Determination of respiration and photosynthesis fractionation coefficients for atmospheric dioxygen inferred from a vegetation-soil-atmosphere analog of the terrestrial biosphere in closed chambers

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Abstract

The isotopic composition of dioxygen in the atmosphere is a global tracer which depends on the biosphere flux of dioxygen toward and from the atmosphere (photosynthesis and respiration) as well as exchanges with the stratosphere. When measured in fossil air trapped in ice cores, the relative concentration of \(^{16}\text{O}, {^{17}\text{O}}\) and \(^{18}\text{O}\) of O\(_2\) can be used for several applications such as ice core dating and past global productivity reconstruction. However, there are still uncertainties about the accuracy of these tracers as they depend on the integrated isotopic fractionation of different biological processes of dioxygen production and uptake, for which we currently have very few independent estimates. Here we determined the respiration and photosynthesis fractionation coefficients for atmospheric dioxygen from experiments carried out in a replicated vegetation-soil-atmosphere analog of the terrestrial biosphere in closed chambers with growing Festuca arundinacea. The values for \(^{18}\text{O}\) discrimination during soil respiration and dark respiration in leaf are equal to -12.3 ± 1.7 ‰ and -19.1 ± 2.4 ‰, respectively. We also found a value for terrestrial photosynthetic fractionation equal to +3.7 ± 1.3 ‰. This last estimate suggests that the contribution of terrestrial productivity in the Dole effect may have been underestimated in previous studies.
1. Introduction

The oxygen cycle represents the most important biogeochemical cycle on Earth: oxygen is the second most important gaseous component in the atmosphere. Oxygen is an essential component for life on Earth as it is consumed by all aerobic organisms through respiration and produced by autotrophic organisms through photosynthesis.

The analysis of the oxygen isotopic composition classically expressed as \(\delta^{18}O\) and \(\delta^{17}O\) of \(O_2\) in air bubbles trapped in ice cores is currently used to provide information on the variations of low latitude water cycle and the productivity of the biosphere during the Quaternary (Bender et al., 1994; Luz et al., 1999; Malaisé et al., 1999; Severinghaus et al., 2009; Blunier et al., 2002; Landais et al., 2010). \(\delta^{18}O\) of \(O_2\) is also a very useful proxy for ice core dating through the resemblance of its variations with the variations of precession or summer insolation in the northern hemisphere (Shackleton, 2000; Dreyfus et al., 2007). These tracers are however complex and their interpretation relies on the precise knowledge of the fractionation factors in the oxygen cycle.

First, the interpretation of variations of \(\delta^{18}O\) of \(O_2\) (or \(\delta^{18}O_{\text{atm}}\)) in the old air trapped in ice core in term of low latitude water cycle (e.g. Severinghaus et al., 2009; Landais et al., 2010; Seltzer et al., 2017) is still debated because of the multiplicity of the processes involved. Dole et al. (1954) has shown that the \(\delta^{18}O_{\text{atm}}\) is enriched compared to the \(\delta^{18}O\) of water of the global ocean (taken here as the Vienna Standard Mean Ocean Water, VSMOW) with a value of 23.88 ‰ (Barkan and Luz, 2005). This Dole effect is the result of several fractionations in the biosphere that enrich the \(\delta^{18}O_{\text{atm}}\) relative to the oceanic one. First measurements have shown that the photosynthesis itself is not associated with a strong fractionation and produces oxygen with an isotopic composition which is close to the isotopic composition of the consumed water (Guy et al., 1993). For the oceanic biosphere, the isotopic composition of \(O_2\) produced by photosynthesis is very close to the isotopic composition of the ocean. However, in terrestrial biosphere the \(\delta^{18}O\) of water consumed by photosynthesis (leaf water) is highly variable both spatially and temporally because of the decrease of \(\delta^{18}O\) of meteoric water toward higher latitudes and the enrichment in heavy isotopes in leaf water during evaporation. The enrichment of the mean leaf water isotopic composition has been estimated within 4.5 – 6 ‰ with respect to the isotopic composition of the mean global ocean (Bender et al., 1994; Hoffmann et al., 2004). On top of this enrichment, the terrestrial and oceanic Dole effects are mostly explained by the respiratory isotopic discrimination of the order of magnitude of + 18 ‰ (Bender et al., 1994).

Because of the isotopic enrichment in leaf water, the terrestrial Dole effect has been initially estimated to be 5 ‰ higher than the oceanic Dole effect and \(\delta^{18}O_{\text{atm}}\) used to estimate changes in the balance between land and marine productivity (Wang et al., 2008; Bender et al., 1994; Hoffmann et al., 2004).
However, the evidence by Eisenstadt et al. (2010) of isotopic discrimination up to + 6‰ for marine phyttoplankton photosynthesis rather suggests that the marine and terrestrial Dole effects are of the same order of magnitude. In this case, the past variations of $\delta^{18}O_{\text{atm}}$ would be related to low latitude water cycle influencing the leaf water $\delta^{18}O$ consumed by photosynthesis (most important in the low latitude vegetated regions). This is supported by orbital and millennial variations of $\delta^{18}O_{\text{atm}}$ in phase with calcite $\delta^{18}O$ in Chinese speleothem, a proxy strongly related to the intensity of hydrological cycle in the South-East Asia (Severinghaus et al., 2009; Landais et al., 2010; Extier et al., 2018). The aforementioned studies show that qualitative and quantitative interpretation of $\delta^{18}O_{\text{atm}}$ relies strongly on the estimate of O$_2$ fractionation factors in the biological cycle but data to constrain the fractionation factors associated with respiration and photosynthesis for the different ecosystems are sparse.

In addition to the use of $\delta^{18}O_{\text{atm}}$, the combination of $\delta^{17}O$ and $\delta^{18}O$ of O$_2$ provides a way to quantify variations in past global productivity (Luz et al., 1999). This method relies on the fact that O$_2$-fractionating processes in the stratosphere and within the biosphere lead to different relationships between $\delta^{17}O$ and $\delta^{18}O$ of O$_2$. The biosphere fractionating processes are mass-dependent such that the $^{17}O$ enrichment is about half the $^{18}O$ enrichment relative to $^{16}O$. On the contrary, oxygen is fractionated in a mass-independent manner in the stratosphere producing approximately equal $^{17}O$ and $^{18}O$ enrichments (Luz et al., 1999). We thus define a $\Delta^{17}O$ anomaly as:

$$\Delta^{17}O = \ln(1 + \delta^{17}O) - 0.516 \times \ln(1 + \delta^{18}O)$$

$\Delta^{17}O$ of O$_2$ is equal to 0 by definition in the present-day troposphere (the standard for isotopic composition of atmospheric oxygen is the present-day atmospheric value). $\Delta^{17}O$ of O$_2$ is negative in the stratosphere and increase in biosphere productivity leads to an increase of $\Delta^{17}O$ of O$_2$. As for the interpretation of $\delta^{18}O_{\text{atm}}$, the quantitative link between $\Delta^{17}O$ of O$_2$ and biosphere productivity depends on the exact fractionation factors associated with biosphere processes (Brandon et al., 2020).

Several studies have been conducted to estimate the fractionation factors during biosphere processes of O$_2$ production and consumption. These fractionation factors are then implemented in global modeling approaches involving the use of models of global vegetation and oceanic biosphere for interpretation of $\Delta^{17}O$ of O$_2$ and $\delta^{18}O_{\text{atm}}$ in term of environmental parameters (Landais et al., 2007; Blunier et al., 2012; Reutenauer et al., 2015; Brandon et al., 2020). Most of the fractionation factors used in these modeling approaches were obtained from studies conducted at the cell level: cyanobacterium (Helman et al., 2005), E. coli (Stolper et al., 2018), microalgae (Eisenstadt et al., 2010).
In these studies, the underlying assumption is that the fractionation factor associated with \( \text{O}_2 \) measured at the cell level can be applied at the ecosystem scale. Yet, results from studies conducted at a larger scale, e.g. at the soil scale by Angert et al. (2001) found a global terrestrial respiratory \( ^{18}\text{O}/^{16}\text{O} \) of \( \text{O}_2 \) discrimination for soil microorganisms varying between -12\,‰ and -15\,‰. This is lower than the -18\,‰ discrimination classically used for respiration, with diffusion in soil playing a role in addition to biological respiration fractionation. Later, Angert et al. (2003) found an even larger spread of \( \text{O}_2 \) isotopic discrimination in soil and showed that temperate and boreal soils have higher fractionation, respectively -17.8\,‰ and -22.5\,‰, because they engage the AOX (alternative oxidase) pathway which strongly discriminates \( ^{18}\text{O} \), unlike tropical soils (-10.8\,‰). These contrasting results show the interest of making measurements at a larger scale than at the cell level to correctly interpret global variations of the isotopic composition of oxygen.

In this study we developed a vegetation-soil-atmosphere simplified analog of the terrestrial biosphere in closed chamber of 120 liters with the aim of estimating the fractionation coefficients of atmospheric dioxygen due to soil respiration, plant respiration and photosynthesis. With this setup we carried out several experimental runs with soil only and soil with plants in order to estimate the fractionation of the different compartments and check values obtained at the cell level. The implications for our interpretation of the Dole effect are also discussed.

2. Material and Methods

2.1. Growth chamber and closed system

2.1.1. Plant growth and experimental setup

a)
Seeds of *Festuca arundinacea* (Schreb.), also commonly called tall fescue, were first sown in a commercial potting soil (Terreau universel, Botanic, France. Composition: black and blond peat, wood fibre, green compost and vermicompost manure, organic and organo-mineral fertilizers and micronutrient fertilizers). During 15 to 20 days, they were then placed in a growth chamber of the Microcosms experimental platform of the European Ecotron of Montpellier (https://www.ecotron.cnrs.fr) under diurnal cycles of enlightenment, air temperature set at 20°C (*T*<sub>air</sub>), air relative humidity (*RH*) at 80% and CO<sub>2</sub> atmospheric concentration close to ambient air (concentration of CO<sub>2</sub> = 400 ppm).

Twelve pots (8 cm × 8 cm × 12 cm with 180 to 200 g of dry soil) containing approximately 25 to 30 fescue mature plants were used for each experimental run. All plants were placed in a plastic tray filled with tap water, inside an airtight transparent chamber manufactured from welded polycarbonate (10 mm wall thickness and 120 liters volume) similar to the chambers used by Milcu et al. (2013) (Fig. 1). The sealing of the closed chamber was checked before each experiment using helium.
To control temperature and light intensity inside the closed chamber, this one was placed in a larger controlled environment growth chamber. Light was provided by two plasma lamps (GAVITA Pro 300 LEP02; GAVITA) with PAR = 200 μmol·m⁻²·s⁻¹ and air temperature inside the closed chamber was regulated at 19 ± 1 °C by adjusting the growth chamber temperature.

The closed chamber (Fig. 1) was used as a closed gas exchange system with controlled, and continuously monitored, environmental parameters. Air and soil temperature (CTN 35, Carel), air relative humidity (PFmini72, Michell instrument, USA) and CO₂ atmospheric concentration (GMP343, Vaisala, Finland) were measured and recorded using the growth chamber datalogger (sampling rate = 1 min). O₂ concentration was continuously monitored using an optical sensor (Oxy1-SMA, Presens, Germany).

Air relative humidity was regulated between 80 % and 90 % using a heat exchanger (acting as a condenser) connected to a closed cycle water cooling system. The condenser was positioned in a way to create a water closed cycle in the biological chamber (water vapor from evapotranspiration condense back into irrigation water). In order to keep the CO₂ mixing ratio close to 400 ppm during the light periods, photosynthetic CO₂ uptake was compensated with injections of pure CO₂ using a mass flow controller (F200CV, Bronkhorst, The Netherlands). During the dark periods, a soda lime trap connected to a micro-pump (NMS 020B, KNF, Germany) was used to remove the excess CO₂ coming from respiration. CO₂ atmospheric concentration during the night was kept below 200 ppm.

To ensure atmospheric pressure stability in the closed chamber, a pressure compensation system, made of two connected 10 liters gas tight bags (multi-layers foil bags, Restek, USA), was installed. Each bag was half full of atmospheric air, the first one was installed in the closed chamber while the second one was outside this chamber. This way, each bag inflates or deflates in response to pressure variation either due to O₂ or CO₂ uptake or release. The pressure difference between the closed chamber and the atmosphere was regularly measured using a differential sensor (FD A602-S1KAlmemo, Ahlborn, Germany).

Finally, the enclosed air was mixed and considered homogeneous using seven brushless fans.

2.1.2. Gas sampling

To measure the isotopic composition along the experiment, small samples of gas were collected in 5 mL glass flasks, made of two Louwers H.V. glass valves (1-way bore 9mm Ref. LH10402008, Louwers Hanique, The Netherlands) welded together. Those flasks, previously evacuated, were mounted on PFA tubing (1/4") using two 1/4" UltraTorr fitting (SS-4-UT-9, Swagelok, USA). Two manual valves (SS-4H, Swagelok, USA) were also installed on the PFA tubes to open or close the circuit. A micro-pump
(NMS 20B, KNF, Germany) was finally turned on during air sampling to ensure closed chamber atmosphere circulation through the flask.

2.2. Isotopic measurements

2.2.1. Water extraction from leaf and isotopic analysis

After each experiment, the plant leaves were collected, placed in airtight flasks and immediately frozen at -20°C for at least 24 hours to make sure there was minimal loss of water through vaporization when the vial was opened later. The extraction of water from leaves was done according to the procedure detailed in Alexandre et al. (2018). The vial was fixed onto a cryogenic extraction line and was first immersed in a liquid nitrogen Dewar to prevent any sublimation of the water. The water extraction line was emptied of most of its air (<10⁻⁵ Pa). Once this pressure has been reached, the pump was turned off and a valve was closed in order to keep a constant static void within the system. The "reception" vial was then immersed in a liquid nitrogen Dewar (which will act as a water trap) and the sample vial was immersed in water maintained at 75°C. The system was kept in these conditions for no less than six hours, so that all the water present in the leaf and stems was extracted. Afterwards, in order to remove all of the organic compounds of the extracted water, an active charcoal was placed in the extracted water and left under agitation for the night.

For analysis of δ¹⁷O and δ¹⁸O of water, leaf water was converted to O₂ using a fluorination line for reaction of H₂O with CoF₃ heated to 370°C at LSCE. The isotopic composition of the dioxygen was measured by IRMS equipped with dual inlet (Thermo Scientific MAT253 mass spectrometer). The standard that was chosen was an O₂ standard calibrated against VSMOW. The precision was 0.015‰ for δ¹⁷O, 0.010‰ for δ¹⁸O and 6 ppm for Δ¹⁷O (for more details, refer to Landais et al. 2006).

2.2.2. O₂ purification and isotopic analysis

The air samples collected in the closed chambers were transported to LSCE for analyses of isotopic composition of O₂. The flasks were connected on a semi-automatic separation line inspired from Barkan and Luz (2003) which was made up of 8 ports in which 2 standards (outside air) and 6 samples were analyzed daily (Brandon et al., 2020). After pumping the whole line, the air was circulated through a water trap (ethanol at -100°C) and then through a carbon dioxide trap immersed in liquid nitrogen at -196°C. After collection of the gas samples on a molecular sieve trap cooled at -196°C, a helium flow carried it through a chromatographic column which was immersed in a water reservoir at 0°C to
separate the dioxygen and the argon from the dinitrogen. After separation of the dioxygen and argon from helium, the gas was collected in a stainless-steel manifold immersed in liquid helium at -269°C. After collection, the samples were analyzed by the IRMS previously mentioned for leaf water analyses. The following ratios were measured: $^{18}\text{O}/^{16}\text{O}$, $^{17}\text{O}/^{16}\text{O}$ and $\text{O}_2/\text{Ar}$ (as an indicator of the $\text{O}_2$ concentration since $\text{Ar}$ is an inert gas). $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ of each sample were obtained through 3 series of 24 dual inlet measurements against a standard made of $\text{O}_2$ and $\text{Ar}$. This sequence was followed by 2 peak jumping analyses of the $\text{O}_2/\text{Ar}$ ratio including separate measurements of the $\text{O}_2$ and $\text{Ar}$ signals for both the standard and the sample. The uncertainty associated with each measurement was obtained from the standard deviation of the three runs and from the repeated peak jumping measurement for $\delta\text{O}_2/\text{Ar}$. The uncertainty values for $\Delta^{17}\text{O}$, $\delta^{18}\text{O}$ and $\delta\text{O}_2/\text{Ar}$ were respectively 10 ppm, 0.05 ‰ and 0.5 ‰.

Each day, we performed measurements of the dioxygen isotopic composition and $\text{O}_2/\text{Ar}$ ratio on two samples of outside air which is the standard for isotopic composition of $\text{O}_2$ (Hillaire-Marcel et al., 2021). So that the calibrated $\delta^{18}\text{O}$ value for our sample was calculated as in equation 2:

$$
\delta^{18}\text{O}_{\text{calibrated}} = \left[ \frac{\delta^{18}\text{O}_{\text{measured}}/1000 + 1}{\delta^{18}\text{O}_{\text{outside air}}/1000 + 1} - 1 \right] \times 1000
$$

(2)

2.3. Experimental runs

2.3.1. General strategy

Our goal was to calculate the fractionation factor associated with $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ for soil respiration, dark respiration and photosynthesis using the microcosm described above. In order to quantify the fractionation factors, we needed to work in closed and controlled conditions. Given the volume of the closed chamber (120 l, hence about 1.12 moles of $\text{O}_2$) and the order of magnitude of dark respiration (order of magnitude of 0.08 µmol $\text{O}_2$ s$^{-1}$ for soil respiration) and net photosynthetic fluxes (order of magnitude of 0.45 µmol $\text{O}_2$ s$^{-1}$) inside the chamber, we calculated that experiments should last from 23 days to more than 2 weeks so that more than one tenth of the $\text{O}_2$ in the chamber can be recycled by the plant and soil. This recycling allows the creation of sufficiently large isotopic signals (especially $\Delta^{17}\text{O}$ of $\text{O}_2$) to be detected and measured. We set up two different experiments in the closed chamber, each experiment being repeated 3 or 4 times to address experimental repeatability of the system.
The first experiment (repeated 4 times, i.e. in 4 sequences) aimed at studying the fractionation coefficients during soil respiration. The second experiment (repeated 3 times, i.e. in 3 sequences, each sequence being divided into several periods with or without light) aimed at studying the fractionation coefficients during dark respiration and photosynthesis of plants.

Prior to the aforementioned experiments, measurements were carried out on a closed empty chamber to check the absence of leaks as well as the absence of isotopic fractionation (Table S1).

2.3.2. Soil respiration experiment

To conduct the soil respiration experiment, 2.6 kg of soil (Terreau universel, Botanic) were placed in 12 different pots. The light was turned off during this experimental run. During this dark period, CO₂ from soil respiration accumulates in the biological closed chamber. To have a stable concentration of CO₂ during the whole dark period, the CO₂ was trapped using soda lime. Four sequences were performed with respective durations of 53, 51, 43 and 36 days.

2.3.3. Photosynthesis and dark respiration experiment

We used the same soil with plants (Festuca arundinacea) grown before the start of the three sequences of the photosynthesis and dark respiration experiment. In order to obtain a significant change of the $\Delta^{13}$O of O₂ signal in our closed 120 L chambers, the 3 experiments were run during 1 to 2 months. CO₂ level was controlled by a CO₂ trap and CO₂ injections. This was done to ensure that the CO₂ in the chamber did not reach levels too far from the atmospheric composition. This could have affected the physiology of the plant. The enlightenment was controlled to alternate between day (photosynthesis and respiration) and night conditions (respiration).

2.4. Quantification of fractionation coefficients

We detail below how we used the results from our experiments to quantify the associated fractionation coefficients. Notations used below are gathered in Table 1.

The isotopic fractionation coefficient of oxygen is expressed through the fractionation coefficient $\alpha$.

$$18\alpha = \frac{R_{O_{\text{product}}}^{18}}{R_{O_{\text{substrat}}}^{18}}$$  \hspace{1cm} (3)
where $\alpha$ is the fractionation coefficient and $R^{18}O$ is the ratio of the concentration $R^{18}O = \frac{n(^{18}O)}{n(^{16}O)}$ with $n$ the number of moles of $O_2$ containing $^{18}O$ or $^{16}O$. $R^{18}O$ is linked to the $\delta^{18}O$ value through:

$$\delta^{18}O = \left(\frac{R^{18}O_{sample}}{R^{18}O_{standard}} - 1\right) \times 1000 \quad (4)$$

The isotopic discrimination is related to the isotopic fractionation coefficient through:

$$\epsilon^{18} = \frac{18}{\alpha} - 1 \quad (5)$$

The same equations (3), (4) and (5) can be proposed for $\delta^{17}O$ and the relationship between the fractionation coefficients $^{17}\epsilon$ and $^{18}\epsilon$ is written as:

$$\gamma = \frac{\ln^{17}\epsilon}{\ln^{18}\epsilon} \quad (6)$$

### 2.4.1. Soil respiration

Respiration is associated with isotopic fractionation. The light isotopes, $^{16}O$, are more easily integrated by microorganisms than the heavy isotopes, $^{18}O$, which hence remain in the atmosphere. We express the fractionation coefficient for soil respiration as:

$$^{18}\alpha_{soil\_respi} = \frac{R^{18}O_{breathed}}{R^{18}O_{air}} \quad (7)$$

In our experiment, the respiratory process took place in a closed reservoir so that we could calculate the fractionation coefficients from the evolution of the concentration and isotopic composition of dioxygen in the chamber. The evolution of the number of molecules of dioxygen in the air of the closed chamber, $n(O_2)$, between time $t$ and time $t+dt$ can be written as:

$$n(O_2)_t = n(O_2)_{t+dt} + dn(O_2) \quad (8)$$

with $dn$ the number of dioxygen molecules respired during the time period $dt$. A similar equation can be written for the number of dioxygen molecules containing $^{18}O$ remaining in the air of the chamber:

$$R^{18}O_t \times n(O_2)_t = R^{18}O_{t+dt} \times n(O_2)_{t+dt} + R^{18}O_{t+dt} \times ^{18}\alpha_{soil\_respi} \times dn(O_2) \quad (9)$$
Combining equations Eq. (8) and Eq. (9) and integrating from $t_0$ (starting time of the experiment when the chamber is closed) to $t$ leads to:

$$18\varepsilon_{\text{soil respi}} = 18\varepsilon_{\text{soil respi}} - 1 = \ln \left( \frac{n^{18}O_{\text{produced}}}{n^{18}O_{\text{lw}}} \right)$$

$$\ln \left( \frac{n^{18}O_{\text{lw}}}{n^{18}O_{\text{atm}}} \right)$$

(10)

Since argon is an inert gas, we can link $n^{18}(O_2)$ to $\delta \left( \frac{O_2}{Ar} \right)$, so that:

$$\frac{n^{18}(O_2)}{n^{18}(O_2)_{\text{atm}}} = \frac{\delta(\frac{O_2}{Ar})}{\delta(\frac{O_2}{Ar})_{\text{atm}}}$$

(11)

2.4.2. Dark respiration

In order to calculate the fractionation associated with soil and plant respiration during dark period, we followed the same calculation as for the soil respiration (section 2.4.1). In this case, we selected only night periods from each sequence of the photosynthesis and dark respiration experiment.

2.4.3. Photosynthesis

During photosynthesis, the oxygen atoms in the dioxygen produced by the plant comes from the oxygen atom of water consumed by photosynthesis in the leaves so that the fractionation coefficient during photosynthesis can be expressed as:

$$18\varepsilon_{\text{photosynthesis}} = \frac{R^{18}O_{\text{produced}}O_2}{R^{18}O_{\text{lw}}}$$

(12)

where lw stands for leaf water.

Photosynthesis occurs during the light periods. However, it should be noted that dark respiration, photorespiration and Mehler’s reaction (Mehler, 1951) occur at the same time. Thus, at each stage,
dioxygen is both produced by photosynthesis and consumed by the aforementioned O₂ uptake processes (hereafter total_respi) by the plant according to the mass conservation equation:

\[ n(O_2)_t = n(O_2)_{t+dt} + dn_{total_respi} + dn_{photosynthesis} \]  \hspace{1cm} (13)

where \( dn_{total_respi} \) is the number of molecules of O₂ consumed by dark respiration, photorespiration and Mehler’s reaction between time t and t+dt, and \( dn_{photosynthesis} \) is the number of molecules of O₂ produced by photosynthesis between t and t+dt.

The budget for \(^{18}\)O of O₂ can be written as:

\[ R^{^{18}}O_t \times \frac{n(O_2)_t}{n(O_2)_0} = R^{^{18}}O_{t+dt} \times \frac{n(O_2)_{t+dt}}{n(O_2)_0} + R^{^{18}}O_{t+dt} \times 18\alpha_{total_respi} \times \frac{dn_{total_respi}}{n(O_2)_0} + R^{^{18}}O_{lw} \times 18\alpha_{photosynthesis} \times \frac{dn_{photosynthesis}}{n(O_2)_0} \]  \hspace{1cm} (14)

where \( 18\alpha_{total_respi} \) is the fractionation coefficients associated with each O₂ consuming process periods throughout the whole experiment.

We introduced the normalized fluxes of photosynthesis and total respiration as:

\[ F_{photosynthesis} = \frac{dn_{photosynthesis}}{n(O_2)_0 \times dt} \]  \hspace{1cm} (15)

\[ F_{total_respi} = \frac{dn_{total_respi}}{n(O_2)_0 \times dt} \]  \hspace{1cm} (16)

\[ aR^{^{18}} = \left| \frac{dR^{^{18}}O}{dt} \right| \]  \hspace{1cm} (17)

This led to the following expression of \( 18\alpha_{photosynthesis} \):

\[ 18\alpha_{photosynthesis} = - aR^{^{18}} + F_{photosynthesis} - F_{total_respi} + 18\alpha_{total_respi} \times F_{total_respi} \]  \hspace{1cm} (18)

\( 18\alpha_{photosynthesis} \) depends on the values of \( 18\alpha_{total_respi} \) and of \( F_{total_respi} \), themselves dependent on the values of \( 18\alpha_{Mehler} \) (fractionation factor associated with Mehler reaction), \( F_{Mehler} \) (flux of
oxygen related to Mehler reaction), $^{18}\alpha_{\text{dark_respi}}$, $F_{\text{dark_respi}}$, $^{18}\alpha_{\text{photorespi}}$ (fractionation factor associated with photorespiration) and $F_{\text{photorespi}}$ (photorespiration flux of oxygen). These last 4 parameters could not be determined in our global experiment. Our determination of $^{18}\alpha_{\text{photosynthesis}}$ will thus rely on assumptions for the estimations of $^{18}\alpha_{\text{Mehler}}$, $F_{\text{Mehler}}$, $^{18}\alpha_{\text{photorespi}}$ and $F_{\text{photorespi}}$.

To separate the $^{18}\alpha_{\text{dark_respi}}$ from the other fractionation factors, we defined:

$$^{18}\alpha_{\text{total_respi}} = ^{18}\alpha_{\text{photorespi}} \times f_{\text{photorespi}} + ^{18}\alpha_{\text{Mehler}} \times f_{\text{Mehler}} + ^{18}\alpha_{\text{dark_respi}} \times f_{\text{dark_respi}}$$

(19)

with

$$F_{\text{total_respi}} = F_{\text{dark_respi}} + F_{\text{photorespi}} + F_{\text{Mehler}}$$

(20)

$f$ indicates the fraction of the total oxygen uptake flux corresponding to each process (dark respiration, photorespiration and Mehler’s reaction) so that:

$$f_{\text{dark_respi}} + f_{\text{photorespi}} + f_{\text{Mehler}} = 1$$

(21)

$$F_{\text{dark_respi}} = f_{\text{dark_respi}} \times F_{\text{total_respi}}$$

(22)

$$F_{\text{photorespi}} = f_{\text{photorespi}} \times F_{\text{total_respi}}$$

(23)

$$F_{\text{Mehler}} = f_{\text{Mehler}} \times F_{\text{total_respi}}$$

(24)

In the absence of further constraints, we used here as first approximation the global values from Landais et al. (2007) for $f_{\text{dark_respi}}$ (0.6), $f_{\text{photorespi}}$ (0.3) and $f_{\text{Mehler}}$ (0.1). Values for $^{18}\alpha_{\text{photorespi}}$ and $^{18}\alpha_{\text{Mehler}}$ were based on the most recent estimates of Helman et al. (2005).

### Table 1. List of variables used to quantify fractionations.

| Symbol          | Description                  |
|-----------------|------------------------------|
| $^{18}\alpha$   | Fractionation coefficient    |
| Symbol | Description                                                                 |
|--------|-----------------------------------------------------------------------------|
| \( \alpha_{\text{dark\_respi}} \) | Fractionation coefficient of soil and plant respiration during night periods |
| \( \alpha_{\text{dark\_leave\_respi}} \) | Fractionation coefficient of respiration of leave during night periods       |
| \( \alpha_{\text{Mehler}} \) | Fractionation coefficient associated with Mehler respiration                 |
| \( \alpha_{\text{photorespi}} \) | Fractionation coefficient associated with photorespiration                   |
| \( \alpha_{\text{photosynthesis}} \) | Fractionation coefficient associated with photosynthesis                     |
| \( \alpha_{\text{soil\_respi}} \) | Fractionation coefficient associated with soil respiration                   |
| \( \alpha_{\text{total\_respi}} \) | Fractionation coefficient associated with total respiration during light period |
| \( \varepsilon \) | Isotopic discrimination                                                     |
| \( \varepsilon_{\text{dark\_respi}} \) | Isotopic discrimination of soil and plant respiration during night periods   |
| \( \varepsilon_{\text{dark\_leave\_respi}} \) | Isotopic discrimination of respiration of leave during night periods          |
| \( \varepsilon_{\text{photosynthesis}} \) | Isotopic discrimination associated with photosynthesis                       |
| \( \varepsilon_{\text{soil\_respi}} \) | Isotopic discrimination of soil respiration associated with soil respiration experiment |
| \( \gamma \) | Ratio of \( \ln(17\alpha) \) to \( \ln(18\alpha) \)                        |
| \( \gamma_{\text{dark\_respi}} \) | Ratio of \( \ln(17\alpha_{\text{dark\_respi}}) \) to \( \ln(18\alpha_{\text{dark\_respi}}) \) |
| \( \gamma_{\text{dark\_leave\_respi}} \) | Ratio of \( \ln(17\alpha_{\text{dark\_leave\_respi}}) \) to \( \ln(18\alpha_{\text{dark\_leave\_respi}}) \) |
| \( \gamma_{\text{photosynthesis}} \) | Ratio of \( \ln(17\alpha_{\text{photosynthesis}}) \) to \( \ln(18\alpha_{\text{photosynthesis}}) \) |
| \( \gamma_{\text{soil\_respi}} \) | Ratio of \( \ln(17\alpha_{\text{soil\_respi}}) \) to \( \ln(18\alpha_{\text{soil\_respi}}) \) |
| Symbol          | Description                                                                 |
|-----------------|-----------------------------------------------------------------------------|
| $aN$            | Linear regression coefficient of the evolution of $n(O_2)$ as a function of time |
| $aR$            | Linear regression coefficient of the evolution of $R^*O$ as a function of time |
| $dn_{\text{photosynthesis}}$ | Number of molecules of O$_2$ produced by photosynthesis between $t$ and $t+dt$ |
| $dn_{\text{total respi}}$ | Number of molecules of O$_2$ consumed by total respiration during light periods between time $t$ and $t+dt$ |
| $F_{\text{dark respi}}$ | Dark respiration flux (normalized vs number of O$_2$ molecules at the start of the experiment) |
| $F_{\text{Mehler}}$ | Mehler flux (normalized vs number of O$_2$ molecules at the start of the experiment) |
| $F_{\text{photorespi}}$ | Photorespiration O$_2$ flux (normalized vs number of O$_2$ molecules at the start of the experiment) |
| $F_{\text{photosynthesis}}$ | Photosynthesis O$_2$ flux (normalized vs number of O$_2$ molecules at the start of the experiment) |
| $F_{\text{total respi}}$ | Total respiration O$_2$ flux during light period (normalized vs number of O$_2$ molecules at the start of the experiment) |
| $f_{\text{dark respi}}$ | Fraction of the dioxygen flux corresponding to dark respiration process |
| $f_{\text{Mehler}}$ | Fraction of the dioxygen flux corresponding to Mehler process |
| $f_{\text{photorespi}}$ | Fraction of the dioxygen flux corresponding to photorespiration process |
| $n(O_2)$ | Number of moles of O$_2$ |
| $R^*O$ | Ratio of heavy (18O or 17O) isotope to light isotope (16O) |
| $R^*O_{lw}$ | $R^*O$ of leaf water |

3. Results

3.1. Soil Respiration

3.1.1. Experimental data
Fig. 2. Evolution of the different concentrations and isotopic ratios in the sequence 2 of the soil respiration experiment. (a) $\delta^{18}$O of O$_2$ (red) variations. (b) $\Delta^{17}$O of O$_2$ (blue) variations. (c) Dioxygen concentration (purple) and $\delta$O$_2$/Ar variations (green).

During the 4 sequences, the respiration activity led to a decreasing level of the O$_2$ concentration measured by the optical sensor or through the $\delta$O$_2$/Ar evolution from IRMS measurements (Fig. S1). The comparison of the evolution of the O$_2$ concentration during the different sequences showed that respiratory fluxes were different with a maximum factor of 4 between the different sequences (Fig. S1). In parallel to the decrease in O$_2$ concentration, the $\delta^{18}$O increased as expected since respiration preferentially consumes the lightest isotopes: over the 51 days of the 2nd soil respiration sequence, we observed a linear decrease of oxygen concentration by more than 5% while $\delta^{18}$O increased by 8‰ (Fig. 2). A Mann-Kendall test (95%) showed that the $\Delta^{17}$O of O$_2$ does not show any trend within 95% over the 4 sequences (Fig. S2).

3.1.2. Fractionation coefficients

We used the 15 to 20 samples obtained during each sequence of soil respiration experiment to draw the relative evolution of $\ln(R^{18}O_2/R^{18}O_{eq})$ vs $\ln((\delta^{18}O_{Ar}/1000 + 1)/(\delta^{18}O_{Ar}/1000 + 1))$ following Eq. (10) (Fig. 3). The slope of the corresponding regression line provided the isotopic discrimination $^{18}\epsilon_{soil,resp}$ and hence the fractionation coefficient $^{18}\alpha_{soil,resp}$ for each sequence (Table S2). It could be observed that despite differences in respiratory fluxes for the different sequences.
sequences, the relationship between $\delta^{18}$O of O$_2$ and O$_2$ concentration (or $\delta$O$_2$/Ar) and hence the calculated fractionation factor associated with respiration is not much affected. The observed differences between respiratory fluxes could be explained by the small variations in organic carbon and by a different development of microbial populations during the different experiments.

Fig.3 Determination of $^{18}$O/$^{16}$O fractionation coefficients in the 4 respiration sequences. $^{18}\alpha_{soil\_respi\_1}$ (brown), $^{18}\alpha_{soil\_respi\_2}$ (green), $^{18}\alpha_{soil\_respi\_3}$ (blue), $^{18}\alpha_{soil\_respi\_4}$ (purple) are respectively respiratory fractionation coefficients associated with sequences 1 to 4.

Using the results of the 4 sequences, we determined the values for the mean isotopic discrimination $^{18}\epsilon_{soil\_respi}$ ($\sim$ 12.3 ± 1.7 ‰), the mean isotopic discrimination $^{17}\epsilon_{soil\_respi}$ ($\sim$ 6.4 ± 0.9 ‰) and the average $\gamma_{soil\_respi}$ (0.5164 ± 0.0005).

3.2. Photosynthesis and dark respiration

3.2.1. Experimental data
Fig. 4. Example of the evolution of the different concentrations and isotopic ratios in the sequence 1 of photosynthesis and dark respiration experiment in the closed chamber over 30 days. Grey rectangles correspond to night periods and white rectangles to light periods. (a) $\delta^{18}O$ of $O_2$ (red) variations. (b) $\Delta^{17}O$ of $O_2$ variations (blue) and regulation of carbon dioxide flux (purple). (c) Dioxygen concentration (purple) and $\delta$O$_2$/Ar variations (green).

During the night periods, when only respiration occurred, we observed a decrease in $O_2$ concentration by 1% within 3 days and a $\delta^{18}$O increase by 1% during the same period (Fig. 4). The evolution was qualitatively similar with that of soil respiration experiments with higher fluxes. We observed the same trends for the evolution of $\delta$O$_2$/Ar during the night periods as for the respiration experiment. During light periods, there was a marked decrease in $\delta^{18}$O (2%) and a marked increase in the flow of oxygen released (1%) during 1 day. This result was consistent with previous studies of Guy et al. (1993) and Eisenstadt et al. (2010) showing that photosynthesis produces oxygen with the $\delta^{18}$O value close to the $\delta^{18}$O of the leaf water, leaf water $\delta^{18}$O being lower than atmospheric $\delta^{18}$O of $O_2$. We observed the same trends for the evolution of $\delta$O$_2$/Ar during the night periods as for the respiration experiment.

The Mann-Kendall test (95%) showed a significative increasing trend of the $\Delta^{17}$O of $O_2$ over sequences 1 and 2 (Fig. S3) ($\simeq$ 100 ppm in 30 days for sequence 1, $\simeq$ 100 ppm in 40 days for sequence 2) while no significant increase of $\Delta^{17}$O of $O_2$ is observed over sequence 3 (Fig. S3).

3.2.2. Fractionation coefficients

Dark respiration
The average of the isotopic discrimination for dark respiration $\varepsilon_{\text{dark respi}}$ and $\varepsilon_{\text{dark respi}}$ were calculated over the 9 night periods and we obtained values of respectively - 17.0 ± 2.0 %o and - 8.5 ± 0.8 %. The average of $\gamma_{\text{dark respi}}$ during the experiment was equal to 0.5124 ± 0.0084 (Table S3).

The dark respiration of this experiment includes respiration of both soil and leave. Because soil respiration fractionation coefficient has been determined above, it is possible to estimate here the fractionation coefficient for the dark leave respiration:

$$F_{\text{dark respi}} = F_{\text{soil respi}} + F_{\text{dark leave respi}}$$

(25)

$$18\alpha_{\text{dark respi}} = f_{\text{soil respi}} \times 18\alpha_{\text{soil respi}} + f_{\text{dark leave respi}} \times 18\alpha_{\text{dark leave respi}}$$

(26)

with $F_{\text{dark leave respi}}$ the flux of leave respiration during the night, $f_{\text{soil respi}}$ the fraction of soil respiration during night periods ($F_{\text{soil respi}} / F_{\text{dark respi}}$) and $f_{\text{dark leave respi}}$ the fraction of dark leave respiration during night periods ($F_{\text{dark leave respi}} / F_{\text{dark respi}}$).

$$18\alpha_{\text{dark leave respi}} = \frac{18\alpha_{\text{dark respi}} - f_{\text{soil respi}} \times 18\alpha_{\text{soil respi}}}{f_{\text{dark leave respi}}}$$

(27)

The isotopic discriminations $18\varepsilon_{\text{dark leave respi}}$ and $17\varepsilon_{\text{dark leave respi}}$ were respectively equals to - 19.1 ± 2.4 %o and - 9.7 ± 0.9 %o. The average of the gamma value was equal to 0.5089 ± 0.0777. The standard deviations ($\sigma$) was calculated by a Monte Carlo method from the individual uncertainties of the $18\alpha_{\text{dark respi}}$, $18\alpha_{\text{soil respi}}$, $F_{\text{soil respi}}$ and $F_{\text{dark respi}}$.

**Photosynthesis**

In order to calculate an average value for fractionation coefficient associated with photosynthesis from Eq. (18), we first calculated the averages of the flux of the $O_2$ consuming processes and of the fractionation coefficients associated with each sequence: $(F_{\text{total respi}})$ and $(18\alpha_{\text{total respi}})$. We also calculated the net $O_2$ flux during light periods, $aN = F_{\text{photosynthesis}} - F_{\text{total respi}}$, as the linear regression, $aN$, of $\frac{n(O_2)}{n(O_2)_{lo}}$ with time. $aR_{18}$ is also obtained as a linear regression of $R_{18}O$ with time over each light period. Our data support our assumption that the regime was stationary over time, i.e.
that $R^{18}O$ and $n(O_2) / n(O_2)_{t0}$ evolved linearly over time, which is why we were able to do linear regressions.

\[
18\alpha_{\text{photosynthesis}} = \frac{-aR^{18} + aN + (18\alpha_{\text{total resp}}) \times (F_{\text{total resp}})}{R^{18}O_{\text{air}} \times F_{\text{photosynthesis}}} \tag{28}
\]

We finally estimated the values of $18\epsilon_{\text{photosynthesis}}$ and $17\epsilon_{\text{photosynthesis}}$ as $3.7 \pm 1.3 \%$ and $1.9 \pm 0.6 \%$, respectively. The average of the gamma value was equal to $0.5207 \pm 0.0537$, a value which depends on the value taken for the $\delta^{16}O$ value of atmospheric air vs VSMOW (Sharp and Wostbrock, 2021), see Table 2. Sensitivity tests (Tables S4, S5 and S6) on values of the $O_2$ flux and associated fractionation coefficients for photosynthesis and Mehler reaction resulted in uncertainty estimates of $0.0012$ and $0.0007$ for the values of $18\alpha_{\text{photosynthesis}}$ and $17\alpha_{\text{photosynthesis}}$ (Table S6).

The value of isotopic fractionation associated with the light period of period 1 of sequence 1 appeared clearly out of range. Following the Dixon’s outlier detection test (Dixon, 1960), this value was considered an anomaly (likelihood > 99 %) and was removed from further analysis (Table S7). The individual determination is presented on Table S7.

4. Discussion

4.1. $\Delta^{17}O$ of $O_2$

The $\Delta^{17}O$ of $O_2$ is equal to 0 by definition for atmospheric air, and hence it should be equal to zero at the beginning of each experiment. The observed change during an experiment can only be driven by biological processes since the interaction with stratosphere is not possible in the closed chambers.

During the soil respiration experimental run, the $\Delta^{17}O$ of $O_2$ was constant. This directly reflects the $\gamma_{\text{soil,resp}}$ value of $0.5164 \pm 0.0005$ found for respiration (Table 2) since $\Delta^{17}O$ of $O_2$ is defined with a slope of 0.516 between $\ln(1 + \delta^{17}O)$ and $\ln(1 + \delta^{18}O)$ (Eq. 1). This result is in good agreement and within the uncertainties given by Helman et al. (2005) with the $\gamma$ value of $0.5174 \pm 0.0003$ obtained with respiration experiments on several micro-organisms.

During the experiment involving both oxygen uptake and photosynthesis, the $\Delta^{17}O$ of $O_2$ has a globally increasing trend with values reaching about 100 ppm after one month. Such behavior is expected and was already observed by Luz et al. (1999) with $\Delta^{17}O$ of $O_2$ values reaching 150 ppm after a 200-day experiment within a closed terrarium. This increase cannot be explained by respiration since respiration does not modify $\Delta^{17}O$ of $O_2$. It is hence mainly due to photosynthesis producing oxygen
with a $\Delta^{17}O$ of O$_2$ different from the atmospheric one. Previous analyses have shown that the $\Delta^{17}O$ of H$_2$O of VSMOW (close to mean oceanic water) expressed vs isotopic composition of atmospheric O$_2$ has a value between 134 to 223 ppm (using a definition of $\Delta^{17}O$ of H$_2$O = $\ln(1+\delta^{17}O)$ - 0.516 × $\ln(1+\delta^{18}O)$) (Sharp and Wostbrock, 2021). Within the water cycle, the slopes of $\ln(1+\delta^{13}O)$ vs $\ln(1+\delta^{18}O)$ for the meteoric line, evaporation and evapotranspiration lines are larger than 0.516 (Li and Meijer, 1998; Landais et al., 2006) so that $\Delta^{13}O$ of water consumed by the plants during photosynthesis should be slightly lower than the $\Delta^{17}O$ of VSMOW expressed vs isotopic composition of atmospheric O$_2$ but still higher than the $\Delta^{17}O$ of atmospheric O$_2$. The photosynthesis is thus responsible for the $\Delta^{17}O$ of O$_2$ increase in the closed chamber.

4.2. Fractionation factors associated with $\delta^{18}O$ of O$_2$ and implications for the Dole effect

Table 2. Summary of the mean values of the isotopic discriminations and gamma values of all sequences of (1) the soil respiration experiment and of (2) the respiration and photosynthesis experiment and the number of data on which they were calculated. ** is the value for $\gamma_{\text{photosynthesis}}$ that depends on the determination of the $\delta^{17}O$ of atmospheric O$_2$ vs $\delta^{17}O$ of VSMOW. We provide here the two different possible estimates using either 12.03 %o (Luz and Barkan, 2011) or 12.08 %o (Barkan and Luz, 2005): value determined with $\delta^{17}O$ = 12.03 %o / value determined with $\delta^{17}O$ = 12.08 %o.

|                               | Average (%) | Standard deviation (%) | Number of data |
|-------------------------------|-------------|------------------------|----------------|
| $\epsilon_{\text{soil_respi}}$ | -12.3       | 1.7                    | 4              |
| $\epsilon_{\text{soil_respi}}$ | -6.4        | 0.9                    | 4              |
| $\gamma_{\text{soil_respi}}$  | 0.5164      | 0.0005                 | 4              |
| $\epsilon_{\text{dark_respi}}$| -17.0       | 2.0                    | 9              |
| $\epsilon_{\text{dark_respi}}$| -8.5        | 0.8                    | 9              |
| $\gamma_{\text{dark_respi}}$  | 0.5124      | 0.0084                 | 9              |
| $\epsilon_{\text{dark_respi}}$| -19.1       | 2.4                    | 9              |
| $\epsilon_{\text{dark_respi}}$| -9.7        | 0.9                    | 9              |
| $\gamma_{\text{dark_respi}}$  | 0.5089      | 0.0777                 | 9              |
| $\epsilon_{\text{photosynthesis}}$ | 3.7        | 1.3                    | 8              |
| $\epsilon_{\text{photosynthesis}}$ | 1.9        | 0.6                    | 8              |
| $\gamma_{\text{photosynthesis}}$ | 0.5207/0.5051** | 0.0537/0.0504**       | 8              |
The isotopic discrimination $\varepsilon_{\text{soil, respi}} = -12.3 \pm 1.7\%o$ for the soil respiration experiments is comparable to the average terrestrial soil respiration fractionation found by Angert et al. (2001) of -12\%. Still, among the diversity of soils studied by Angert et al. (2001), the soils showing the $^{18}\varepsilon$ values closest to our values are clay soil ($^{18}\varepsilon = -13\%o$) and sandy soil ($^{18}\varepsilon = -11\%o$). These soils are different from our soil which was enriched in organic matter. Further experiments are then needed to understand the variability in $^{18}\varepsilon$ associated with soil respiration.

The isotopic discrimination for dark respiration in leaf, $\varepsilon_{\text{dark, leave, respi}} = -19.1 \pm 2.4\%o$ is associated with a large uncertainty and would benefit from additional experiments with a higher sampling and measurement rate. Still, even if it was obtained on different organism and experimental set-up, this value is in agreement with the values for isotopic discrimination for dark respiration determined by Helman et al. (2005) on bacteria from the Lake Kinneret ($^{18}\varepsilon = -17.1\%o$) and Synechocystis ($^{18}\varepsilon = -19.4\%o$ and -19.5\%).

The average $^{18}\varepsilon_{\text{photosynthesis}}$ is $+3.7 \pm 1.3\%o$ which goes against the classical assumption that terrestrial photosynthesis does not fractionate (Guy et al., 1993). This value for the isotopic discrimination is smaller than the photosynthetic fractionation in marine environment $^{18}\varepsilon = +6\%o$ found by Eisenstadt et al. (2010). Still, this result suggests that the terrestrial Dole effect may be higher than currently assumed and challenge the assumption that terrestrial and oceanic Dole effects have the same values (Luz and Barkan, 2011).

### 4-Conclusion

Using a simplified analog of the terrestrial biosphere in a closed chamber we found that the fractionation factors of soil respiration and dark respiration of leaf at the chamber level agree with the previous estimates derived from studies at micro-organism level. This is an important confirmatory step for the fractionation factors previously used to estimate the global Dole effect. More importantly, we document for the first time a significant $^{18}$O fractionation during terrestrial photosynthesis ($+3.7\%o \pm 1.3\%o$). If confirmed by future studies, this can have a substantial impact on the calculation of the Dole effect, with important consequences for our estimates of the past global primary production.

Our study showed the usefulness of closed chamber to quantify the fractionation factors associated with biological processes in the oxygen cycle at the plant level. The main limitation of our present study was the low sampling rate during our experiments which hamper the precision of the determined fractionation factors. Future work should use this validated set-up to multiply such experiments to
improve the precision of fractionation factors and to explore the variability of fractionation factors for
different plants and hence different metabolisms. A good application would be to study the difference
between C3 and C4 plants because C4 plants do not photorespiration.

Data availability

All individual fractionation coefficients for each experiment are given in the Supplement.

Author contributions

AL and CPI designed the project. CPI, JS and SD carried out experiments at ECOTRON of Montpellier
and FP, CPa, RJ, AD and OJ at LSCE. CPa, NP and AL analyzed the data. CPa and AL prepared the
manuscript with contributions from NP, CPI, JS and AM.

Competing interests

The authors declare that they have no conflict of interest.

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