances and understanding their mode of action. Among bioassays developed, the use of multiwell plates is particularly common (Trepos *et al.* 2014, Hellio *et al.* 2015). However, biofilms developed in flow-cells could also be useful to evaluate the anti-microalgal biofilm activity of compounds (Table 1). Data obtained with both bioassays are listed in Table 2 and it appeared clearly that study in flow cells led deeper analysis.

In this context, a reference compound has been tested using both bioassays: multi-well plates and flow-cell system. A hemibastadin analog named dibromohemibastadin-1 was selected. It has been shown an anti-bacterial biofilm and anti-quorum sensing activities without toxic activity (Le Norcy *et al.* 2017a). DBHB can inhibit the settlement of cyprid larvae of *Balanus improvisus* and byssus formation of *Mytilus edulis* without toxicity on nauplii of *Artemia salina* (Bayer *et al.* 2011, Niemann *et al*. 2015, Ortlepp *et al.* 2007). Moreover, on four species tested, DBHB affected the growth of two microalgae (*C. closterium* and *Exanthemachrysis gayraliae)* with a MIC of 80 µM after 5 days (Le Norcy 1017b).

263

264 *3.2.1 Study of DBHB by multi-well plates*

265 DBHB activity (at concentration from 0.016 to 80 µM) was evaluated towards microalgal adhesion
266 and growth and MIC values were determined. MIC is defined as the lowest concentration that
267 produces a significant reduction in growth or adhesion (Hellio *et al.* 2015). Strains showed different
268 sensitivity to DBHB. DBHB exhibited an inhibitory activity against *C. closterium* (80 µM) in terms
269 of both growth and adhesion whereas *P. pupureum* displayed a lower sensitivity towards DBHB
270 (no inhibition up to 80µM). This result correlated well with previous studies using the same
271 bioassay for the screening of natural antifoulants: *P. purpureum* was described as a resisting strain
272 (Moodie *et al.* 2018).

273

274 *3.2.2 Impact of DBHB on adhesion of planktonic cells by flow-cells*

275 From results obtained using multi-well plates, the evaluation of DBHB activity against microalgal
276 adhesion was realized by using flow-cell system. It was operated at two concentrations: 16 and 80
277 µM. At 16 µM, no impact on the microalgal growth was observed in multi-well plates whereas 80
278 µM corresponding to the MIC.

279 The impact of DBHB on *C. closterium* and *P. purpureum* is evaluated (Figure S3). For this purpose,
280 DBHB (16 or 80 µM) was injected in the flow cell at the same time as microalgal cells. After 24
281 hours of static adhesion, a difference of adhesion was observed between both strains. DBHB did
282 not affect the *P. purpureum* adhesion at the tested concentrations. Concerning, *C. closterium* a
283 significant ($p<0.01$) decrease of adhesion was observed for a concentration of 80µM whereas for
284 16 µM, no significant effect ($p>0.05$) was observed on the number of adhered cells. All results
285 corroborated with those mentioned above. However, it could be pointed out that for *C. closterium*,
286 in the presence of 80 µM, the inhibition rates diverged: inhibition percentages of 100% and 40%
287 (comparatively to control) were quantified for multi-well plates and flow-cell bioassay,
288 respectively. The adhesion of cells was influenced by several parameters. For example, the
289 adhesion surface (polystyrene versus glass), the physiological state of cells during experiment (5
290 days versus 24 hours), the inoculation cellular concentration (10^3 *vs* 10^5 cells/mL), the washing

291 method (pipette versus peristaltic pump) were parameters that could impact microalgal adhesion
292 evaluation.

293

294 *3.2.3 Impact of DBHB on adhesion strengths in flow-cells*

295 The use of flow-cells allowed the determination of the impact of DBHB on the adhesion strengths
296 of microalgae. For that, after 72 hours of adhesion in flow cells with DBHB, a continuous flow
297 (330 µL/min, 8.10^{-3} Pa) was applied during 30 minutes. The concentration of 16 µM was applied
298 to be lower than the MIC (80µM). The number of adhered cells on glass slide was quantified before
299 and after activation of flow and the removal percentage was determined. These data provided key
300 information about adhesion strengths of cells on the glass surface which increased with time as
301 observed in previous studies (Schultz et al. 2000, Holland *et al.* 2004, Finlay *et al.* 2013, Alles and
302 Rosenhahn 2015, Nolte et al 2017). Concerning *C. closterium*, a significant ($p<0.01$) increase of
303 removal percent was observed after 72 hours in the presence of DBHB (from 24 ± 4% to 41 ± 2%):
304 the adhesion strengths were lesser. This result was particularly interesting because *C. closterium* is
305 a large diatom. It has been shown that the magnitude of interactions (as electrostatic, van de Waals
306 and acid base) increased with increasing cell size. Moreover, the drag forces acting on cells
307 augmented with increasing cell size in systems involving medium flow (Ozkan *et al.* 2013b). For
308 *P. purpureum*, no significant difference was observed between the standard and the experiment
309 containing DBHB. DBHB did not act in the same way against *C. closterium* than *P. purpureum*.
310 The adhesion of microalgae was strongly linked to the physico-chemical attraction between
311 microalgal cells containing bound extracellular polymeric substances and the substratum (Klein *et*
312 *al.* 2014, Ozkan *et al.* 2013a). Hypothetically, DBHB could affect these parameters disturbing not
313 only the number of adhered cells but also their adhesion strengths. Hence, flow cell bioassay could
314 inform not only on the ability of bioactive compounds to inhibit the adhesion but also on
315 mechanisms of action of bioactive compounds.

316

317 *3.2.4 Impact of DBHB on growth of adhered cells in flow cell*

318 To evaluate the impact of DBHB on the growth of adhered cells, the compound in solution (at 16
319 µM) was added in the growth medium. After application of a continuous flow, the biovolume

developed on glass slide was quantified. Two different behaviors were observed depending on the strains.

For *C. closterium*, no cell was observed after 24 hours. Cells previously adhered in flow cell system were probably detached from glass surface in the presence of DBHB and the application of flow. Results confirmed those mentioned above: DBHB decreased *C. closterium* adhesion strengths.

For *P. purpureum* no effect of DBHB was observed. Whatever the incubation time tested, the biovolume developed in the presence of 16 µM DBHB was not significantly different ($p>0.05$) from control (Figure S4). This result was in accordance with results obtained in multi-well plates: DBHB did not affect *P. purpureum* growth.

4. Conclusion

The results of our experiments have shown the ability of microalgae to form biofilms in flow-cells. *P. purpureum* cells adhered in higher number on glass surfaces than *C. closterium* cells. Nevertheless, *C. closterium* formed thicker biofilms. The results corroborated those obtained with the reference method (multiwell plate). However, flow-cell system was able to discriminate the adhesion of planktonic cells and the growth of adhered cells contrary to multi-well plate assay. Additional data could also be obtained as the impact of compounds on adhesion strengths and the morphology of biofilms. The flow-cell system appeared to be a complementary method to multi-well plate assay, which remains a first essential step for the screening of potential antibiofilm compounds. This method is more informative but it is nonetheless more difficult to implement, requires more compound and especially requires a confocal laser scanning microscope to provide biofilms structural observations and digital data as biovolume. The method can be applied in the research of antibiofilm/antifouling compounds (natural substances, commercial organic biocides) used in antifouling paint as well as in other domains impacted by biofilms development (water treatment, fish farming, industrial processes…). Biochemical process and mechanisms could be evaluated in further works to improve the current knowledge.

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Declaration of interest: none

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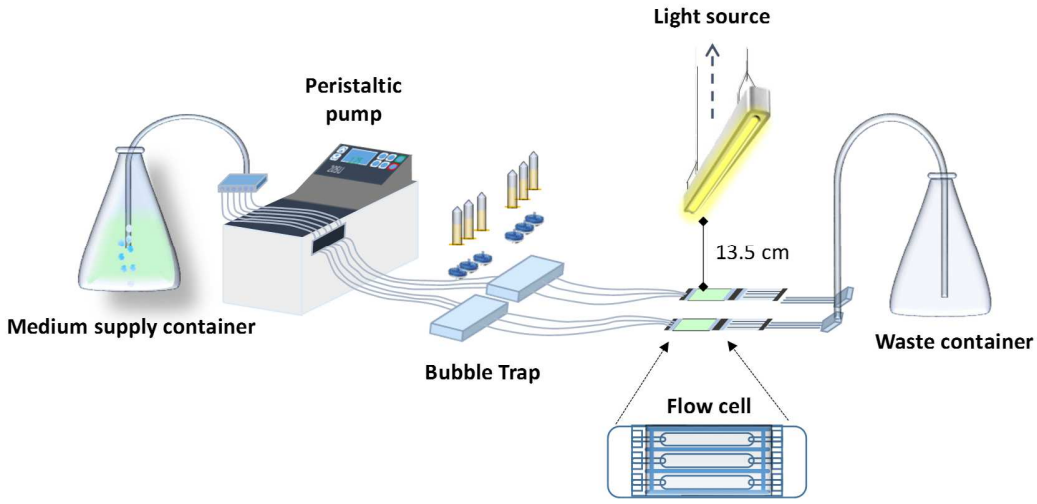
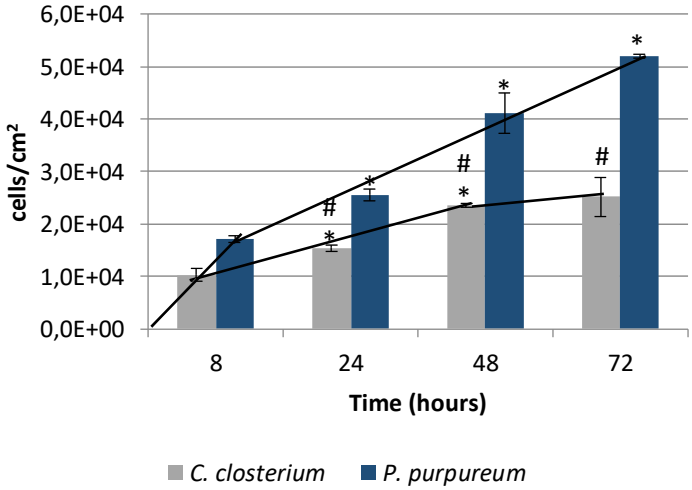


Figure 1. Flow cell system adapted from Tolker-Nielson and Steinberg (2011)



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551 Figure 2: Adhesion kinetics on flow cell during 72h for *C. closterium* and *P. purpureum*. ANOVA
552 *p <0.01, compared to the precedent time #p <0.01; compared between microalgae. The errors bars
553 represent standard deviations calculated from three independent experiments.
554

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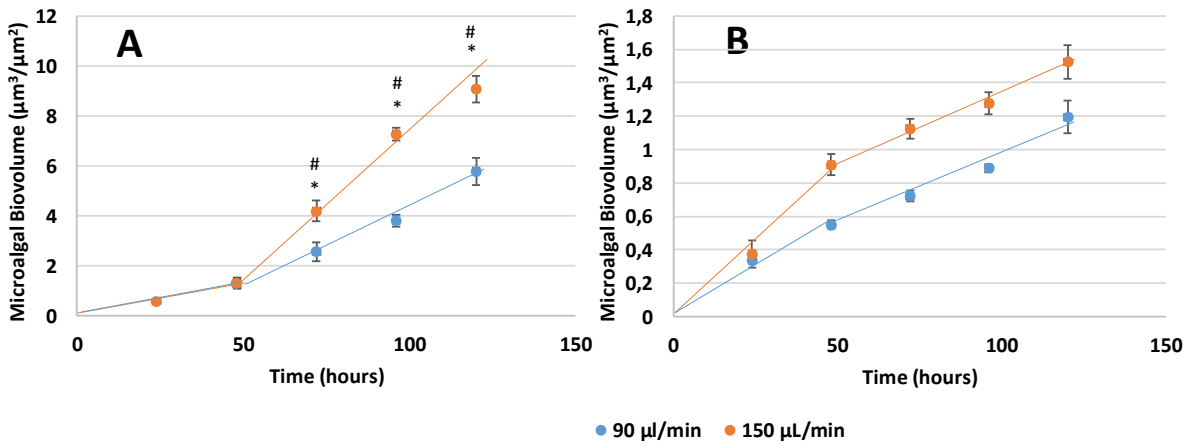

Figure 3: Effect of medium flow on microalgal biomass during biofilm maturation (A) *C. closterium*, (B) *P. purpureum.* ANOVA *p <0.01, compared to the precedent time #p <0.01; compared between flows. The errors bars represent standard deviations calculated from three independent experiments.

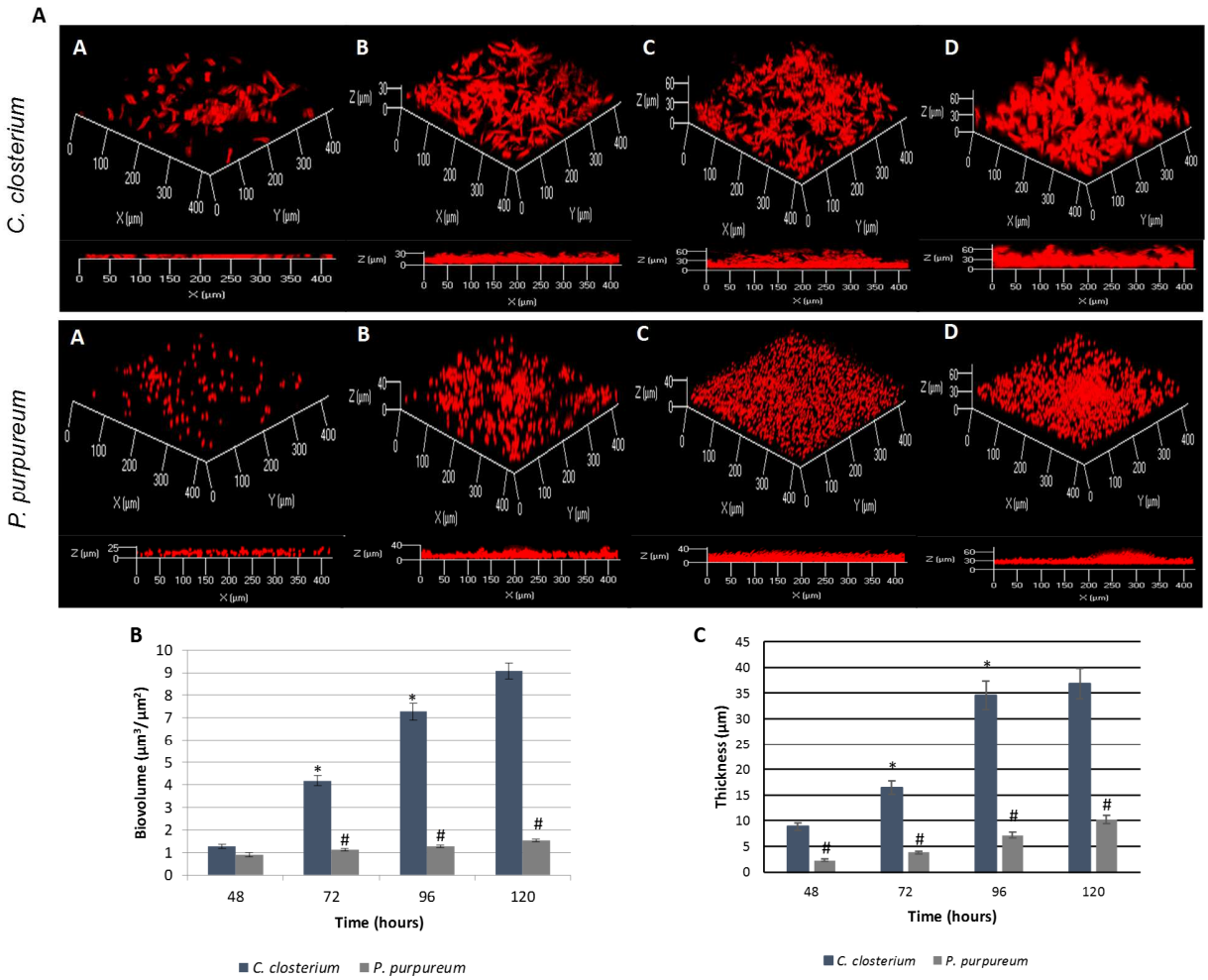

Figure 4: (A) CLSM observations of biofilm formation pour *C. closterium* and *P. purpureum* with a flow of 150 µL/min A. 48h, B. 72h, C. 96h, D. 120h, (B) Biovolumes and (C) Average thicknesses for *C. closterium* and *P. purpureum* during maturation stage. ANOVA, *p <0.01, compared to the precedent time, #p <0.01, compared between microalgae

570 Table 1. Overview of the classical *in vitro* method used to study antimicroalgal activity of
571 molecules and flow cells method

	Multi-well plates[a]	Flow-cells
Nutrient availability	Closed system (static)	Open system (dynamic)
Adherence	Wells (polystyrene)	Glass of coverslip
Time incubation	5 days	Between 2 hours and 6 days
Biofilm vizualisation	-	Direct real-time
Structural analysis	-	Analysis of structural parameters
Compound incubation	In wells	Added to the medium bottle and circulated through the flow cell for the required time

572 [a] Hellio et al. 2015, Treppos et al. 2014, Reddy et al. 2017

573

574 Table 2. Comparison of data evaluated with multi-well plates and flow-cells system to evaluate
575 bioactive compounds

	Multi-well plates	Flow-cells
Parameters	▪ Planktonic growth ▪ Biofilm from planktonic and adhered cells	▪ Adhesion ▪ Biofilm from adhered cells ▪ Adhesion strength
Variables	▪ Screening of compounds ▪ Concentration range	▪ One compound ▪ One concentration ▪ One strain ▪ Variability of surfaces
Results	▪ Growth MIC ▪ Adhesion MIC	▪ Cell enumeration by surface unit ▪ Biofilm morphology ▪ Biovolume, biofilm thickness ▪ Adhesion strength ▪ Extracellular Polymeric Substances observation

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