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Accurate physiological monitoring using lab-on-a-chip platform for aquatic micro-organisms growth and optimized culture

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Abstract

The present work was dedicated to the development of a lab-on-chip microsystem for the monitoring of microalgal photosynthetic activity. Thanks to integrated electrochemical microcells, dissolved oxygen O₂ concentrations due to photosynthetic microalgal activity were measured in a continuous way and oxygen production rates of microalgae cultures were finally determined in the frame of artificial night/day cycles. Application was performed by studying the physiological metabolisms of the green microalga *Chlamydomonas reinhardtii*. First, the different growth dynamics of microalgal cultures were characterized, enabling the determination of the induction, "exponential growth", "declining growth rate" and stationary phases. Then, the influences of carbon-based nutrients, such as sodium bicarbonate HCO₃Na and methanol CH₃OH, as well as of pollutants, such as silver nitrate AgNO₃, were studied, evidencing contradictory behaviors according to the competition between nutritional properties, toxicity effects and acclimation phenomena. This paves the way to the development of analysis microsystems for the understanding of microalgal metabolisms as well as for the improvement of microalgae growth processes and associated industrial production.

Keywords

Biosensor, electrochemical sensor, lab-on-chip, green microalgae culture, algal metabolisms

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1. Introduction

The number of microalgae species is estimated at several hundreds of thousands [1]. This vast diversity represents a strong potential for science and industry. Since 2010, various studies have shown that microalgae are a rich source of carbon compounds, which can be used in biofuels, health supplements, pharmaceuticals and cosmetics [2]. However, this fast increasing involves the cultivation of aquatic micro-organism biomass for generating high value product. This production is done either in closed photo-bio-reactors (PBR) or, more traditionally, in open systems which constitute a simple and mature technology already deployed at industrial scale, but without any integrated monitoring unit to deal with microalgal growth. In both cases, it is important to ensure that the conditions are the best to ascertain a perfect microalgae growth. This production and development require different key parameters to be measured: nutrients quantity and quality, light, pH, turbulence, salinity and temperature [2].

In general, variables to be monitored in microalgal growth processes can be categorized as physical, chemical and biological. For this last category, the monitoring process relies mostly on principles developed and validated using laboratory-scale systems because of difficulties in developing suitable online sensors. Recently, Havlik *et al.* reviewed some technologies commonly-used for online, in situ monitoring of process parameters associated to microalgae cultures, with a focus on the monitoring of physical (temperature, illumination), chemical (pH, pO₂, pCO₂, nitrogen-based or phosphorous-based nutrients) and biological (biomass composition and concentration, photosynthetic activity...) parameters [3]. Moreover, microalgae themselves may also be used as living biosensors since changes in photosynthetic activity and fluorescence allow to go back to the presence of pollutants in the analyzed water. In this frame, microfluidics and lab-on-a-chip (LOC) developments propose low-cost, multi-parameter, real-time, continuous monitoring methods [4,5] while focusing on electrochemical [6] and optical [7] detection techniques. In order to deal with selectivity, beyond the use of several microalgae, the functional integration of electrochemical and optical methods will be required. Indeed, both detection principles are adapted to the analysis of microalgal bio-metabolisms (for example through the measurement of acidity/basicity, dissolved oxygen concentration, photosynthetic activity and fluorescence) but are concerned by different interferences. They can therefore cope more easily with water sample complexity in the frame of water pollution analysis. As a result, several works have proposed the development of lab-on-a-chip with both electrochemical and optical integrated microdevices in order to study microalgae cultures [8-14]. In this context, the use of such LOC platform will tackle off some

bottlenecks associated to microalgae cultures. Among others, response times, a major parameter for improving process feedback, could be greatly improved, leading to the development of real-time analysis approach.

This work describes the use of our integrated lab-on-a-chip recently developed [14] to accurately track the physiological behavior of the green microalga *Chlamydomonas reinhardtii* (Cr). It is shown how it is possible to monitor growth rate or substrate/pollutant effect on microalgal populations in microvolumes, focusing on the physiological adaptation of aquatic micro-organisms to induced stress in solution.

2. Materials and methods

2.1 Chemical

The lab-on-a-chip detection platform was developed in previous works [10,14]. Its microfabrication process is based on microelectronic mass production techniques in cleanroom. It leads to low-cost, easy to use microsystems and reproducible properties from one component to another. Specific B33 glass substrates were chosen in order to be cope with optical measurement [8]. Indeed, in addition to the integration of electrochemical devices described in this paper, the optical properties (transparency, low absorption) of this type of support make it possible to perform observations under microscope for the counting of micro-organisms, to initiate microalgae photosynthesis by optical illumination, and/or to consider alternative optical measurements required for the chosen culture.

The electrochemical microdevices were fabricated according to the ElecCell (Electrochemical microcell) technological platform previously developed [15]. A double physical vapour deposition (PVD) step allowed producing fully integrated electrochemical microcells: a first titanium/platinum (Ti/Pt) thin film formed the working micro-electrode and the counter electrode as well as the sub-structure of the pseudo-reference electrode. A second silver (Ag) PVD film completed the integration of this last electrode. This layer was oxidized in a KCl solution through a post-process step to obtain a silver/silver chloride pseudo-reference electrode. A fully integrated three electrodes electrochemical microcell were therefore realized. As described in previous works [16,17], a silicon nitride SiN_x wafer-level passivation was performed using a low-temperature PVD process in order to define electrode surface and to ensure their long-term electrochemical stability.

Finally, the microfluidic systems were made by lamination of dry films [18]. The so-obtained microchannels and associated microchambers make it possible to obtain displacements of fluids that do not require the use of micropumps or any other fluid actuation devices. In order to achieve a robust microsystem, the cover was made by wafer bonding using again B33 glass substrate. After dicing and individualization of the chips, the electrical connections were ensured by the welding of a flex, allowing the final realization of the lab-on-a-chip platform (Fig. 1). As a matter of fact, the LOC final analysis volume was estimated to one hundred cubic millimeters or microliters.

Our hybrid microsystem was finally completed by commercial blue-light emitting diodes (LED) (KP-3216QBC-D from Kingbright) that were chosen according to size ($3.2 \times 1.6 \text{ mm}^2$), appropriate wavelength (460–475 nm) and luminance (290 cd/m^2), as well as to a specific excitation filter (ET470/40x from ChromaTechnology Corp). This light source was close to the microalgae microchambers in order to initiate and control the photosynthetic activity.

2.2 Electrochemical apparatus

All electrochemical measurements were carried out by voltammetry with a multi-channel VMP3 potentiostat from biologic in a dark Faraday cage. In the frame of algal solutions, the electroactive species to generate the electrical signal was oxygen (O_2) which is electrochemically reduced on the platinum (Pt) working microelectrode for a potential of -0.7 V versus integrated Ag/AgCl. Thus, dissolved oxygen was finally monitored electrochemically using chronoamperometry techniques.

2.3 Algae cultivation and sample preparation

The green microalga *Chlamydomonas reinhardtii* (Cr) CC-125 was obtained from the "Ecotoxicology of Aquatic Micro-organisms Laboratory" from the Quebecker "Département des Sciences Biologiques (GRIL – TOXEN, Université du Québec, Montréal, Canada). Cr was cultivated in 200 mL Erlenmeyer flasks at 25°C in mineral, liquid high salt medium (HSM) [19] with $\text{pH } 6.9 \pm 0.1$. Media and flasks were autoclaved (120°C , 1.5 bar, 20 min) before inoculation and algal solutions are transplanted once a month under sterile conditions. The cultures were aerated with the atmospheric air containing 2.5% (v/v) of carbon dioxide CO_2 , which is passed through a bacteriological filter, and illuminated by a white light source with irradiance of 30 or 60 cd/m^2 according to culture experiments. Algal solutions were modified

by adding the carbon concentration as a nutrient substrate for the algae: sodium bicarbonate HCO_3Na and methanol CH_3OH (Dow Chemicals Company). Silver nitrate AgNO_3 (Dow Chemicals Company) was also used as a disturbing element of microalgal physiology.

The algal cell growth was synchronized by alternating light and dark periods (L/D – 14/10 h) using the commercial blue-light LED as excitation source for photosynthesis. Two microalgae solution concentrations were studied with 1×10^6 and 6×10^6 cells/mL, noted C1 and C6 respectively, to evaluate the detection properties of the electrochemical sensor according to cell density. The C1 pristine solution study is here performed in order to demonstrate the possibility to use the sensor without any sample pre-treatment. To obtain the C6 concentration, 6 mL of initial algal suspension in the exponential growth phase C1 was centrifuged at 800G and re-suspended in 1 mL of the culture medium. The cell count was carried out by the Fast Read 102[®], which is a plastic slide that allows the simultaneous reading of ten chambers with a microscope [20]. Microalgae samples (volume: 1 mL) were finally analyzed by measuring the evolution of their photosynthetic activity in microvolumes while using LOC-based microsystems [10, 14].

All the experiments concerning the influence of the carbon-based substrates (sodium bicarbonate HCO_3Na , methanol CH_3OH) or pollutants (silver nitrate AgNO_3) on the aquatic micro-organism growth were operated by dilution in fresh algal solutions that were collected the same day. Microalgae cultures in the exponential growth phase were specifically chosen to ensure their long-term viability in LOC-based microsystems [21]. Different solutions (nutritive or toxic according to cases) were prepared for the growth of the aquatic micro-organisms while studying separately the influences of the different substrates/pollutants by varying concentrations. Since the sampling procedure required a five-minute duration, all the measurements were done after at least five minutes to provide the effect on bio-organisms.

3. Results and discussion

Using the same kind of LOC-based sensing platform, previous works concerned the results obtained when determining growth key parameters like pH, concentration of chloride (Cl^-) ions (related to the medium salinity), as well as adapted luminance (290 cd/m^2) for optimized culture of microalgae [14]. Here are developed the monitoring of the microalgae growth carried by measuring the evolution of the photosynthetic activity in microvolumes associated to the realized lab-on-a-chip. Briefly, it can be said that in the presence of light, photosynthetic

activity is deduced from the rate of oxygen production. In the LOC, this rate is determined by measuring the dissolved oxygen (O_2) thanks to the electrochemical microcell integrated in the tank. Figure 2 shows the evolution of the O_2 reduction current obtained on the platinum working electrode during the day/night cycles (period: 60 s).

The "light off" curve slope provides information on the respiratory activity of the algae. This value is used to determine the number of live and active algae in the sample inserted in the LOC. Thanks to this measurement, the "light on" curve slopes can be normalized in order to determine the photosynthetic activities of different algal samples, whatever their concentration [10]. According to previous studies and experiments, the detection limit for the oxygen production rate measurements was estimated to 6 pA/s.

It should also be noticed that this method, compared to conventional counting methods, makes it possible to discriminate the effectively functional algae. Thus, during the "light on" phase, the reduction negative current slope provides finally information on the photosynthetic rate and activity of the micro-organisms, enabling the real-time determination of optimal growth conditions. Finally, thanks to the small volumes in the LOC platform, the total response time is no longer than couples of seconds.

3.1 Growth dynamics

The growth of an axenic culture of microalgae is characterized by five phases. According to the handbook of microalgal culture: biotechnology and applied phycology [2], the theoretical growth curve of a population of microalgae as a function of time is represented according to figure 3, evidencing five different phases of algae growth in batch cultures experiences:

- 1) lag or induction phase: during this initial phase, little increase in cell density occurs: the lag is attributed to the physiological adaptation of the cell metabolism to growth.
- 2) exponential growth phase: during this second phase, the cell density increases as a function of time according to an exponential function, and the specific growth rate is mainly dependent on algal species, light intensity and temperature.
- 3) phase of declining growth rate: cell division slows down when nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to limit growth.
- 4) stationary phase: in the fourth stage, the limiting factor and the growth rate are balanced, which results in a relatively constant cell density

5) death, "crash" or lethal phase: during the final stage, water quality deteriorates and nutrients are depleted to a level incapable of sustaining growth, and cell density decreases rapidly and the culture eventually collapses (in practice and more generally, culture crashes can be caused by a variety of reasons, including nutrients depletion, oxygen deficiency, overheating, pH disturbance or contamination).

3.2 Electrochemical sensing for tracking the speed and growth rate of algal cultures

As explained previously, in the LOC-based microsystem case, the microalgae population growth is studied by monitoring the evolution of the dissolved oxygen concentration during "light-on" phases. Each measurement was made while considering an analysis volume around 10 μL (10 mm^3). During the growth process, the algal solutions (concentrations C1 and C6) were sampled on several different days, and measurements of oxygen production rate were carried out in pico-amperes per second (pA/s). Values are reported on figure 4 and growth phase durations are estimated on table 1.

It should be noticed that, for the pristine solution C1, no measurement was possible until three days because oxygen production rates related to the collected microalgae were below the electrochemical detection limit (6 pA/s), preventing us for checking the induction phase. Results demonstrate that the higher algae concentration, the higher oxygen production rate, and that the oxygen production rate is increasing with time. Nevertheless, similar growth phase durations were estimated for both C1 and C6 solutions. It is assumed that the induction and exponential phases of the microalgae culture growth were evidenced during the first two days and the following eight days. Then, the "declining growth rate" phases occurs as expected (duration: ~ 10 days), followed by the stationary phase (duration: 4 days and more). Since *Chlamydomonas reinhardtii* was characterized by a long stationary duration in certain culture conditions [22], it was chosen not to study the lethal phase.

As a matter of fact, the analysis of microalgae growth metabolism was shown for measurement times around few minutes while considering pristine cultures. Such result should be associated to the use of microvolumes that allows high concentration variations for a given biological activity and/or metabolism [4, 21, 23]. This demonstrates that LOC-based microsystems allow the characterization of environmental samples in real conditions, as well as the determination of the different growth phases.

3.3 Effect of carbon-based substrates on algal culture growth

The composition of the culture medium in carbon-based substrate is supposed to influence on the photosynthetic activity of microalgae like *Chlamydomonas reinhardtii*, and therefore to be intimately linked to the algal growth rate. Here, by varying the intake concentration, effects of methanol CH₃OH or sodium bicarbonate NaHCO₃ supplies, which directly impact algal activity, were assessed for the pristine solution C1 (figures 5a and 5b).

Different starting points were evidenced for the oxygen production rate. Since measurement are normalized according to the microalgae concentration [10], this phenomenon is related to sample preparation. Indeed, even if higher illumination (60 cd/m²) were used during culture in order to decrease preparation duration, the exponential phase was logically responsible for discrepancy of the initial value (about 70 and 35 pA/s for methanol and sodium bicarbonate respectively). Nevertheless, it was decided to discuss the true variations of the oxygen production rate rather than "normalized" values related to the maximal one.

In both cases, the oxygen production rate, and therefore the microalgae photosynthetic activity, is found to increase for the lowest carbon input concentrations and to decrease for the highest ones, evidencing the presence of an optimum. For methanol, this maximal value is obtained for a 75 mM concentration and is associated to a low amplification factor around 1.1. This result is in accordance with S. Stepanov and E. Zolotareva, whose works evidence a maximal amplification ratio of 1.3 for a 50 mM concentration [24]. For sodium bicarbonate, a higher amplification ratio (value: 1.5) was obtained for a concentration of 0.5 mM. Such amplification phenomena were already evidenced for other microalgae such as *Chlorella vulgaris*, *Chlorococcum humicola* and *Desmococcus olivaceum* [25], and are here confirmed for *Chlamydomonas reinhardtii*. As a result, it can be concluded that sodium bicarbonate is characterized by higher growth enhancement, and therefore lower toxicity, than methanol.

Thus, the measurement of the optimum supply of carbon source so as to increase the physiological activity of aquatic bio-organisms was finally evidenced for the pristine solution. As a matter of fact, such results demonstrate the possibility of using LOC-based microsystems to determine quickly and simply the optimal amount of inputs for improving microalgae crops.

3.4 Monitoring of toxicity on algal culture growth

Finally, the influence of silver nitrate AgNO₃ on *Chlamydomonas reinhardtii* (Cr) physiology was analyzed in order to study the alga physiological adaptation to the toxic stress induced by silver ions Ag⁺, well known as an anti-biofouling agent [26,27]. Thus, the study of a 1 μM

AgNO₃ exposition on Cr photosynthetic activity was undertaken using a pristine solution (figure 6). A decreasing photosynthetic activity is shown during the lowest exposure durations, evidencing the toxic effect of silver nitrate. Nevertheless, after about an hour, the behavior of the curve changes drastically. At this time, the oxygen production rate starts to rise again. This can be explained by the assimilation of cellulose from dead cells by algae for photosynthesis [28] and the final acclimation of Cr microalgae to the presence of pollutants for long exposure. This phenomenon was already evidenced by B. Novicka *et al.* for macrovolumes (75 mL) and therefore longer exposures (4 days or about 100 hours) [29]. Indeed, this duration discrepancy should be associated to the use of microvolumes in the case of a LOC-based microsystem, inducing the wide improvement in term of response times.

Such low exposure durations allowed finally to go further and to finally study the toxicity of silver nitrate concentration. Thus, Cr cultures were exposed at different AgNO₃ concentrations levels and, according to our previous results, oxygen production rate was determined after the intrinsic five-minute exposure duration (figure 7). It was shown that the presence of silver ions Ag⁺ in solution decreases the photosynthetic activity, evidencing a linear variation with concentration and characterizing fully the silver nitrate toxicity (sensitivity: 3.0 pA.s⁻¹.μM⁻¹).

4. Conclusion

A portable lab-on-a-chip (LOC) microsystem was developed for the monitoring of microalgae photosynthetic activity while monitoring electrochemically the microalgal oxygen production rate thanks to integrated electrochemical microcells (ElecCell) in the frame of artificial night/day cycles. Demonstration was performed by studying the physiological metabolisms of the green microalga *Chlamydomonas reinhardtii*, dealing with growth phenomena as well as nutrient/pollutant metabolic influences. Thus, the monitoring of physiological activity based on the evolution of photosynthetic activity makes it possible to estimate and differentiate the different growth modes of microalgal cultures, enabling the determination of the induction, "exponential growth rate", "declining growth rate" and stationary phases. Moreover, the influences of carbon-based nutrients, such as sodium bicarbonate HCO₃Na and methanol CH₃OH, as well as of pollutants, such as silver nitrate AgNO₃, were studied, evidencing contradictory behaviors according to the competition between nutritional properties, toxicity effects and acclimation phenomena. Specific attention was brought to the discrepancies associated to the use of microvolumes (around 10 μL or mm³) rather than macrovolumes, evidencing lower durations for microalgal metabolic changes and therefore faster analysis. As

a matter of fact, the proposed integrated lab-on-a-chip is adapted to the use of pristine cultures, small volumes of microalgae samples and small amounts of products/chemicals in order to optimize growth rate. It can therefore be concluded that the use of LOC-based analysis systems will allow to monitor in a simple and rapid way the change of parameters which can affect the microalgae cultures, making possible its optimization. Indeed, dealing with the optimization of bio-organisms culture processes (used for food or antibiotic production for example), the lab-on-a-chip contribution to rapid response times will be an added value in order to manage growth parameters as well as to detect toxic stresses or pollutant influences. It should be possible to check very precisely the growth stage, and to fix almost instantaneously the optimal harvest date. By monitoring growth phenomena and having a precise information of the culture state, it should also be possible to prevent any unsuitable behavior or any contamination of a given culture batch, giving the opportunity to operate a feedback on culture processes. This paves the way to the development of analysis microsystems for the understanding of microalgal metabolisms as well as for the improvement of microalga growth processes and associated agri-business industrial production.

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Figure captions

Table 1: Growth phase durations of algal solutions estimated by oxygen production rate measurements in LOC-based microsystems (C1: pristine solution and C6: six-times concentrated solution, culture illumination: 30 cd/m²)

Figure 1: LOC platform used for the algal physiology monitoring

Figure 2: Evolution of the recorded current through time, reflecting oxygen concentration variation

Figure 3: Five growth phases of microalgae cultures: 1) induction phase, 2) exponential growth phase, 3) declining growth rate phase, 4) stationary phase, 5) lethal phase

Figure 4: Temporal variations of the oxygen production rate of fresh algal solutions (C1: pristine solution and C6: six times concentrated solution, culture illumination: 30 cd/m²)
All data are mean experimental values and error bars are calculated standard deviations

Figure 5: Evolution of the oxygen production rate with carbon nutrient concentration for the pristine solution C1: left) methanol CH₃OH and right) sodium bicarbonate HCO₃Na
All data are mean experimental values and error bars are calculated standard deviations

Figure 6: Evolution of the oxygen production rate with exposure duration to a 1 μM AgNO₃ concentration solution (pristine solution C1)
All data are mean experimental values and error bars are calculated standard deviations

Figure 7: Evolution of the oxygen production rate with silver nitrate concentration (exposure duration: five minutes, pristine solution C1)
All data are mean experimental values and error bars are calculated standard deviations

phase	induction	exponential growth	declining growth rate	stationary	lethal
C1 solution	not detectable	days 3 to 10	days 10 to 20	days 20 to 24+	not studied
C6 solution	days 0 to 2	days 2 to 10	days 10 to 20	days 20 to 24+	not studied

Table 1: Growth phase durations of algal solutions estimated by oxygen production rate measurements in LOC-based microsystems (C1: pristine solution and C6: six-times concentrated solution, culture illumination: 30 cd/m²)

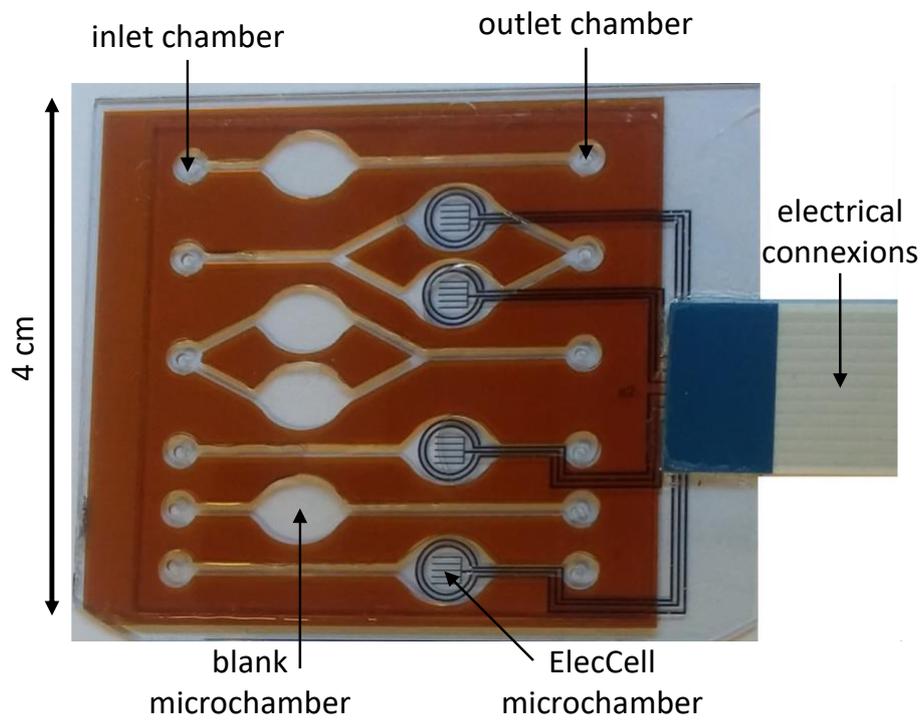


Figure 1: LOC platform used for the algal physiology monitoring

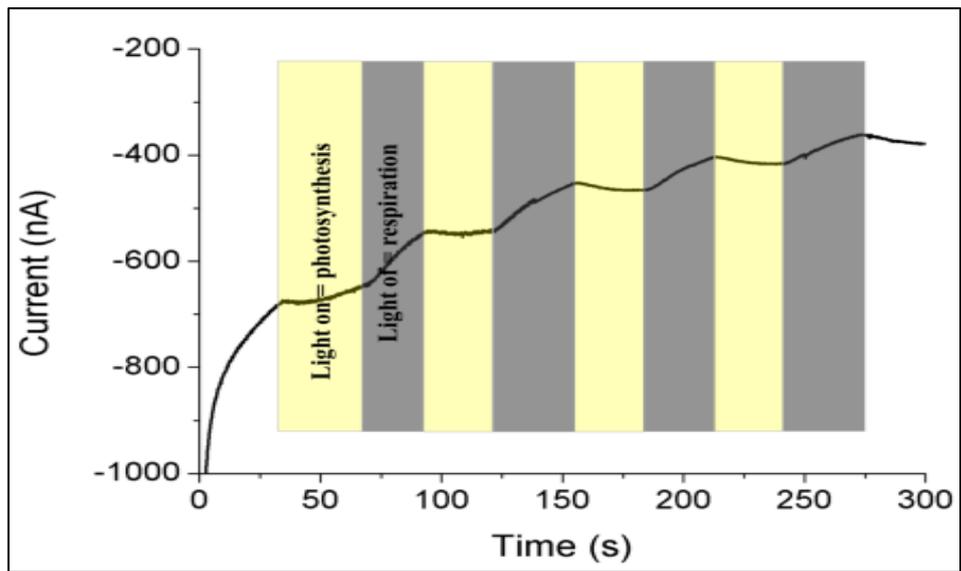


Figure 2: Evolution of the recorded current through time, reflecting oxygen concentration variation

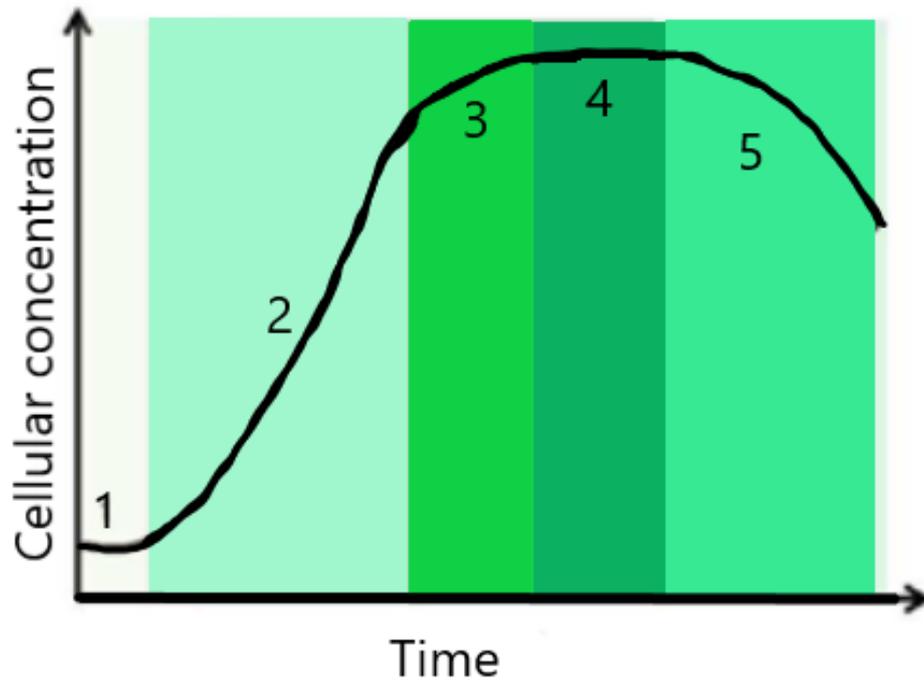


Figure 3: Five growth phases of microalgae cultures: 1) induction phase, 2) exponential growth phase, 3) declining growth rate phase, 4) stationary phase, 5) lethal phase

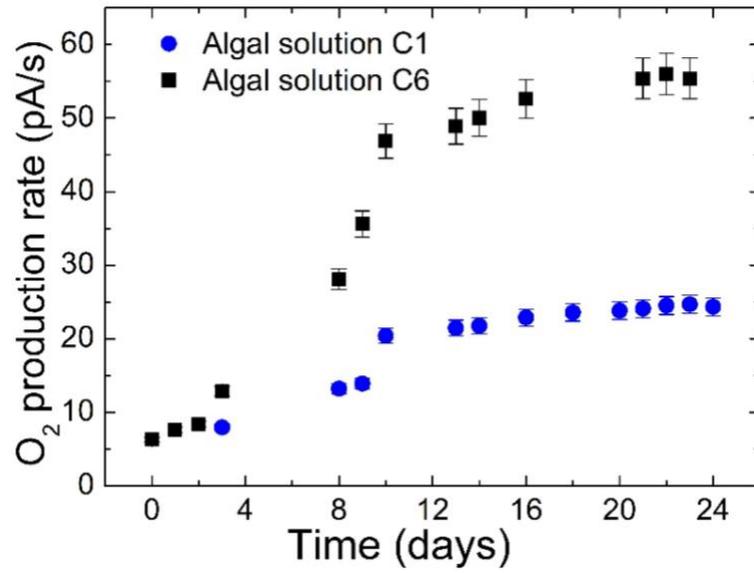


Figure 4: Temporal variations of the oxygen production rate of fresh algal solutions (C1: pristine solution and C6: six-times concentrated solution, culture illumination: 30 cd/m²)
 All data are mean experimental values and error bars are calculated standard deviations

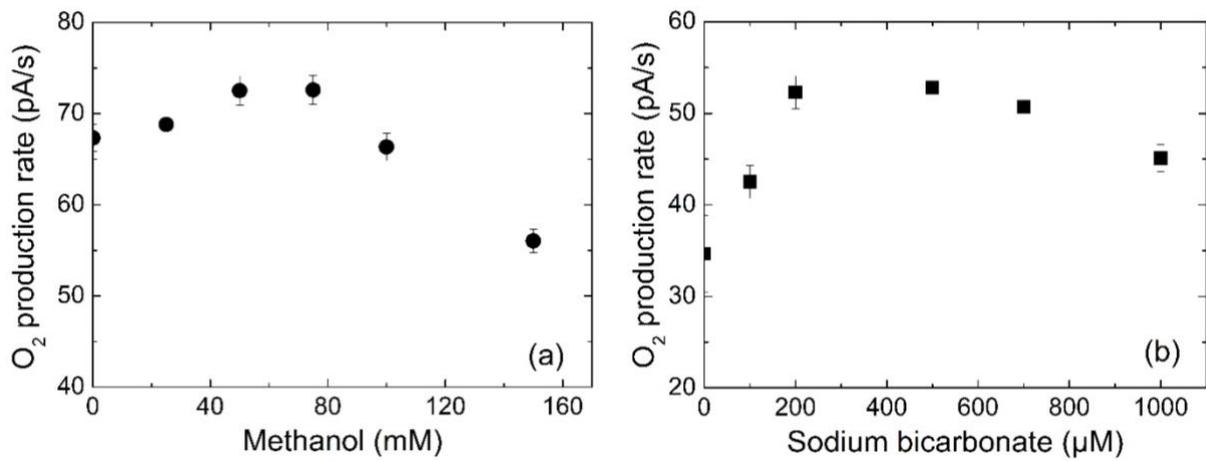


Figure 5: Evolution of the oxygen production rate with carbon nutrient concentration for the pristine solution C1: left) methanol CH_3OH and right) sodium bicarbonate HCO_3Na . All data are mean experimental values and error bars are calculated standard deviations.

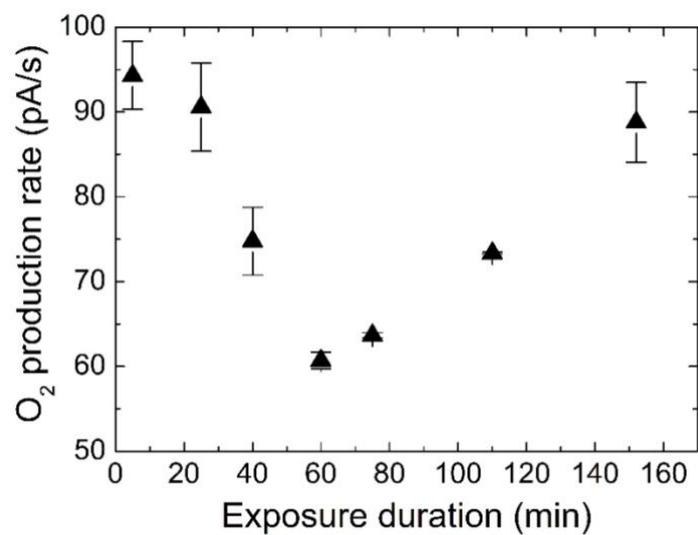


Figure 6: Evolution of the oxygen production rate with exposure duration to a 1 μM AgNO_3 concentration solution (pristine solution C1)
All data are mean experimental values and error bars are calculated standard deviations

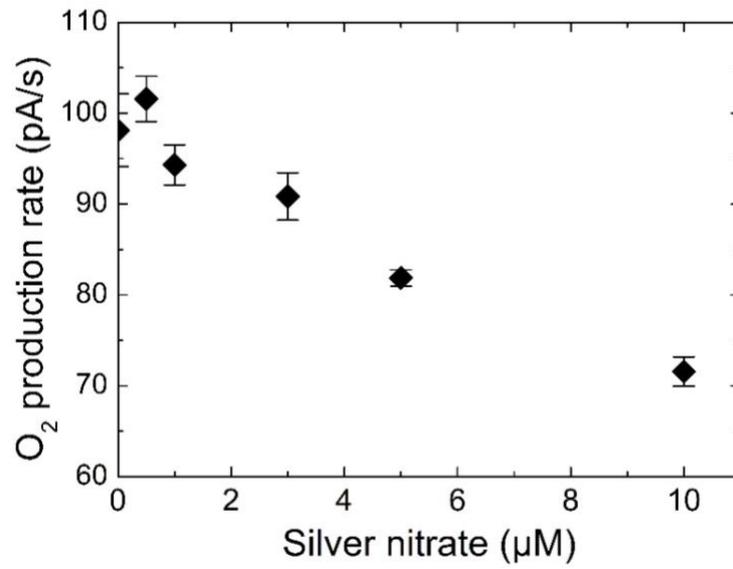


Figure 7: Evolution of the oxygen production rate with silver nitrate concentration
(exposure duration: five minutes, pristine solution C1)

All data are mean experimental values and error bars are calculated standard deviations