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Membrane Inlet Mass Spectrometry: A Powerful Tool for Algal Research

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Since the first great oxygenation event, photosynthetic microorganisms have continuously shaped the Earth's atmosphere. Studying biological mechanisms involved in the interaction between microalgae and cyanobacteria with the Earth's atmosphere requires the monitoring of gas exchange. Membrane inlet mass spectrometry (MIMS) has been developed in the early 1960s to study gas exchange mechanisms of photosynthetic cells. It has since played an important role in investigating various cellular processes that involve gaseous compounds (O₂, CO₂, NO, or H₂) and in characterizing enzymatic activities *in vitro* or *in vivo*. With the development of affordable mass spectrometers, MIMS is gaining wide popularity and is now used by an increasing number of laboratories. However, it still requires an important theory and practical considerations to be used. Here, we provide a practical guide describing the current technical basis of a MIMS setup and the general principles of data processing. We further review how MIMS can be used to study various aspects of algal research and discuss how MIMS will be useful in addressing future scientific challenges.

Keywords: gas exchange, photosynthesis, carbonic anhydrase, CO₂ concentrating mechanism, O₂ evolution, H₂ production, microalgae, cyanobacteria

HIGHLIGHTS

Photosynthetic microorganisms are major actors shaping the Earth's atmosphere and limiting global warming by fixing CO₂. We hereby describe practical and theoretical state of the art of the most polyvalent technique to measure gas exchange in microorganisms.

INTRODUCTION

Since its formation, the Earth's atmosphere has continuously been shaped by living organisms. Among all biological processes, oxygenic photosynthesis has dramatically changed the atmosphere composition by massively capturing CO₂ and producing O₂ during the Great Oxygenation Event that began 2.4 billion years ago with the emergence of cyanobacteria (Hohmann-Marriott and Blankenship, 2011). Nowadays, microalgae and cyanobacteria account for more than 50% of global photosynthesis (Field et al., 1998) and have a great importance in the regulation of atmospheric CO₂ levels and global warming. Understanding biological mechanisms underlying CO₂ capture or

production of other greenhouse gases such as nitrous oxide (N_2O) by microbial photosynthesis is of utmost relevance to better assess the impact of global changes on oceanic carbon sinks. It is also crucial to explore the limits of biomass productivity of algae and give some hints to assess the impact of microalgae-based biofuels on the environment (Burlacot et al., 2020a).

In the early 60s, Hoch and Kok (1963) designed a membrane inlet system coupled to a mass spectrometer (MIMS), which allowed the direct measurement of concentrations of dissolved CO_2 and O_2 in a microalgal suspension. In the MIMS setup they developed, a thin plastic membrane (polyethylene or Teflon) permeable to gases allowed part of the dissolved gases to pass from the microalgal suspension to the mass spectrometer. Coupled to the use of ^{18}O -labeled O_2 , MIMS allowed *in situ* measurement of O_2 uptake processes occurring during microalgal and cyanobacterial photosynthesis (Hoch and Kok, 1963; Gerster et al., 1974; Radmer and Kok, 1976; Gerster et al., 1977; Radmer and Ollinger, 1980b; Peltier and Thibault, 1985a; Peltier and Thibault, 1985b). The use of isotope-labeled water has also been an important tool for the study of water oxidation mechanisms by photosystem II (PSII) (Radmer and Ollinger, 1980a; Radmer and Ollinger, 1986; Shevela et al., 2011; Koroidov et al., 2014), which is not reviewed here and readers are referred to a recent review (Shevela and Messinger, 2013).

Later on, MIMS has been used to monitor various biological gases in the context of: *i*, inorganic carbon transport (Badger et al., 1994; Yu et al., 1994; Sültemeyer et al., 1998); *ii*, hydrogen production (van der Oost and Cox, 1988; Redding et al., 1999; Cournac et al., 2002; Tamburic et al., 2011); or *iii*, ethylene production (Zavřel et al., 2016). With the development of genetics tools, MIMS has been widely used to characterize various microalgae or cyanobacteria mutants and contributed to the understanding of molecular mechanisms involved in photosynthetic gas exchange (So et al., 1998; Hanson et al.,

2003; Helman et al., 2003; Cournac et al., 2004; Jans et al., 2008). It has also been used for *in vitro* studies of enzymes using gases as a substrate or products, like hydrogenases (Leroux et al., 2008), carbonic anhydrases (Northrop and Simpson, 1998) and the Fatty Acid Photodecarboxylase (Sorigué et al., 2017). In addition, it has also been used to study artificial catalysts for water oxidation (Poulsen et al., 2005; Koroidov et al., 2015) and to follow biogeochemical cycles in the oceans (Chua et al., 2016). Taken together, MIMS has made significant contributions to the fields of photosynthesis research, enzymology, biofuel research, and earth science which was recently reviewed (Burlacot et al., 2020a).

MIMS is nowadays a mature technique implicated in a growing number of applications in the field of algal research. With mass spectrometers being more affordable in the past years, MIMS is increasingly available in many laboratories. However, mounting and running a MIMS setup is technically and theoretically challenging. Previous reviews have focused on some specific aspects of MIMS in the context of photosynthesis research (Degn, 1992; Kotiaho and Lauritsen, 2002; Konermann et al., 2008; Beckmann et al., 2009; Shevela and Messinger, 2013; Cheah et al., 2014; Shevela et al., 2018). The current review intends to provide a guide covering theoretical, technical, and data processing aspects required for a broad usage of MIMS in algal research.

MIMS SETUP, OPTIMIZATION AND DATA PROCESSING

A typical MIMS setup is composed of five parts (**Figure 1**): (i), a reaction vessel containing the liquid medium/algal culture where gas exchange reactions take place; (ii), a membrane separating the liquid phase of the reaction vessel from the high vacuum line;

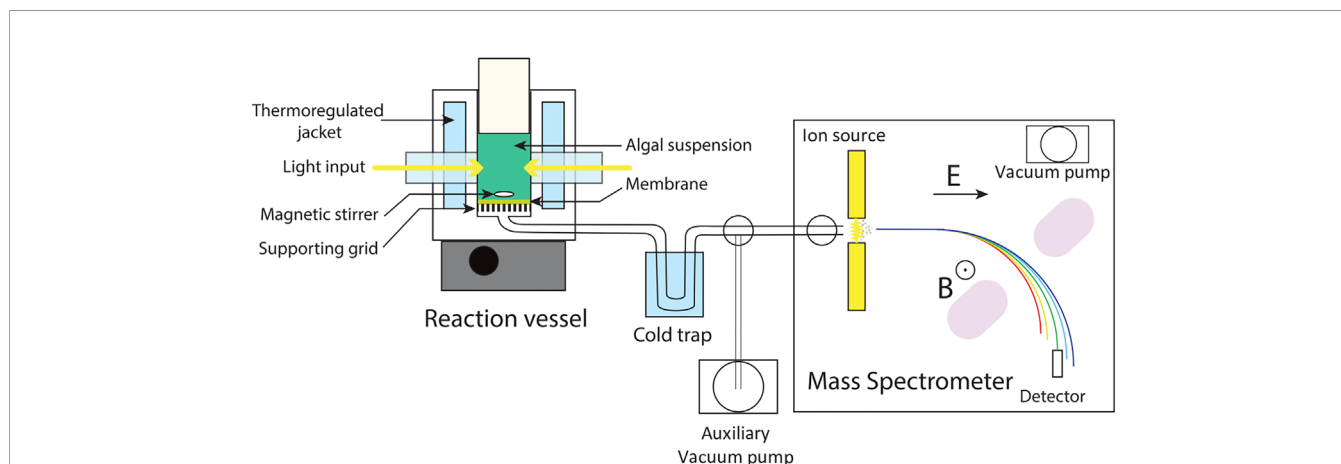


FIGURE 1 | Schematic setup of a Membrane Inlet Mass Spectrometry. The biological sample (cell suspension, cell free extract, or enzyme containing medium) is introduced inside a thermo-regulated reaction vessel equipped with a gas permeable membrane. Dissolved gases contained in the liquid medium pass through the membrane to a high vacuum tubing. After passing through a cold trap protecting the mass spectrometer from water leaks, gases enter the ion source of a mass spectrometer. Upon ionization, charged molecules are accelerated by an electric field (E), deviated by a magnetic field (B), and collected by a detector. An auxiliary vacuum pump allows for making the vacuum on the line with the closed inlet to the mass spectrometer. B, magnetic field; E, electric field.

(iii), a vacuum line connecting the reaction vessel to the mass spectrometer; (iv), a cold trap protecting the mass spectrometer from water leakage; and (v), a mass spectrometer for gas analysis. MIMS allows measurement of any volatile compound, with a molar mass up to a hundred gram per mole, dissolved in liquid phase (Kotiaho and Lauritsen, 2002). MIMS operation requires proper material setup and appropriate data processing. Each of the technical parts needs to be optimized to ensure reliable and stable measurement.

The Membrane Inlet

The membrane is gas permeable and serves as a physical barrier between the liquid phase and the ion source of the mass spectrometer connected by a vacuum line. In the setup described in **Figure 1** the membrane is held at the bottom of the reaction vessel supported by a stainless-steel grid. Note that different membrane inlet setups like microprobes can be used (Kotiaho and Lauritsen, 2002; Konermann et al., 2008). The gas leak through the membrane occurs through a three-step process called permeation: adsorption at the membrane surface, diffusion through the membrane material, and desorption on the other side of the membrane. In this process, diffusion is the limiting factor (Konermann et al., 2008) and is a temperature-dependent process. Thus, membrane permeability is highly influenced by temperature (**Figure 2**), and the reaction vessel must be precisely thermo-regulated (**Figure 1**). The gas leak is crucial since it defines the sensitivity of the method, especially it should remain small compared to the measured reaction rates to limit noise. Gas leakage depends on the membrane properties and is linearly related to the gas concentration inside the vessel (**Methods S1**). Various porous plastic films can be used as a membrane, although polytetrafluoroethylene (PTFE, also known as Teflon) and silicon are the most commonly used (Beckmann et al., 2009; Shevela et al., 2018). The permeability to gases depends on the

nature of the membrane. Silicon membranes are more permeable to gases than PTFE membranes (Lloyd et al., 1983) (PTFE membranes can be found on www.hansatech-instruments.com/ or www.ysi.com). The gas leak depends on both membrane surface and thickness, which must be chosen depending on the required sensitivity and on the duration of the experiments to be carried out. The ion source is overly sensitive to water that interferes with ionization and therefore quantification. In this respect, a PTFE membrane has the advantage over silicone of being seven times less permeable to water relatively to N_2 (Jensen and Cox, 1988), resulting in less noise. A magnetic stirrer is used to maintain algae in suspension and homogenize the gas content of the liquid medium (Lundsgaard et al., 1978). In a case where the stirring occurs on the membrane, the stirring speed must be adjusted to ensure a proper homogenization without compromising membrane integrity. Likewise, a smooth PTFE coated stirring bar can be used to achieve optimal stirring while limiting membrane wear.

The Vacuum Line and the Cold Trap

The vacuum line connecting the space below the membrane to the ion source of the mass spectrometer is, together with the membrane, a key element to consider when optimizing the response time of the experimental setup. Because of high vacuum (10^{-5} – 10^{-8} mbar) in the tubing, gases do not flow like a fluid but perform a random walk inside the tube towards the ion source of the mass spectrometer where gases are ionized. During their random walk, gases can adsorb and desorb at the surface of the tubing, thus slowing down the gas flow. The tubing length and cross-section should be optimized to increase the time response without compromising output signal of the mass spectrometer. Limiting length of the tubing decreases the response time of the system both because of limited inner tubing volume and decreased surface adsorption effects of the tubing. On the other hand, while small tubing cross-section favors the time response by limiting the tubing volume, this effect is counter-balanced by an increased probability of molecule absorption on the tubing surface, a too small cross-section decreasing the signal/noise ratio of the setup. We found an inner diameter of $\frac{1}{4}$ inch to be a good compromise for the type of experiments shown in this paper although smaller tubing down to $\frac{1}{16}$ inch can be suitably used (Konermann et al., 2008).

To limit water vapor entering the mass spectrometer ion source, the vacuum line tubing passes through a cold trap where the water vapor is condensed. Since the cold trap can also condense other gases, the choice of the trap temperature depends on the gas species to be analyzed. For example, cooling the vacuum line with liquid nitrogen (77 K) allows efficiently trapping water but also CO_2 (**Figure 3**). The condensation points of other main gases [N_2 , O_2 , and argon (Ar)] are much lower than that of water and are usually hard to selectively trap (**Figure 3**). A temperature of 200 K allows selective trapping of water and can be obtained using a mix of ethanol and dry ice in the absence of a cooling unit (Bailleul et al., 2017). The cold trap should be situated as close as possible to the vacuum pump of the mass spectrometer in order to keep its internal pressure as low as possible and limit unintended trapping. For further information on optimization of

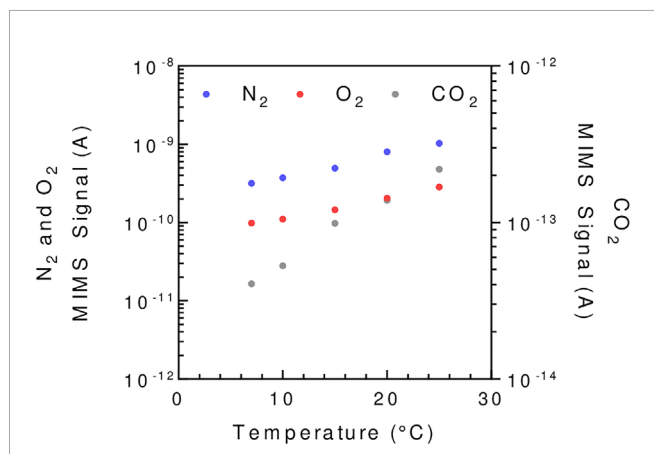
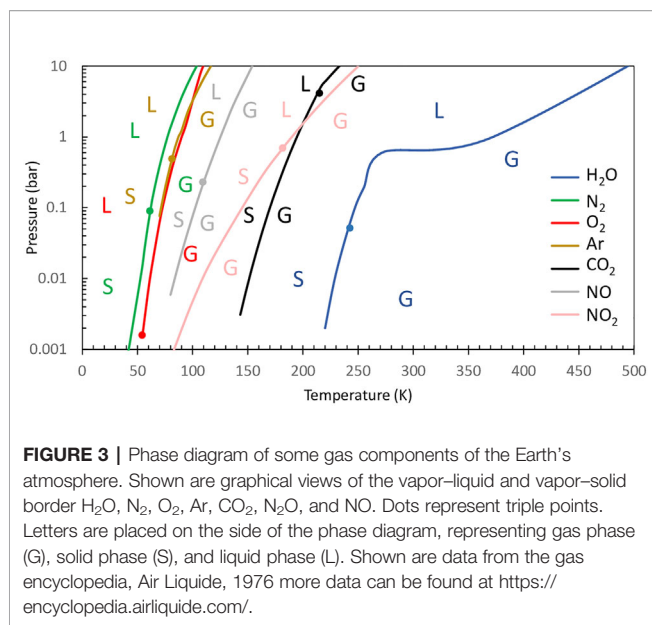


FIGURE 2 | The influence of temperature on the MIMS signal strength illustrated for N_2 , O_2 , and CO_2 . Reaction vessel is filled with *C. reinhardtii* minimal growing medium buffered at pH = 7.2 using 3-(N-morpholino) propane sulfonic acid (MOPS, 20 mM final concentration) and is flushed with air until reaching steady state. A PTFE membrane (13 μ m) is used. To avoid solubility issues, the signal shown is normalized by the Bunsen coefficient (see **Methods 1**). The mass spectrometer signal is given in amperes (A).



the membrane inlet system, readers are referred to (Konermann et al., 2008; Beckmann et al., 2009; Shevela et al., 2018).

Mass Spectrometer

Mass spectrometry is a technique that allows separation and detection of charged molecules in a gas phase depending on their mass over charge (m/z) ratio. During ionization, most reactions produce a single positively charged ion. But most often, the molecule is broken down by ionization, and seldom, double ionization can occur; these effects give rise to two or more fragments. The fragmentation pattern is a signature of the molecule and depends on the ionization method used. In the case of small molecules like gases, the main ion (molecular ion) results from the loss of an electron, other fragments representing a minor part in the fragmentation pattern (Table 1). The mass spectrometer, by measuring the signal intensity of detected ions for m/z of interest, enables the determination of gas amounts (Methods S1). Any mass spectrometer can be used in a MIMS setup with various ion sources (e.g. pulsed ionization, glow discharged ionization) and analyzers (e.g. Time Of Flight, Ion Trap) (Johnson et al., 2000). However, magnetic sector or quadrupole mass spectrometers are the most commonly used for gas analysis in a MIMS setup, although quadrupole has the advantage of portability and low price (Ferrón et al., 2016; Bailleul et al., 2017; Chatton et al., 2017).

Data Management

Because the mass spectrometer is consuming gases, measured variations of gas concentrations must be corrected from the mass spectrometer consumption to determine actual gas exchange rates between the biological sample and the extracellular medium (Methods S2). Although this effect has been well described (Berlier et al., 1985; Kotiaho and Lauritsen, 2002; Konermann et al., 2008) its correction has been overlooked in the recent literature. After calculating gas concentrations inside

TABLE 1 | Typical mass fragmentation pattern of various gases.

	H_2	D_2	H_2O	N_2	NO	O_2	Ar	CO_2	N_2O
Nominal m/z	2	4	18	28	30	32	40	44	44
1	2								
2	100	1.59							
4		100							
12								8.71	
14				13.79	7.51				12.91
15					2.4				0.1
16			0.9		1.5	21.8		9.61	5
17			21.22						
18			100						
19			0.5						
20							14.62		
22								1.9	
28				100				9.81	10.81
29				0.74				0.1	0.1
30					100				31.1
31					0.4				
32					0.2	100			
36							0.3		
38							0.05		
40							100		
44								100	100
45								1.2	0.07
46								0.4	

For each gas, shown is the relative amount of m/z in percentage of the maximal signal. Data are extracted from <https://webbook.nist.gov/chemistry/>. Fragmentations patterns are here given for natural isotopic abundance and therefore include isotopes-specific signal (e.g. $m/z = 45$ for CO_2 reflects natural abundance of $^{13}\text{CO}_2$). Note that the exact fragmentation patterns depend on each ion source and the ionization energy used and must be determined for each instrument.

the reaction vessel, gas exchange rates are calculated by correcting from the mass spectrometer consumption. Fluctuations of physical properties of the setup can result in variations of the flux of gas to the mass spectrometer and be an important source of noise. An additional normalization of gas exchange rates to a non-reactive gas (like N_2 or Ar) can advantageously correct such signal shifts or noise. These artifacts are therefore highly limited although biggest ones remain (Figure S2). Note that prior to any experiment, one must ensure the absence of gas leakage in the system (tubing, connections, inlets), which can be done by flushing helium outside the setup and following the $m/z = 4$ with the mass spectrometer. Details of the calculations have been described in Methods S1 and S2, and we have developed easy-to-run software that allows real time calculation and visualization of MIMS data (Downloadable at: https://github.com/francoisBurlacot/MIMS_Analysis).

MIMS USAGE IN ALGAL RESEARCH

MIMS has been initially developed and is still widely used to measure oxygen exchange during photosynthesis. Its usage has been extended to the study of other cellular mechanisms, such as hydrogen production, carbon concentrating mechanisms, and more recently, nitric oxide (NO) photoreduction into N_2O . We hereby provide some examples of applications of the MIMS in the field of algal biology to measure gas exchanges in the model species *Chlamydomonas reinhardtii* (Figure 4).

Assessment of Photosynthetic Oxygen Exchange

By using ^{18}O -enriched O_2 in illuminated microalgal suspension, Hoch and Kok (Hoch and Kok, 1963) could show that O_2 can be both produced and consumed during photosynthesis. While O_2 is produced by PSII from water splitting, O_2 is simultaneously consumed by different cellular processes (Figures 4 and 5A). Practically, the use of highly enriched O_2 (usually around 99% ^{18}O) allows neglecting $^{18}\text{O}^{16}\text{O}$ species, thus measurements of $^{16}\text{O}^{16}\text{O}$ ($m/z = 32$) and $^{18}\text{O}^{18}\text{O}$ ($m/z = 36$) are used to determine rates of gross O_2 evolution (O_2 Evolution) and O_2 uptake (O_2 Uptake), net O_2 production rate (Net O_2) being the end result of O_2 Evolution and Uptake. Considering that water splitting only produces $^{16}\text{O}_2$ (the natural abundance of ^{16}O being 99.8%, O_2 is produced from H_2O at 99.6% as $^{16}\text{O}_2$) and neglecting isotopic discrimination between $^{18}\text{O}_2$ and $^{16}\text{O}_2$ by uptake mechanisms, the following equations modified from (Radmer and Kok, 1976; Peltier and Thibault, 1985b) can be used:

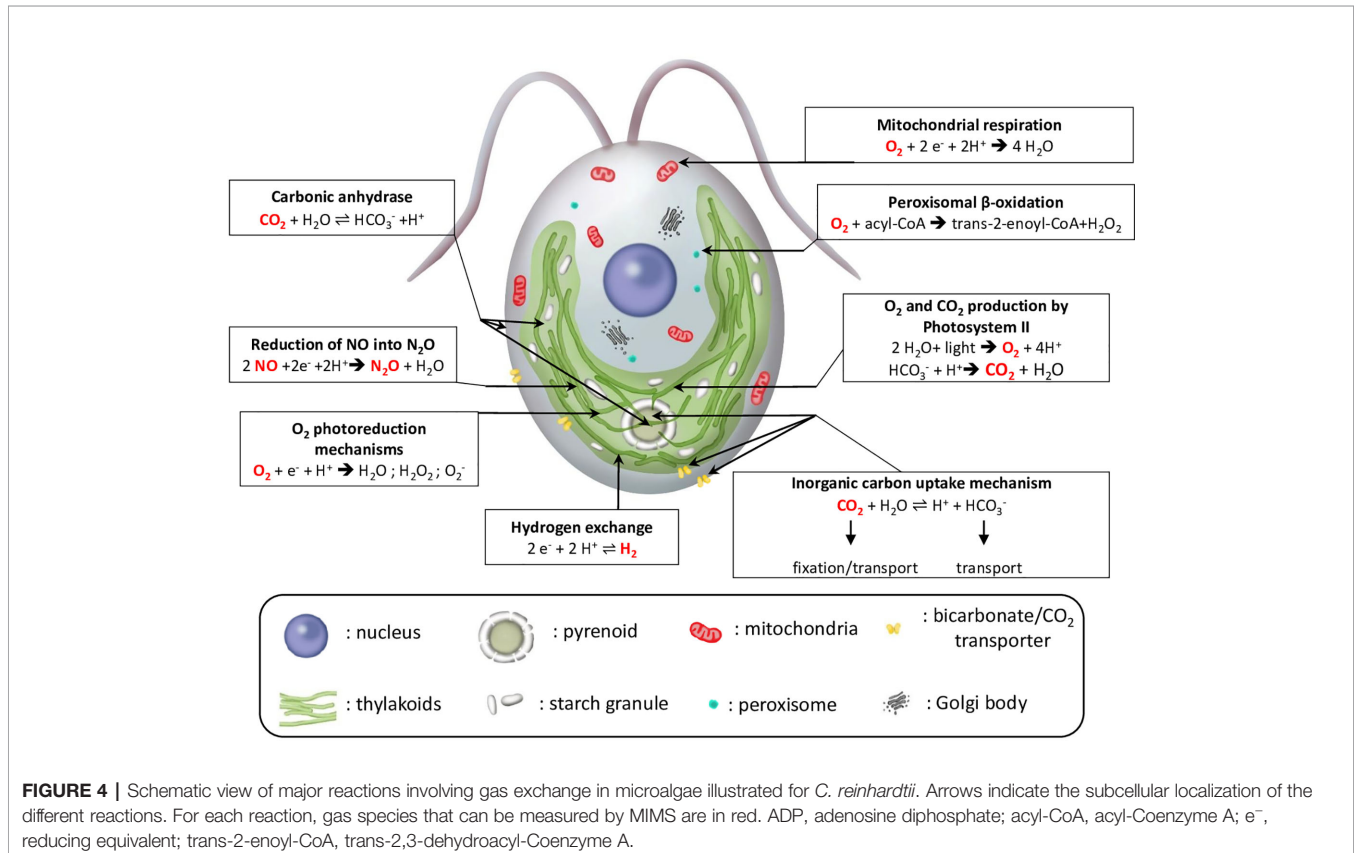
$$\text{O}_2 \text{ Uptake} = v_{^{18}\text{O}_2} \times \left(1 + \frac{C_{^{18}\text{O}_2}(t)}{C_{^{18}\text{O}_2}(t)}\right) \quad (1)$$

$$\text{O}_2 \text{ Evolution} = v_{^{16}\text{O}_2} - v_{^{18}\text{O}_2} \times \frac{C_{^{16}\text{O}_2}(t)}{C_{^{18}\text{O}_2}(t)} \quad (2)$$

$$\text{Net O}_2 = \text{O}_2 \text{ Evolution} + \text{O}_2 \text{ Uptake} \quad (3)$$

where $v_{^{18}\text{O}_2}$ and $v_{^{16}\text{O}_2}$ are the gas exchange rates of $^{18}\text{O}_2$ and $^{16}\text{O}_2$ respectively; $C_{^{18}\text{O}_2}(t)$ and $C_{^{16}\text{O}_2}(t)$ represent the gas concentrations of $^{18}\text{O}_2$ and $^{16}\text{O}_2$ respectively (see Methods S1 and S2). Typical patterns of $^{18}\text{O}_2$ and $^{16}\text{O}_2$ concentration, gross O_2 evolution, O_2 uptake, and O_2 net production rates as well as cumulated O_2 exchanges measured in *C. reinhardtii* cells during a dark to light transient are shown in Figures 5B–D.

Measuring O_2 exchange by MIMS in microalgae and cyanobacteria allowed dissecting molecular players involved in the O_2 uptake process, initially by using various inhibitors or characterizing the dependency on O_2 or CO_2 concentrations (Badger et al., 2000). (Radmer and Kok, 1976) early proposed that a highly efficient O_2 photoreduction process was present in cyanobacteria and microalgae. The persistence of mitochondrial respiration in the light was shown to contribute (Peltier and Thibault, 1985b), and the existence of a CO_2 -dependent O_2 uptake component was evidenced (Badger, 1985; Sültemeyer et al., 1987). With the development of genetic approaches, the nature and contribution of the different players were better characterized. For example, the contribution of mitochondrial respiration to O_2 uptake is enhanced in the absence of the proton gradient regulation like 1 (PGRL1)-mediated cyclic electron flow (CEF), thus showing the functional complementarity between these pathways in the production of intra-cellular ATP (Dang et al., 2014). Redox communication between chloroplast and



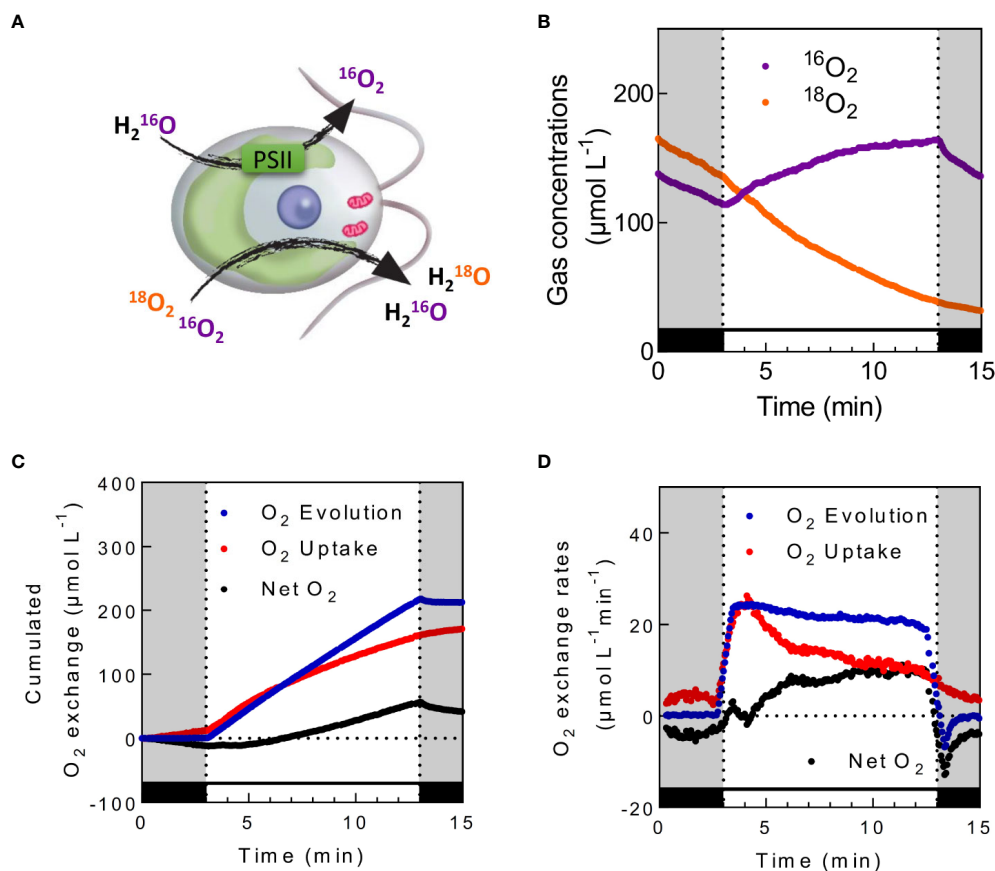


FIGURE 5 | *In vivo* measurements of photosynthetic O_2 exchange in the presence of ^{18}O -labeled O_2 . **(A)** Schematic view of oxygen exchange illustrated in *C. reinhardtii*. While photosystem II (PSII) produces unlabeled O_2 from the photolysis of H_2O , oxygen uptake mechanisms consume both ^{16}O -labeled and unlabeled O_2 . **(B)** $^{16}O_2$ and $^{18}O_2$ concentrations measured in *C. reinhardtii* cells during dark–light transients. **(C, D)** Calculated cumulated O_2 exchanges **(C)** and the corresponding O_2 exchange rates **(D)** for the same experiment. Cells were grown photoautotrophically in air, centrifuged and resuspended in fresh medium at a concentration of $20 \mu\text{g Chl ml}^{-1}$. Upon addition of 5mM HCO_3^- , $^{18}O_2$ was injected inside the cell suspension, and the reaction vessel was closed. After 5 min of dark adaptation, green light was turned on ($500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) for 10 min. Levels of $^{16}O_2$ and $^{18}O_2$ were recorded at respective $m/z = 32$ and 36 . O_2 Uptake (red), O_2 Evolution (blue), and Net O_2 production (black) were calculated as described; cumulated gas exchange were calculated by directly integrating obtained exchange rates. To limit noise on the exchange rates graphic, data shown in **(D)** are integrated with a sliding average of 30 s wide.

peroxisome (Kong et al., 2018) or with mitochondria in diatoms (Bailleul et al., 2015) was also evidenced from O_2 exchange measurements. The involvement of Flavodiiron proteins (Flvs) in light-dependent O_2 uptake was further established in cyanobacteria (Helman et al., 2003; Allahverdiyeva et al., 2013), and more recently in microalgae (Chaux et al., 2017). MIMS is nowadays widely used to understand the fate of the photosynthetic electron flow in various environmental conditions and mutant of cyanobacteria (Ermakova et al., 2016; Boatman et al., 2018; Luimstra et al., 2019), microalgae (Fisher and Halsey, 2016), or coral reef symbiosis (Einbinder et al., 2016).

If laboratory studies performed on model species allowed recognizing O_2 photoreduction as a major alternative photosynthetic electron sink crucial for cell acclimation to various environmental conditions (Curien et al., 2016), the relative contribution of different O_2 uptake mechanisms in natural environments remains largely unexplored (Bailleul et al., 2017). Despite its performance in measuring gross

photosynthesis, MIMS has long remained a cumbersome technique, not suitable for field experiments. On the other hand, chlorophyll fluorescence measurements using pulse-amplitude modulated (PAM) fluorimeter have been widely used for estimating PSII yield in natural environments such as in the ocean (Falkowski and Kolber, 1995). Chlorophyll fluorescence however, faces some limitations when used to determine gross O_2 production since the estimation of electron transport rates requires the measurement of cell absorbance (Genty et al., 1989; Godaux et al., 2015), which is difficult to realize in outdoor conditions. Recently, the miniaturization of mass spectrometers allowed *in situ* measurements of O_2 exchange in phytoplankton from the north Pacific (Ferrón et al., 2016) or in planktonic blooms from the north Atlantic (Bailleul et al., 2017), starting thus a new era for expanding research obtained in the laboratory on model species to species in their natural environments.

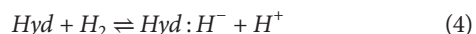
Indeed, fluorescence measurements have often been used together with MIMS CO_2 and O_2 exchange measurements to

study the link between CO₂ fixation and non-photochemical quenching of chlorophyll fluorescence (Sültemeyer et al., 1989; Fratamico et al., 2016; Ware et al., 2020). Furthermore, in conditions where the use of ¹⁸O₂ is not possible (e.g. anaerobiosis), coupling PSII quantum yield with gas exchanges has recently allowed determining gross O₂ production and inferring the existence of an O₂ uptake process (Burlacot et al., 2018).

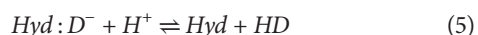
Hydrogen Production and Hydrogenase Activity Measurements

In microalgae and cyanobacteria, hydrogenases (H₂ases) catalyze the reversible formation of hydrogen (H₂) by direct reduction of protons (H⁺). In microalgae, the electron donor to the [Fe–Fe] H₂ase is ferredoxin (Florin et al., 2001), which can be reduced by the photosynthetic electron transport chain or by fermentative pathways (Catalanotti et al., 2013). Experimentally, H₂ production can be measured by different techniques, including modified O₂ electrodes (Wang, 1980; Godaux et al., 2015), gas chromatography (Hunt and Smith, 1961; Nagy et al., 2018), nuclear magnetic resonance spectroscopy (NMR) (Xu et al., 2016; Manz et al., 2017), or MIMS. Among these techniques, MIMS and modified O₂ electrodes allow *in vivo* quantitative measurement of hydrogen in a time-resolved manner. Under natural conditions, hydrogen photoproduction by microalgae is a transient phenomenon, generally considered as a safety valve avoiding over-reduction of PSI electron acceptors under anaerobiosis (Ghysels et al., 2013). Hydrogen photoproduction is limited by the O₂ sensitivity of H₂ase, O₂ being produced by PSII during illumination (Erbes et al., 1979). When using experimental conditions maintaining anaerobic conditions (thus limiting the H₂ase inhibition) like sulfur deprivation (Melis et al., 2000), low illumination (Liran et al., 2016) or O₂ quenchers like glucose and glucose oxidase/catalase (Godaux et al., 2015), a limitation of the supply of electrons to the H₂ase can be evidenced. The use of MIMS and of various *C. reinhardtii* mutants allowed identifying biological bottlenecks limiting the supply of electrons to the H₂ase (Tóth and Yacoby, 2019; Burlacot et al., 2020a). Lately, MIMS was used in the development of a very promising H₂ photoproducing protocol using flashing light as the light source in photoautotrophic self-anaerobic conditions (Kosourov et al., 2018).

MIMS has early been used for *in vitro* and *in vivo* measurements of H₂ase activity (Jouanneau et al., 1980; Vignais et al., 1982; Berlier et al., 1985). In the presence of H₂, H₂ase spontaneously splits H₂ (Hoberman and Rittenberg, 1943; Rittenberg and Krasna, 1955), forming one proton with the reversible reaction:



where Hyd is the binding site of H₂ase. When supplying deuterium (D₂), HD is formed during the back reaction (6) in the presence of protons in the reaction mixture:



This reaction directly depends on the turnover rate of H₂ases (*i.e.* H₂ase activity) (Vignais, 2005). In the absence of H₂

production or uptake, following kinetics of D₂, HD and H₂ with MIMS allows measuring the H₂ase activity (H₂ase activity) by the H/D exchange rate (V_{exch}):

$$\text{H}_2\text{ase activity} = V_{\text{exch}}(t) = \frac{1}{\tau} (2v_{\text{H}_2}(t) + v_{\text{HD}}(t)) \quad (6)$$

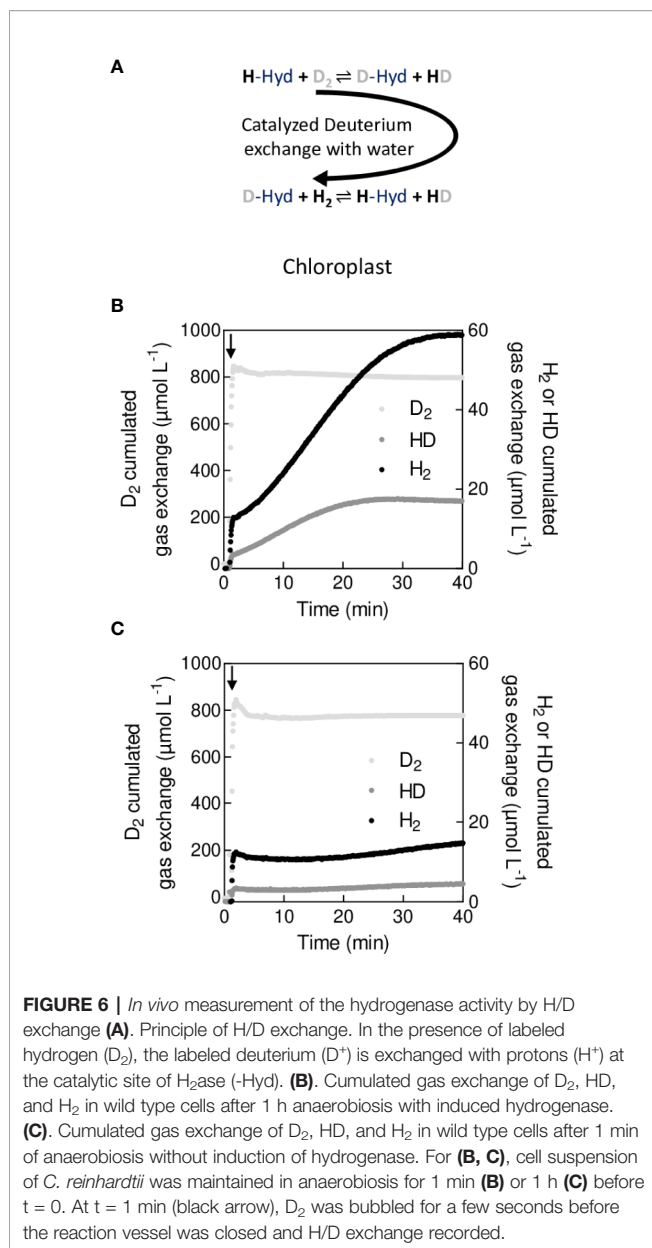
with

$$\tau = \frac{C_{\text{D}_2}(t) + \frac{C_{\text{HD}}(t)}{2}}{C_{\text{D}_2}(t) + C_{\text{H}_2}(t) + C_{\text{HD}}(t)} \quad (7)$$

where C_{D₂}(t), C_{H₂}(t) and C_{HD}(t) are the concentrations of D₂, H₂ and HD respectively and v_{H₂}(t), v_{HD}(t) being the gas exchange rates of H₂ and HD, respectively (see **Methods S1** and **S2**) (Cournac et al., 2004). A few microalgal species such as *C. reinhardtii* harbor H₂ases (Burlacot and Peltier, 2018) under anaerobic conditions. Measuring the H/D exchange allowed monitoring of H₂ase induction or inhibition *in vivo* (Vignais et al., 2002; Tolleter et al., 2011; Burlacot et al., 2018). Typical patterns of D₂, H₂ and HD exchange measured upon injection of D₂ before and after induction of H₂ase in *C. reinhardtii* are shown on **Figure 6**. Note that in conditions where H₂ase produces H₂ during illumination the H₂ase activity, needs to be corrected from the increase in total hydrogen species (H₂, HD, D₂) (Cournac et al., 2004). Although gas chromatography or NMR has also the potential to differentiate D₂, HD and H₂ (Hunt and Smith, 1961; Xu et al., 2016), MIMS allows performing such measurements *in vitro* and *in vivo* in a time resolved manner. If H/D exchange measurements using a MIMS allow determining the catalytic constant of H₂ase, it can also be used to determine the resistance of gas diffusion between the active H₂ase site and the reaction medium *in vitro* (Leroux et al., 2008). *In vitro* H/D exchange measurements have been used to study enzymatic properties of native H₂ase (Abou Hamdan et al., 2012; Gauquelin et al., 2018), including O₂-tolerant H₂ases (Liebgott et al., 2011), as well as H₂ases modified by site-directed mutagenesis in order to limit O₂ diffusion to the active site (Cano et al., 2014). H₂ photoproduction by microorganisms has recently regained huge interest for biofuel production due to recent improvements in strains and experimental protocols (Tóth and Yacoby, 2019). The use of MIMS should help in evaluating the upcoming combination of newly developed H₂ production protocols (Kosourov et al., 2018; Nagy et al., 2018) and previously characterized mutants photoproducing more H₂ (Tolleter et al., 2011; Eilenberg et al., 2016; Burlacot et al., 2018; Ben-Zvi et al., 2019). This has recently started using *flv* mutants (Jokel et al., 2019) and is promising for future bio H₂ production developments.

Assessing NO and N₂O Gas Exchange

On Earth, 6% of the radiative forcing is due to N₂O (IPCC, 2013), whose greenhouse effect is 300 times that of CO₂. N₂O is produced from the reduction of nitric oxide (NO) by bacteria (Goreau et al., 1980), fungi (Maeda et al., 2015), and microalgae (Guieysse et al., 2013). The measurement of N₂O in a time resolved manner can help understand the dynamics of N₂O



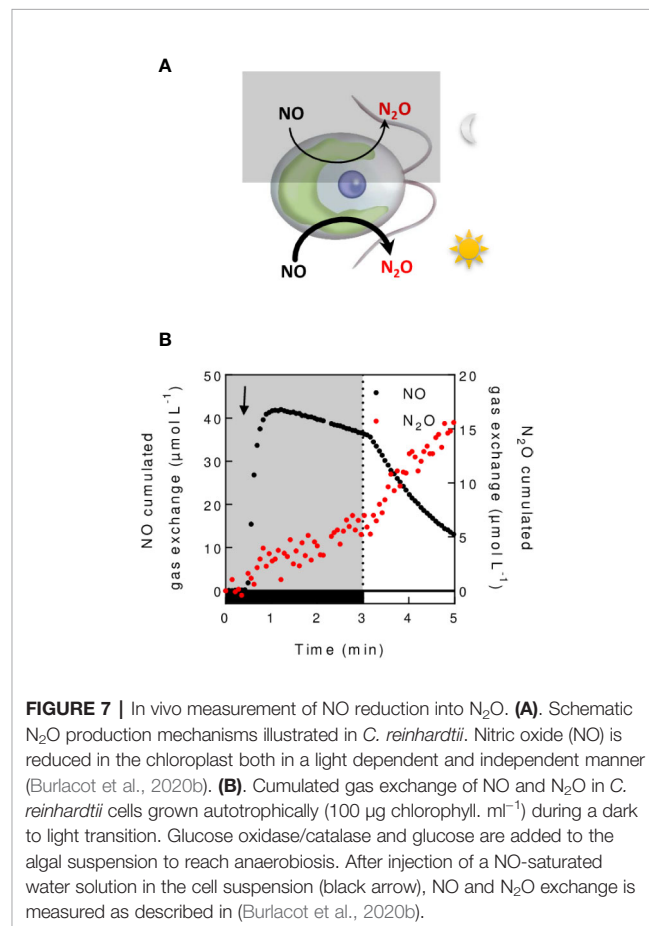
formation in the environment or in isolated organisms. In green microalgae, MIMS has recently been used to dissect the molecular mechanisms involved in the conversion of NO into N₂O (Figure 7) (Burlacot et al., 2020b). In practical terms, the detection of N₂O requires specific calculations because its mass spectrum overlaps that of CO₂ (see Methods S1). However, due to a relatively high detection limit for N₂O (around 18 μM) (Chatton et al., 2017), MIMS is not suitable for detecting low N₂O amounts, such as in water in equilibrium with ambient air (4.3 μM) (IPCC, 2013).

NO is an important intracellular signaling molecule in algae like in most living organisms. While NO is crucial for growth of *C. reinhardtii* under anaerobiosis and for its acclimation to nitrogen, sulfur or phosphate deprivation (Hemschemeier et al., 2013; Wang and Spalding, 2014; De Mia et al., 2019;

Filina et al., 2019), mechanisms of NO production remain blurry. The use of MIMS in algae fed with nitrates or nitrites, allowed to evidence the role of NO reduction mechanisms in the regulation of NO homeostasis (Burlacot et al., 2020b). Following NO production can also be based on imaging fluorescent chemical probes specifically reacting with NO (Li and Wan, 2015) or on the use of specific electrodes (Csonka et al., 2015). However, MIMS by supplying quantitative, time resolved, and stable measurements of NO (Bethke et al., 2004; Conrath et al., 2004), with further simultaneous measurement of NO reduction products such as N₂O, should help deciphering mechanisms participating in NO homeostasis.

Inorganic Carbon Affinity in Microalgae and Carbonic Anhydrase Activity

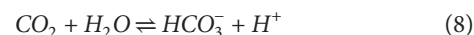
When grown under low CO₂ concentration, many microalgae or cyanobacteria induce an active import of inorganic carbon (C_i = CO₂ and HCO₃⁻) (Figure 8A) from the extracellular medium to the active site of CO₂ fixation (Badger et al., 1980; Badger and Andrews, 1982; Sültemeyer et al., 1991; Giordano et al., 2005; Reinfelder, 2011). This mechanism, called Carbon Concentrating Mechanism (CCM), principally operates by concentrating HCO₃⁻ inside cells (Price et al., 2007). In microalgae, HCO₃⁻ is converted into CO₂ inside the pyrenoid, at the vicinity of the carbon-fixing enzyme (RuBisCO) thus increasing the local CO₂



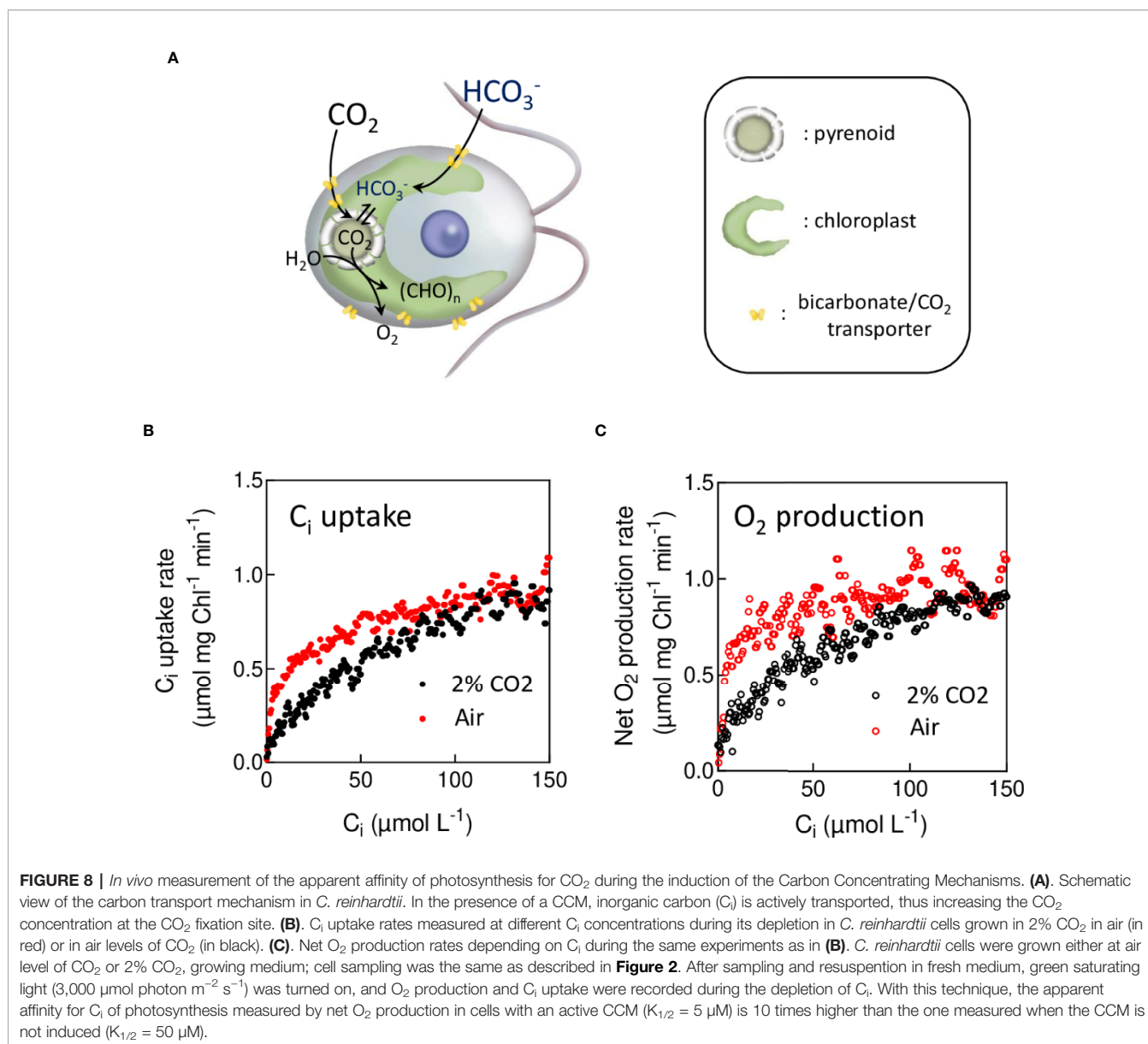
concentration (**Figure 8A**) (Mackinder, 2018). The CCM ensures high C_i fixation rates by photosynthesis under low C_i concentration (Badger et al., 1980). When active, the CCM results in an increased apparent affinity of photosynthesis for C_i , which can be assessed by measuring O_2 production rates at various C_i concentrations, either using an O_2 electrode (Badger et al., 1980) or a MIMS (Sültemeyer et al., 1993). MIMS has the advantage of simultaneously measuring O_2 and CO_2 . Since the CO_2 decreases during the time course of the experiment due to the activity of photosynthesis, it is possible to determine in one single experiment net O_2 or CO_2 exchange rates at different CO_2 concentrations ($v_{O_2}(C_{CO_2}(t))$ or $v_{CO_2}(C_{CO_2}(t))$) during CO_2 fixation by photosynthesis (Douchi et al., 2019). **Figures 8B, C** show typical experiments in which CO_2 uptake and O_2 production rates have been determined as a function of the CO_2 concentration in air-grown and CO_2 -grown cells of *C. reinhardtii*. Note that in such

experiments the cell concentration must be kept low enough to ensure that photosynthetic gas exchange kinetics are sufficiently slow as compared to the response time of the MIMS setup. Using this technique (**Figure 8**), the apparent affinity for C_i of photosynthetic O_2 production of cells with an active CCM is about 10 times higher than in cells with no active CCM as previously reported (Badger et al., 1980; Sültemeyer et al., 1998).

During the induction of CCM, carbonic anhydrases (CAs) are also induced and catalyze the reversible hydration of CO_2 by water which is summarized in reaction (8):



In microalgae, different CA isoforms are present in the different cellular compartments, thus limiting the disequilibrium between transported and consumed C_i species (**Figure 4**)



(Sültemeyer et al., 1990; Mackinder et al., 2017). Inside the pyrenoid, CA rapidly converts HCO_3^- into CO_2 , the substrate of RuBisCO, thus ensuring a high CO_2 concentration at the catalytic site of the enzyme (Mackinder, 2018).

When their CCM is active, cyanobacteria and microalgae can take both CO_2 and HCO_3^- in the medium (Sültemeyer et al., 1991; Badger et al., 1994). A way to assess the net flux of both species is to use the disequilibrium between inorganic species (see equation 8) induced by the preferential uptake of one species. Note that this approach is not possible in the presence of extracellular CA, which has limited its usage in microalgae (Badger et al., 1994; Palmqvist et al., 1994). By comparing net O_2 production (proportional to the overall net C_i uptake) to the net

CO_2 uptake and knowing the uncatalyzed rate of CO_2 and HCO_3^- interconversion (8), it is possible to calculate the net HCO_3^- uptake (Badger et al., 1994). Using the disequilibrium method with MIMS has shown that HCO_3^- was preferentially taken during CCM in cyanobacteria (Sültemeyer et al., 1998).

In the presence of doubly ^{18}O -labeled CO_2 , the CA activity which catalyzes the exchange of oxygen isotopes between CO_2 and H_2O results in a progressive dilution of ^{18}O from CO_2 (Gerster, 1971) (**Figure 9A**). To limit the background level of $m/z = 44$ ($^{12}\text{CO}_2$) due to naturally present CO_2 in the algal suspension, the assay can be done using ^{18}O -enriched $^{13}\text{CO}_2$ (Radmer and Kok, 1976), following the $m/z = 45$ ($^{13}\text{C}^{16}\text{O}_2$), 47 ($^{13}\text{C}^{18}\text{O}^{16}\text{O}$) and 49 ($^{13}\text{C}^{18}\text{O}_2$). After injection of $^{13}\text{C}^{18}\text{O}_2$

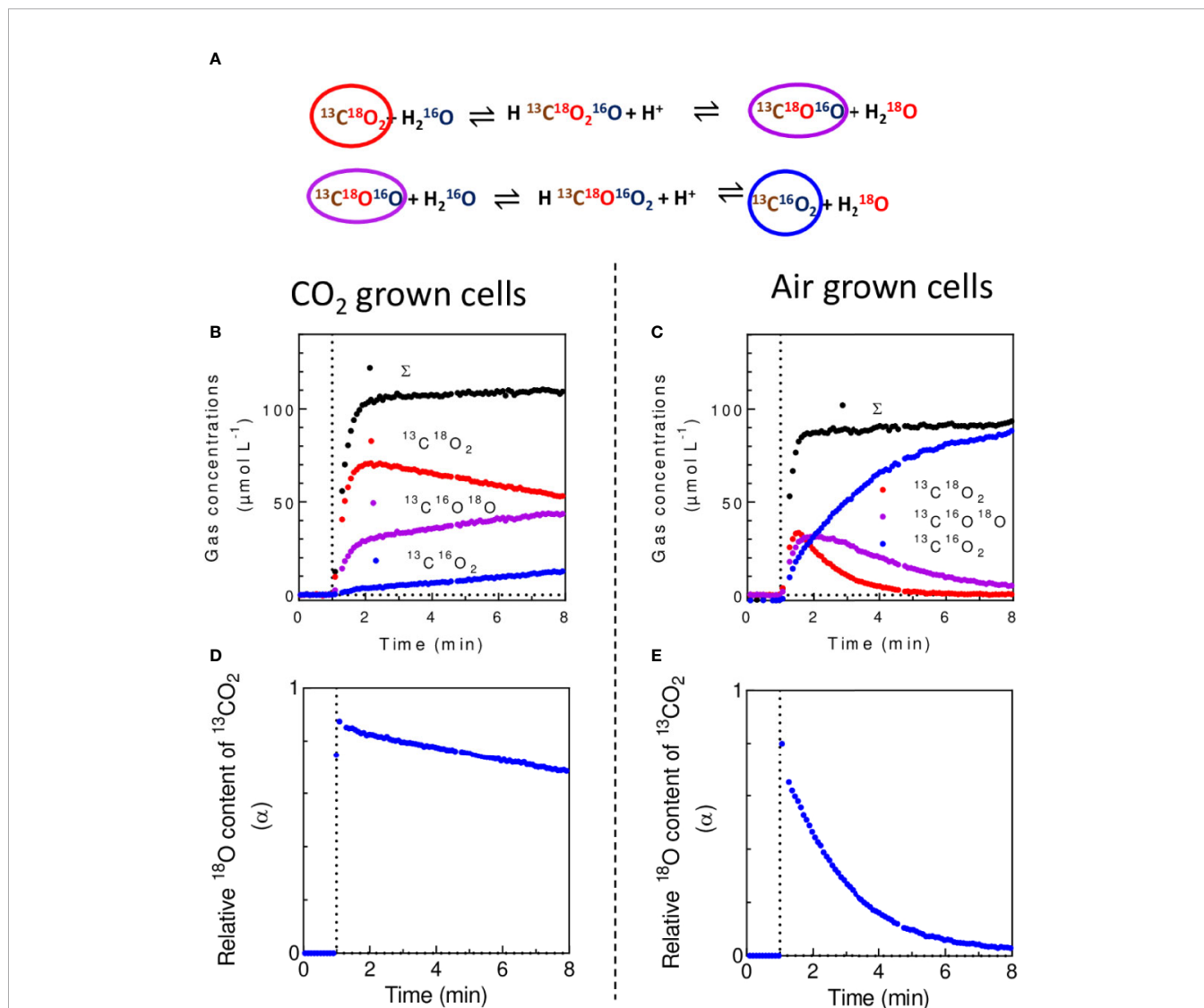


FIGURE 9 | *In vivo* measurement of carbonic anhydrase (CA) activity. **(A)** Cascade of reactions leading to the unlabeled of ^{18}O -enriched CO_2 in solution in H_2O . **(B, C)** Concentrations of $^{13}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{18}\text{O}^{16}\text{O}$, $^{13}\text{C}^{18}\text{O}_2$ and total ^{13}C -labeled carbon form (Σ) upon injection of $^{13}\text{C}^{18}\text{O}_2$ in a suspension of *C. reinhardtii* cells cultured in 2% CO_2 **(B)** or at air level of CO_2 **(C)**. **(D, E)** Isotopic ^{18}O content (α) of $^{13}\text{CO}_2$ during experiments of **(B, C)** respectively. ^{13}C and ^{18}O -enriched CO_2 was used to avoid the mass spectrometric background on $m/z = 44$ due to naturally present CO_2 , therefore enhancing the signal to background ratio (Radmer and Kok, 1976). $^{13}\text{C}^{18}\text{O}_2$ was injected after 1 min of darkness at 0.1 mM final concentration (vertical dotted line).

(supplied as $\text{H}^{13}\text{C}^{18}\text{O}_3^-$ in a buffered reaction medium) a typical pattern of the progressive unlabeled of $^{13}\text{CO}_2$ in *C. reinhardtii* cells is shown (**Figures 9B–E**). The isotopic content of ^{18}O in $^{13}\text{CO}_2$ during the progressive unlabeled is given by:

$$\alpha = \frac{2 \times C_{^{13}\text{C}^{18}\text{O}_2}(t) + C_{^{13}\text{C}^{18}\text{O}^{16}\text{O}}(t)}{C_{^{13}\text{C}^{18}\text{O}_2}(t) + C_{^{13}\text{C}^{18}\text{O}^{16}\text{O}}(t) + C_{^{13}\text{C}^{16}\text{O}_2}(t)} \quad (9)$$

where $C_{^{13}\text{C}^{18}\text{O}_2}(t)$, $C_{^{13}\text{C}^{18}\text{O}^{16}\text{O}}(t)$ and $C_{^{13}\text{C}^{16}\text{O}_2}(t)$ are the concentrations of $^{13}\text{C}^{18}\text{O}_2$, $^{13}\text{C}^{18}\text{O}^{16}\text{O}$ and $^{13}\text{C}^{16}\text{O}_2$ respectively (**Methods 1**). On purified CA, the isotopic enrichment decays exponentially:

$$\alpha = a_1 \times e^{-\theta t} \quad (10)$$

where θ is the rate constant of the exchange of ^{18}O with water (Gerster, 1971; Silverman, 1982). Note that in some cases like a concomitant use of ^{18}O -labeled O_2 , the presence of ^{18}O and C^{16}O_2 in the ion source of the mass spectrometer can spontaneously generate $\text{C}^{18}\text{O}^{16}\text{O}$ which needs to be corrected (Cournac et al., 1993). *In vivo*, a CA isotope exchange assay measures the global contribution of all CAs present in the biological sample with an additional effect due to diffusion/transport of C_i through the membranes (Sültemeyer and Rinast, 1996; Tolleter et al., 2017). When a periplasmic CA is present, as it is the case in *C. reinhardtii* cells with an active CCM, its activity dominates the exchange kinetics (Sültemeyer and Rinast, 1996). MIMS is so far the most reliable method for CA activity measurements in biological samples and is particularly suitable for *in vivo* measurements on algae (Dang et al., 2014; Benlloch et al., 2015; Tolleter et al., 2017), cyanobacteria (Whitehead et al., 2014), corals (Tansik et al., 2015), and plants (Peltier et al., 1995; Clausen et al., 2005; Burén et al., 2011; Benlloch et al., 2015). *In vitro*, the use MIMS and $\text{H}^{13}\text{CO}_3^-$ has also allowed unraveling the existence of a light-induced CO_2 production by the PSII (Koroidov et al., 2014; Shevela et al., 2020).

FUTURE DEVELOPMENTS AND PERSPECTIVES

Although MIMS is more than 50 years old, its usage has only recently become popular (Ketola and Lauritsen, 2016). The recent development in setups and analytical protocols and its popularity helped pushed the limits of our knowledge in the biology of photosynthetic microorganisms at various scales (Burlacot et al., 2020a).

Gas exchange measurements, chlorophyll fluorescence (Maxwell and Johnson, 2000), and electrochromism measurements (Bailleul et al., 2010) are the three main tools available to measure photosynthetic activity in microalgae and cyanobacteria on intact organisms. Simultaneous measurements of gas exchange by MIMS and chlorophyll fluorescence have allowed for correlating energy dissipation processes with CO_2 and O_2 photoreduction occurring during photosynthesis (Sültemeyer et al., 1989; Burlacot and Peltier, 2018; Ware et al., 2020). However, our understanding is still limited by the functional redundancy of many mechanisms and their interaction with cryptic mechanisms such as cyclic electron flow, which are not easily experimentally accessible. Further coupling of these methods, allowing for instance parallel measurement of gas

exchange by MIMS and cyclic electron flow by electrochromism, together with an increased accessibility to genetic resources, should provide in the future new insights on how the main photosynthetic processes are regulated and interact during acclimation to various environmental situations.

In the perspective of large-scale biofuel production by microalgae or cyanobacteria, recent research has focused on the design and use of photobioreactors coupled to MIMS for the analysis of volatile compounds of interest. These setups have been used to measure real time productions of H_2 (Tamburic et al., 2011; Zhang et al., 2015) or ethylene (Zavřel et al., 2016) during medium and long-term cultivation of microalgae or cyanobacteria. In the future, MIMS could be used to study and optimize the production in photobioreactors of volatile hydrocarbons by engineered photosynthetic cells.

Further miniaturization and decreasing prices of mass spectrometers should enable an even larger number of laboratories to have access to MIMS in the future, thus accelerating our understanding of how photosynthetic microorganisms impact the atmosphere of our planet (Burlacot et al., 2020a). The use or application of MIMS in the field opens a new era of evaluating the occurrence and ecological relevance of molecular mechanisms in natural environment.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AB and GP designed the illustrating experiments. AB performed the experiments. AB and FB designed the software. FB developed the software with supervision from AB. AB, YL-B and GP wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.01302/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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