Leaf-level O_2 and CO_2 measurements with the LI-6800 and Picarro G2207-i

Application Note

The LI-6800 Portable Photosynthesis System probes the carbon fixation reactions of photosynthesis by calculating fluxes for CO_2 (Assimilation) from the difference in measured concentrations of CO_2 and H_2O entering and exiting a leaf cuvette. With the 6800-01A Multiphase Flash Fluorometer, additional information on the light reactions of photosynthesis, including quantum yield of photosystem II (Φ_{PSII}) and electron transport rate (J) can also be calculated. The source of electrons for redox reactions in the chloroplast is H_2O, resulting in the evolution of Oxygen (O_2). While the general stoichiometry of these reactions is 1 mol O_2 evolved per mol CO_2 fixed (von Caemmerer 2000), net fluxes of O_2 can depend on environmental conditions, as O_2 is produced solely during the light reactions, but can be consumed by at least three separate reactions (Canvin et al., 1980). Simultaneous measurements of O_2 exchange, CO_2 exchange and chlorophyll fluorescence can provide unique information regarding leaf biochemistry.

Oxygen concentrations in the atmosphere are two to three orders of magnitude larger than CO_2, yet the leaf fluxes are similar in magnitude. Significant challenges occur when measuring small changes in O_2 concentration on a large nominal background concentration, resulting in potentially larger errors in the O_2 fluxes relative to the CO_2 fluxes. With proper attention to the setup and signal averaging, physiologically significant data can be collected.

Here we describe measurements using a Picarro G2207-i O_2 gas concentration analyzer coupled to the LI-6800 Portable Photosynthesis System to quantify both CO_2 and O_2 fluxes. Best practices for plumbing the instruments together, correction for dilution by foreign gases, and calculation of O_2 fluxes are discussed. A discussion of relative uncertainties between CO_2 and O_2 fluxes and a few example data-sets are also included.

Plumbing

The first consideration for an open flow-through gas exchange measurement should be the concentration stability of the air supply to the measurement cuvette, in this case the air source supplied via the LI-6800 console. In the open atmosphere O_2 concentration is fairly constant over the short-term, but can vary significantly inside a poorly ventilated lab space. In order to achieve a constant reference O_2 concentration, a source of constant O_2 is preferred. For the measurements described here, we used a tank of CO_2-free compressed air (N_2/O_2 mix) supplied to the LI-6800 console.

A recommended plumbing configuration for the LI-6800 and G2207-i is shown in Figure 1. To supply sample air to the G2207-i, the LI-6800 provides sub-sample outlets on both the reference and sample air streams. In the G2207-i, these sample and reference air streams must be sequentially sampled. A needle valve is placed on each of the lines to maintain constant, controlled airflow from the subsample ports. Due to the comparatively low flow rate of the G2207-i (80-110 sccm or 50 – 75 μmol s^{-1}), diverting 100 μmol s^{-1} from each channel is sufficient for the O_2 measurements. Continuous flow from each line even when not being sampled helps to minimize dead volumes and keep the airlines purged. The flow meters at the exhaust ports of the LI-6800 gas analyzers can be used to adjust the needle valve on each subsample line to ensure the proper flow rate. Initial setup of the flow can be done by closing the reference or sample needle valve and monitoring the respective IRGA exhaust flow meter. Then the needle valve can be opened until 100 μmol s^{-1} is being diverted. This flow rate should be continuously monitored throughout the experiments.

By continuously venting the output of both reference and sample air streams, there will be minimal dead volumes present in the system. On the G2207-i side of the valve, it is necessary to include an open split, as the LI-6800 airstream will supply a larger flow rate than required by the G2207-i. The open flow split and identical flow rate in the two airstreams is important for the G2207-i. the G2207-i's vacuum pump acts to maintain cavity pressure by adjusting flow through the system. A change in flow rate through the G2207-i will cause pressure changes up-stream including scrub tubes present in the flow path. Both H_2O and CO_2 should be removed from the airstream (see Box 1 on foreign gas dilution), therefore changes in the pressure can cause differential scrubbing between sample and reference lines. In our experiments, the pressure effect was on the order of 30 –

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50 ppm H_2O (0.03 – 0.05 mmol mol^{-1}), enough to cause significant errors in the O_2 measurements (see *Appendix A: Foreign Gas Dilution on O2*). The order of the scrub tubes is also important. If using soda lime to scrub CO_2 and Drierite® to scrub H_2O, air should pass through the soda lime first. Soda lime must contain moisture to be effective and releases water vapor as a result. Placing Drierite® before the soda lime will not result in a dry airstream for O_2 measurements and will shorten the effective life of the soda lime.

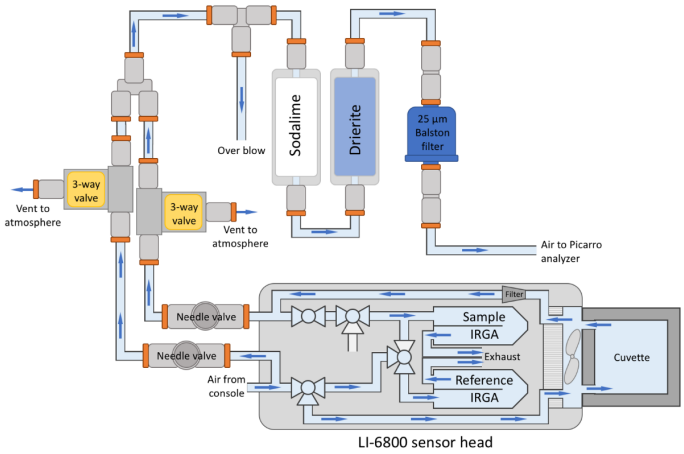

Figure 1. Example Flow configuration for measuring O_2 concentrations combining an LI-6800 and Picarro G2207-i O_2 analyzer. Flow from the reference and sample sub-sample ports on the LI-6800 sensor head are directed through a needle valve to maintain ≈100 µmol/s to the valve, with air vented to the atmosphere while the other channel is sampled, minimizing dead volumes. Tube length between the 3-way valve and 'Y' quick-connect do represent a dead volume and should be minimized to a few cm of tubing. Note that the 3-way valves, if using 5V solenoids such as LI-COR part number 300-07025 (see Table 1) can be automatically controlled by the LI-6800 auxiliary channels, where a switchable 5V power supply is available.

Oxygen Evolution calculations

The flux calculations for O_2 evolution will depend upon the scrubbing scheme chosen. We recommend scrubbing H_2O at all times, but it is up the you to choose whether or not to scrub CO_2. The flux calculation for O_2 evolution will depend on what foreign gases are being scrubbed. A full derivation for either scenario is given in *Appendix B: Mass balance equations for CO2 and O2 flux calculation*. In the case of scrubbing both H_2O and CO_2 the calculation for O_2 evolution (O_E) is:

$$sO_E = \frac{u_i(O_S - O_R)}{1 - O_S} \quad (1)$$

Where s is measured leaf area (m^2), u_i is the flow rate entering the leaf cuvette (mol s^{-1}), O_R and O_S are the O_2 concentrations measured in reference and sample, respectively in ppm, (or mol O_2 mol^{-1}).

If scrubbing H_2O only, the equation for O_2 evolution is:

$$sO_E = \frac{u_i(O_S - O_R)(1 - C_S) - u_i O_s (C_R - C_S)}{1 - (O_S + C_S)} \quad (2)$$

where C_R and C_S are *CO2_r* and *CO2_s* respectively, from the LI-6800. Note that the above equations have been derived to report positive values for O_2 evolution and negative values for O_2 consumption.

Uncertainty in Calculated Fluxes for CO_2 and O_2

The uncertainty in a differential measurement, as computed from two absolute measurements, is the sum of the uncertainties in each absolute measurement. In general, for leaf-level measurements, the expected Δ in gas concentrations between sample and reference air streams in O_2 will be similar to the Δ measured for CO_2. For any given flux value, the Δ in concentration is determined by the flow rate to the cuvette and the amount of leaf material being measured. Figure 2A shows the expected Δ as a function of the flux rate for two different cases – the large 6×6 cuvette encompassing 36 cm^2 of leaf area, and the fluorometer with 6 cm^2 of leaf area. In both scenarios, a cuvette flow rate of 300 µmol s^{-1} was assumed, to allow 100 µmol s^{-1} flowing to the O_2 analyzer and maintaining 200 µmol s^{-1} through the LI-6800 sample gas analyzer. For very low fluxes, for example a respiration rate of 1 µmol m^{-2} s^{-1}, the Δ generated is 2 µmol mol^{-1} and 12 µmol mol^{-1} for a leaf area of 6 cm^2 and 36 cm^2, respectively.

The uncertainty in calculated flux is a function of the uncertainty in the measured Δ between the reference and sample airstreams. Here, we will use the 1-σ standard deviation specification (e.g., 68% confidence interval) provided by the manufacturer (0.1 µmol mol^{-1} on CO_2 for the LI-6800 with 4-second averaging and 2 µmol mol^{-1} on O_2 for the G2207-i at 300 second averaging). The uncertainty in calculated fluxes is calculated as a function of the 1-σ standard deviation and expected Δ. The calculated uncertainty in fluxes is shown in Figure 2B, as % uncertainty from the "true" value. In the case of the fluorometer, with a Δ of 2 µmol mol^{-1}, the uncertainty in CO_2 Assimilation in the LI-6800 is 5%, while in the G2207-i it is > 100%. However, when using the larger 6x6 cuvette with a Δ of 12 µmol mol^{-1}, uncertainties decrease for both measurements, <1% for the LI-6800 and < 20% for the G2207-i.

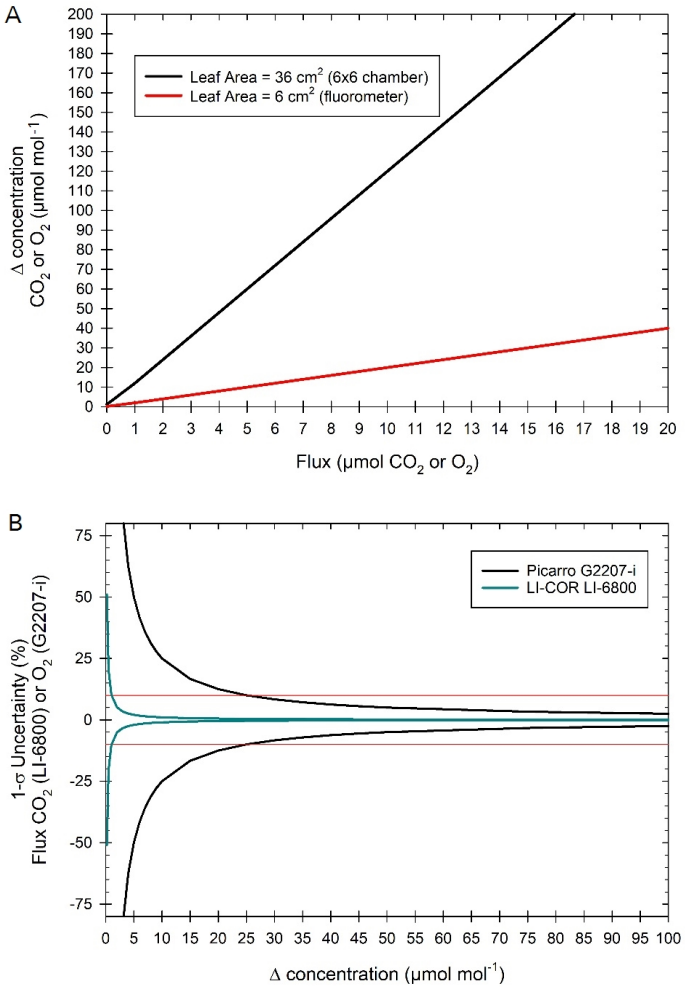

Figure 2. **A**: Δ in concentration between reference and sample airstreams as a function of flux of either CO_2 or O_2. The lines drawn are for leaves completely filling either the large 6×6 cuvette (36 cm^2) or the fluorometer cuvette (6 cm^2), both at a flow rate of 300 µmol s^{-1}. Changes in flow rate or leaf area will impact the Δ gas concentration. **B**: The 1-σ uncertainty in flux (in % difference from true value) as a function of Δ concentration between reference and sample airstreams. The curves were generated by estimating errors in flux using manufacturer supplied analyzer performance (0.1 µmol mol^{-1} 1-σ precision at 4 second averaging for the LI-6800 and 2 µmol mol^{-1} 1-σ precision at 300 second averaging for the G2207-i). Red reference lines indicate ± 10% uncertainty.

While good measurements can be made using any of the LI-6800 cuvettes, caution should be taken when measuring small leaf areas as the errors in the O_2 measurements can become considerable. Best practice is to use the largest leaf area as possible and to operate at low flow rates. This will increase the Δ and reduce uncertainty. Additionally, when leaf area and/or fluxes are small, it is critical to use longer averaging times in the G2207-i (up to 5 minutes) to increase precision.

Example Data

We performed several experiments to validate combined measurements with the LI-6800 and the G2207-i. In order to reduce uncertainties, we used low flow rates and large leaf areas.

Light response curves were performed on *Phaseolus vulgaris* (bean) leaves (Figure 3). Our results are in line with the expected 1:1 stoichiometry for CO_2:O_2 (von Caemmerer 2000). While not studied in detail, the φO_2 in the linear portion of the light response curve is higher than that for φCO_2, agreeing with theoretical expectations (Singsass et al., 2001). The light response curves were performed using the 6x6 cuvette with 36 cm^2 of leaf area. At higher light intensities, ΔO_2 is greater than 100 µmol mol^{-1}, and uncertainty in the measurements is reduced. Near the light compensation point, uncertainty in both CO_2 and O_2 measurements will be high. With the large leaf area in this cuvette, ΔO_2 is reasonably large even during respiration measurements (~9 µmol mol^{-1}, see Figure 2B).

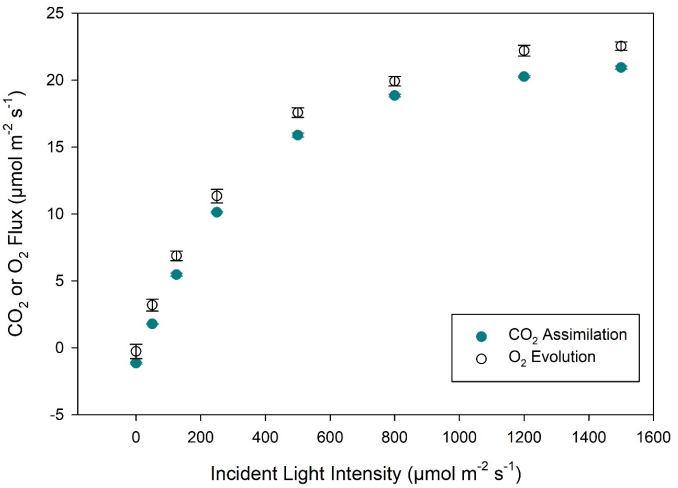

Figure 3. Example light response curve in a C3 plant *Phaseolus spp*. All measurements were made using the 6x6 cuvette with 36 cm^2 of leaf area, error bars are the +/-1 standard deviation of n= 3 replicates.

Additionally, CO_2 response curves were performed on the C4 species *Zea mays* (corn) using the 6800-01A MultiPhase Flash Fluorometer (Figure 4). This cuvette allows for the combined simultaneous measurement of CO_2 gas exchange, O_2 gas exchange and chlorophyll fluorescence. The LI-6800 fluorometer can measure up to 6 cm^2 leaf area. In this experiment, electron transport rate (J) can be calculated from three different measurements: 1) Fluorescence, or $J_f = \phi PS_{II} * Q * \alpha * f_{II}$, where ΦPS_{II} is measured by

chlorophyll fluorescence, Q is incident light intensity, α is leaf absorptance here assumed to be 0.84 and f_{II} is fraction of photons absorbed by photosystem II, here assumed to be 0.5; 2) Gross CO_2 exchange $J_{CO_2} = 4 * A_G$, where A_G is Gross CO_2 Assimilation, or net CO_2 Assimilation + dark respiration; and 3) Gross O_2 exchange, $J_{CO_2} = 4 * O_{EG}$, where O_{EG} is gross O_2 evolution (net O_2 evolution + dark O_2 consumption).

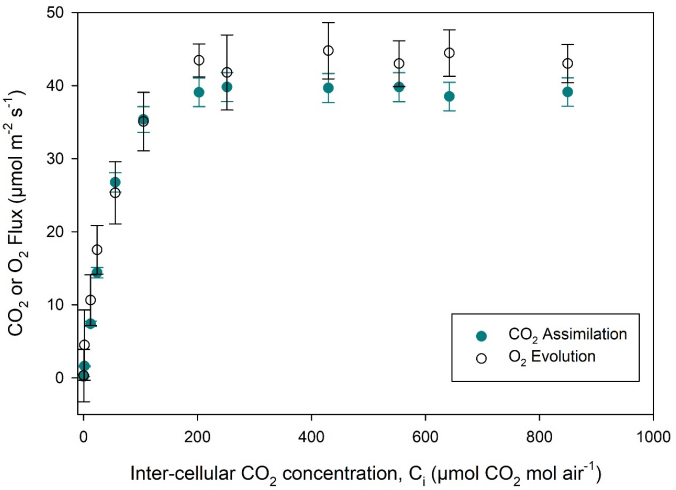

Figure 4. Example CO_2 response curve in a C4 plant *Zea mays*. All measurements were made using the fluorometer cuvette with 6 cm^2 of leaf area, error bars are the +/- 1 standard deviation of n= 3 replicates.

The results of these comparisons are shown in Figure 5. Slopes of ~1 in all cases agree with theoretical expectations and with previous results using isotopic techniques to measure O_2 exchange (Ruuska et al., 2000).

Conclusions

O_2 measurements can be combined with CO_2 and chlorophyll fluorescence to provide unique information for leaf-level physiological research. Issues to consider prior to performing experiments include plumbing the system, dealing with dilution by foreign gases and expected uncertainty in the O_2 flux measurements relative to CO_2 flux uncertainty.

Appendix A: Foreign Gas Dilution on O_2

In a non-reactive mixture of multiple gas species at constant temperature and pressure, the addition of one species results in a commensurate decrease in the mole fraction of all other gas species in the mixture. We refer to this effect as dilution. An equation can be written to describe this dilution effect for O_2 (see Hupp, 2011 for derivation of the dilution correction). First, let's consider the dilution impacts of adding only H_2O on O_2 mole fraction (all units in mol mol^{-1}):

$$O_{dry} = \frac{O_{meas}}{1 - W_{meas}} \quad (3)$$

where O_{meas} is the mole fraction measured, W_{meas} is the water mole fraction in the airstream and O_{dry} is the dilution-corrected O_2 concentration. The high abundance of O_2 in the atmosphere leads to a large dilution effect. For example, at typical atmospheric concentration of 21% (210,000 µmol mol^{-1}), an increase of 5 ppm (0.005 mmol mol^{-1}) water vapor leads to a ~1 µmol mol^{-1} dilution of O_2.

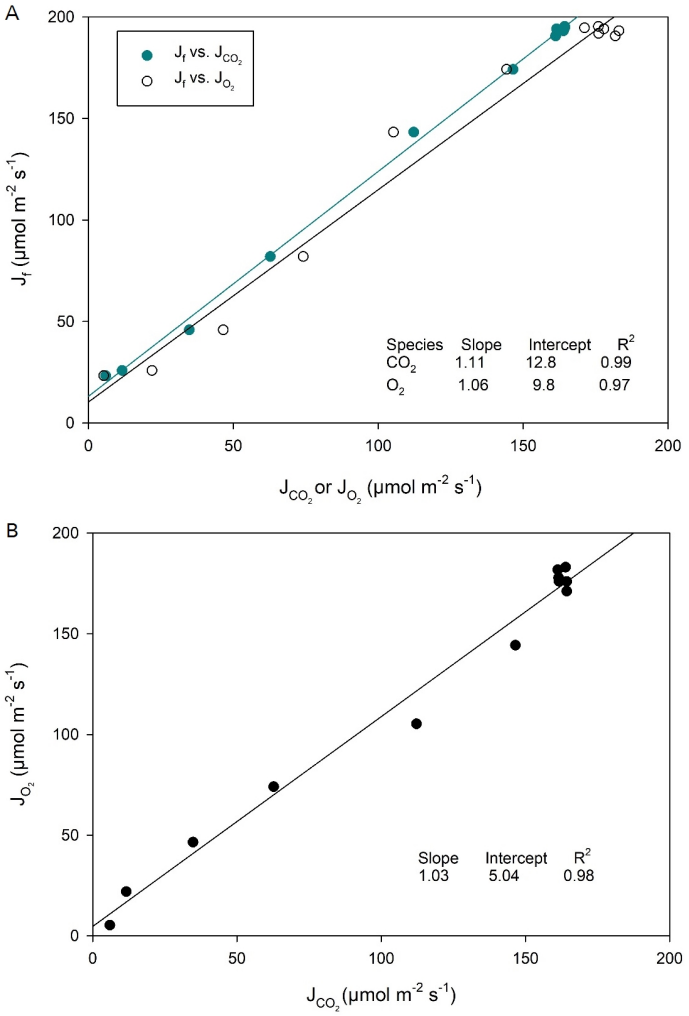

Figure 5. Comparisons of electron transport rate (J) calculated from three independent measurements. See text for equations used. **A**: J calculated from fluorescence measurements compared to those calculated from gross fluxes of CO_2 or O_2. **B**: J calculated from O_2 flux compared to J from CO_2 flux.

In a leaf-level gas exchange system, incoming air must be humidified to prevent stomatal closure during measurements, and the leaf will add H_2O to the airstream. The sample airstream can have significantly larger water mole fraction than the reference airstream. For this reason, the H_2O dilution effect cannot be ignored. The precision of the LI-6800 H_2O gas analyzer is 0.01 mmol mol^{-1} (10 ppm) at 10 mmol mol-1 (1,000 ppm). From equation 3 then, at best the uncertainty in a dilution-corrected O_2 measurement

near 21% is 2 µmol mol^{-1} simply due to uncertainty in the H_2O measurement. This uncertainty can be as large as the induced O_2 change in some cases. Additionally, water sorption on cuvette surfaces and tubing will cause further errors. For these reasons, a dilution corrected O_2 flux measured in wet air streams unacceptably uncertain. To make high precision O_2 measurements it is necessary to calculate small O_2 fluxes, and the H_2O must be scrubbed prior to entering the G2207-i O_2 analyzer physically eliminating the need for an H_2O dilution correction.

The analysis above justifies the necessity of scrubbing H_2O from the airstream before making the O_2 measurements. CO_2 will also differ significantly between reference and sample air streams, enough to require a dilution correction. Just as for H_2O, a 5 µmol mol^{-1} change in CO_2 will cause a 1 µmol mol^{-1} in O_2 near 21% O_2 concentrations. However, for CO_2, the LI-6800 measurement precision is better (<0.1 µmol mol^{-1}). Uncertainties in the CO_2 concentration measurement result in an uncertainty of ~0.02 µmol mol^{-1} in the CO_2 dilution-corrected O_2 concentration. However, if CO_2 is not scrubbed, then the choice of chemicals for scrubbing H_2O becomes more limited, as the H_2O scrubber must not interact with CO_2. In our experience, the chemical of choice for this scenario is magnesium perchlorate ($Mg(ClO_4)_2$), which comes with certain drawbacks; the final state of magnesium perchlorate is a liquid, which needs to be prevented from entering the analyzer and causing damage. Magnesium perchlorate can also be quite expensive. For these reasons, scrubbing both H_2O and CO_2 from the airstream prior to the G2207-i is often the best solution. A single set of chemical columns placed directly in front of the G2207-i, can be used to scrub both sample and reference lines (Figure 1).

Table 1. Useful part numbers.

Part Number	Description
300-15712[a]	Hose barb (metric; M5×0.8 to 1/8" ID)
300-10471[a]	Needle Valve
300-07385[a]	¼" Quick-connect "T" fitting
300-03367	¼" Quick-connect "Y" fitting
300-03123	¼" Quick-connect straight union
8150-250	Bev-a-line tubing (15m roll)
300-01961	Balston air Filter
300-07025	Solenoid valve 6 PSI 5V
314-07215	25-pin D sub connector for LI-6800 console
9960-093	Scrub Tube with ¼" hose barb fittings

[a]Included in the sub-sampling kit part # 9968-210

Appendix B: Mass balance equations for CO_2 and O_2 flux calculation

The LI-6800 system measures both CO_2 and H_2O and corrects for the mole fraction dilution of CO_2 by H_2O. Uncertainties in H_2O measurements as well as the kinetics of H_2O surface interactions make using the dilution correction problematic (see *Appendix A: Foreign Gas Dilution on O2*). Thus, the options are to scrub only water vapor and correct the O_2 concentrations for dilution by CO_2, or to additionally scrub CO_2 and H_2O. We will provide mass balances and final flux calculations for both calculations here.

Scrub H_2O and CO_2

The mass balance for O_2 in an open-path gas exchange system can be written as:

$$sO_E = u_o O_S - u_i O_R \quad (4)$$

where s is leaf area (m^2), O_E is O_2 evolution (µmol m^{-2} s^{-1}), u_i and u_o are molar flow rates (mol s^{-1}), entering and exiting the leaf cuvette, respectively, and O_R and O_S are O_2 concentrations (µmol O_2 mol^{-1}) entering and exiting the leaf cuvette, respectively. When scrubbing both H_2O and CO_2 from the airstream prior to measurement by the Picarro G2207-i O_2 analyzer, the output flow rate u_o is altered only by the addition of O_2:

$$u_o = u_i + sO_E \quad (5)$$

Combining equations 4 and 5 and solving for sO_E yields the flux equation

$$sO_E = \frac{u_i(O_S - O_R)}{1 - O_S} \quad (6)$$

Scrub only H_2O

The mass balance for O_2 in the LI-6800 system when H_2O is scrubbed but CO_2 is not, is identical to equation 4

$$sO_E = u_o O_S - u_i O_R \quad (7)$$

However, in this case the flow exiting the cuvette is altered by both the uptake of CO_2 and evolution of O_2

$$u_o = u_i + sO_E - sA \quad (8)$$

where A is CO_2 Assimilation (µmol CO2 mol^{-1} s^{-1}) and other variables as in equation 4. Combining equations 5 and 4 and solving for sO_E yields

$$sO_E = \frac{u_i(O_S - O_R) - sAO_s}{1 - O_S} \quad (9)$$

A similar equation can be written for the mass balance of CO_2

$$sA = \frac{u_i(C_R - C_S) - sO_E C_s}{1 - C_S} \quad (10)$$

Substituting the expression for sA into equation 9 allows us to solve for sO_E in terms of the concentrations. This form of the equation can be used to calculate O_2 evolution in the case where CO_2 is not being scrubbed.

$$sO_E = \frac{u_i(O_S - O_R)(1 - C_S) - u_i O_s(C_R - C_S)}{1 - (O_S + C_S)} \quad 11$$

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