ON-LINE STABLE ISOTOPE MEASUREMENTS DURING PLANT AND SOIL GAS EXCHANGE

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Abstract. Recent techniques for on-line stable isotope measurements during plant and soil exchange of CO_2 and/or water vapor are briefly reviewed. For CO_2, these techniques provide means for on-line measurements of isotopic discrimination during CO_2 exchange by leaves in the laboratory and in the field, of isotopic discrimination during soil respiration and during soil-atmosphere CO_2 exchange, and of isotopic discrimination in O_2 during plant respiration. For water vapor, these techniques provide means to measure oxygen isotopic composition of water vapor during leaf transpiration and for the analysis of sub microliter condensed water vapor samples. Most of these techniques involve on-line sampling of CO_2 and water vapor from a dynamic, intact soil or plant system. In the laboratory, these systems also allow on-line isotopic analysis by continuous-flow isotope ratio mass spectrometry. The information obtained with these on-line techniques is becoming increasingly valuable, and often critical, for ecophysiologial research and in the study of biosphere-atmosphere interactions.

1. INTRODUCTION

The use of stable isotopes in environmental sciences is rapidly increasing. This is because stable isotopes provide powerful tracers of sources and sinks of elements in the biosphere-atmosphere system, as well as useful indicators of processes underlying biospheric response to change. In both cases the isotopic labeling by natural processes of gases such as CO_2, O_2 and H_2O exchanged between plants, soil and the atmosphere is often involved. The process-based mechanisms of such isotopic labeling must be studied in detail for quantitative use of the isotope approach at any scale, from the organism through ecosystem to the global scales. This, in turn, has motivated a great deal of research and development in the stable isotope methodology. One of the major changes in stable isotope analyses in recent years has been the advancement of a suite of on-line methods.

Notably, the term on-line technique has been adopted for two distinct operations. The first refers to on-line isotopic analysis. In this case, the traditional dual inlet of the mass spectrometer, using a sample and reference reservoirs, is replaced with a single inlet that introduces a continuous flow of a carrier gas (usually He). A pulse of sample or of reference gas is injected into the carrier stream. The sample line is first purified through a combination of traps and GC column to separate the gas of interest (e.g. CO_2). The reference gas is usually introduced after the purification step and the gas stream is then introduced to the source of the isotope ratio mass spectrometer (IRMS). The isotopic analysis is carried out by integration of the peak areas of the relevant mass beams (as opposed to beam heights in the dual inlet mode). The main advantages of this operation is in greatly increasing sample throughput and dramatically reducing the required sample size.

The second on-line operation refers to an on-line sampling of the gas of interest for isotopic analysis. Such experimental systems are often mentioned in the ecophysiological literature and are distinct from the on-line analysis in that they are not necessarily connected on-line to the IRMS. The main idea here is that sampling on-line allows measuring the isotopic discrimination of interest in a non-destructive manner in active biological systems. For example, leaf discrimination against $C^{18}O^{16}O$ or $^{13}CO_2$ can be measured repeatedly and in response to changes in environmental conditions by sampling the air passing above an active leaf [1]. From the change in the isotopic composition of the air entering and exiting a chamber in which a leaf (or a patch of soil, etc.) is enclosed, it is possible to accurately infer the isotopie effect associated with the leaf acitivity. The two aspects of the on-line methodology, on-line sampling and on-line analysis are equally important in the context discussed here. While on-line analysis of material collected in a destructive way (say, dried plant material), will make the analysis more efficient, but on-line sampling of the gas exchanged by active leaves in a real

time fashion will provide information that cannot be obtained otherwise. A combined operation is often required since on-line sampling produces small gas samples. Sampling and analysis of atmospheric gas sample such as CO_2 and O_2 in on-line technique takes a 2–5 minutes and requires small air samples of a few μL (considering differences in ambient concentrations, such as between CO_2 and O_2). Analysis of water vapor samples requires equilibration with CO_2 over several hours. But equilibrating water samples with ambient CO_2 in the air samples greatly simplifies the analysis. Alternatively a small water sample can be pyrolize to CO and analyze on-line.

On-line methods such as described here allow us to make investigations of almost undisturbed, in-vivo, systems and to observe short term, dynamic responses to changes in environmental conditions. This is in sharp contrast to original geochemical methods in which samples were stored in containers and analyzed separately from the experimental system and after long delays, in a specialized stable isotope laboratories.

2. ISOTOPIC DISCRIMINATION DURING CO_2 EXCHANGE OF LEAVES: LABORATORY MEASUREMENTS

2.1. Introduction

The ^{18}O of atmospheric CO_2 is influenced by CO_2 exchange with the land surface. In leaves and soil, CO_2 readily dissolve in water via the bicarbonate system and oxygen isotopic exchange occurs. Since in natural system there are always trace amounts of CO_2 and large quantities of water, the water impose its isotopic composition on that of CO_2. Because the isotopic composition of water in leaves and soil is unique, measurements of the ^{18}O in atmospheric CO_2 can provide information on specific fluxes of CO_2 exchanged with the land surface. The quantitative aspects of the ^{18}O exchange between leaf water and CO_2 diffusing in and out of leaves has been studied in laboratory experiments at the single leaf level. In this case, a leaf attached to the plant is sealed within a cuvette with an air stream flowing through it. By sampling and analyzing both the air going into and exiting the cuvette, the interactions of the leaf with the atmosphere inside the cuvette can be investigated.

2.2. Gas exchange system

The gas exchange system is described in Fig. 1 [2]. Synthetic air was mixed from N_2, CO_2 and O_2 cylinders using mass flow controllers, MFC (MKS Instruments, USA), and humidified via bubbling a variable portion of the air stream through water at room temperature ($\delta^{18}O = -4.5‰$, hence vapor ~ −14.5 ‰), acidified with two drops of 80% H_3PO_4. The airflow was split into reference and analysis air streams, the latter flow, range 800–1500 ml min^{-1}, passing to a Parkinson 'conifer pod' leaf cuvette (PLC3C, ADC Scientific, UK) and measured via another MFC. Illumination was from a 250 W projector lamp (GE, USA), passing through 3 cm depth of water to reduce infra-red radiation. Incident radiation on the leaf was controlled by shading with a predetermined number of 'miracloth' filters. Absolute CO_2 and H_2O concentration in reference and analysis streams were monitored alternately via an infra-red gas analyzer, Li-6262 (LI-Cor, USA).

2.3. Isotopic measurement of CO_2

The outflow of the leaf chamber, min 700 cm^3 min^{-1}, after passing through the IRGA, was split and 100 cm^3 min^{-1} was pumped first through a Nafion® dryer, and then a sample loop (0.85 ml) fitted onto a six port, two position valve (Valco Instruments Co, USA — see Fig. 1). CO_2 was trapped at liquid nitrogen temperatures for 30 s. After warming to room temperature, the sample was swept with helium carrier gas (120 mL min^{-1}; Gordon Gas and Chemicals, Israel) through a magnesium perchlorate drying trap and a 2 m. packed column of molecular sieve 5A, 80/100 mesh (Alltech, USA). The large peaks of N_2 and O_2 which eluted first from the column were diluted via a gas diluter (Micromass, UK), followed by the non-diluted sample CO_2. The gas was introduced into the source of

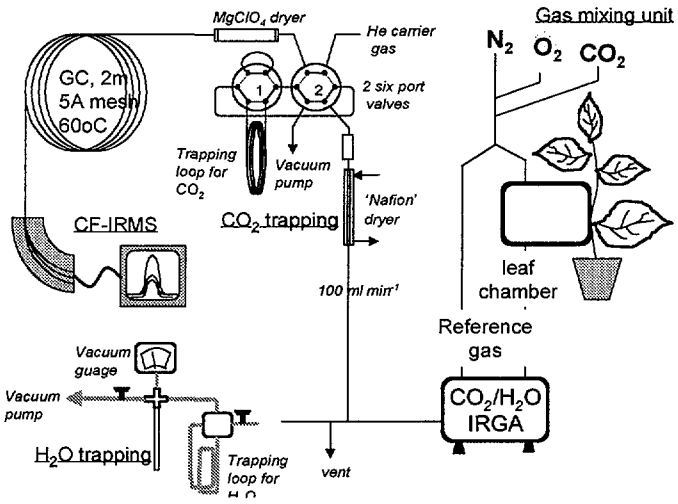

Fig. 1. On-line sampling and analysis system for gases exchanged between leaves and atmosphere.

an OPTIMA mass spectrometer (Micromass, UK) via an open split. $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios were measured from the integrated peak areas of masses 44, 45 and 46, normalized against a 30 second CO_2 reference pulse injected prior to each sample. Sample size was standardized by adjusting the cryogenic trapping time according to the CO_2 concentration in the outflow from the leaf chamber. N_2O was assumed to be constant in air (310 ppb) and absent from 'synthetic' air, thus δ values corrected accordingly [3] and expressed in the small delta notation vs. VPDB (for ^{13}C) and VSMOW (for ^{18}O), derived from calibration of the reference gas with CO_2 of known isotopic composition. Precision for repeated sampling of CO_2 was 0.06‰ ($\delta^{13}C$) and 0.07‰ ($\delta^{18}O$).

2.4. Isotopic measurement of water vapor: Equilibrium with ambient CO_2

The remaining airflow from the leaf chamber was passed at positive pressure to a 1/4 inch i.d. stainless steel vacuum line (pressure $< 1 \times 10^{-3}$ torr), where CO_2 and water vapor were trapped from the air stream (3 min. at 500 mL min^{-1}) in a coil cooled with liquid nitrogen. After trapping, the line was evacuated and the trap was heated with a flame, distilling both CO_2 and H_2O into a Pyrex side-arm immersed in liquid N_2. After quantitative transfer the Pyrex tube was flame sealed. The sample was left for CO_2-H_2O equilibrium at constant temperature (29°C, Labline Instruments Inc, USA) for 72 hrs. The CO_2 was then dried in a vacuum line with an ethanol trap at −70°C, before isotopic analysis on a MAT 250 dual inlet mass spectrometer (Finnigan-MAT, Germany). $\delta^{18}O$ of water vapor was calculated from that of the CO_2, according to Scrimgeour et al. [4], correcting for the amount CO_2 and H_2O (calculated from the concentration, flow rate and time of trapping) and $\delta^{18}O$ of the pre-equilibration CO_2, taken from the corresponding measurement on the continuous flow system. Precision of $\delta^{13}C$ CO_2 and $\delta^{18}O$ water vapor was 0.04‰ and 0.11‰.

2.5. Experimental procedure

Light responses (in which the rate of photosynthesis is gradually modified by changing the light photosynthetic photon flux density PPFD) were conducted from high to low PPFD, in 21% O_2. Collections of CO_2 for isotopic analyses were carried out for 3 minutes, while water vapor was trapped continuously (i.e. two samples of CO_2 and one of water were analyzed per light level). Photosynthesis measurements were averaged for the collection period. At the end of the experiment, the portion of leaf inside the cuvette was excised and placed in a 15ml vacutainer (Becton Dickinson, USA), for extraction of leaf water. The complete light response analysis (ca. 10 determinations) was

done with CO_2 relatively depleted in ^{13}C and ^{18}O ($\delta^{13}C = -30$ ‰ and $\delta^{18}O = +10$‰) to maximize the precision of measurement, or ambient air pumped through a 50 L external buffering volume ($\delta^{13}C = -8$‰ and $\delta^{18}O = +41$‰).

2.6. Isotopic calculations

Isotope ratios were expressed in the delta notation, $\delta‰ = 1000 \cdot (R_a/R_s - 1)$, where R_a and R_s are the rare/common stable isotope ratios for the sample and standard respectively (standards are VPDB, $^{13}C/^{12}C = 0.01118$, and VSMOW, $^{18}O/^{16}O = 0.0020052$ respectively). Instantaneous discrimination, Δ, for ^{13}C and ^{18}O was determined from [1],

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1000 + \delta_o - \xi(\delta_o - \delta_e)}.1000 \tag{1}$$

where $\xi = c_e/(c_e - c_o)$, c_e, c_o and δ_e, δ_o referring to the CO_2 concentration (corrected to the same humidity) and isotopic composition of air entering and leaving the cuvette, respectively. Additionally, $\Delta^{13}C$ was described simply in terms of the enzymatic and diffusive fractionation model [5] as $\Delta_i = a + (b' - a)c_i/c_a$, where c_i and c_a refer to CO_2 concentration in the sub-stomatal cavity and atmosphere respectively, a is the fractionation during diffusion in air (4.4‰) and b' is the fractionation during carboxylations (29‰). The additional reduction in CO_2 concentration from c_i to the chloroplast, c_c, was estimated from the difference between the simple model and the measured discrimination, $\Delta_i - \Delta_{obs}$, [1] as

$$\Delta_i - \Delta_{obs} = \frac{(b' - a_i)}{g_i}.\frac{A}{p_a} \tag{2}$$

where Δ_{obs} is the discrimination measured in equation 1, g_i refers to the total internal conductance, a_i is the combined fractionation (+1.8‰) during dissolution (+1.1‰) and diffusion through the liquid phase (+0.7‰). Internal conductance was derived from the gradient of the $\Delta_I - \Delta_{obs}$ response vs. A/c_a, which avoids introducing error due to the uncertainty of b' and photorespiratory fractionation [6], [7], [8].

Discrimination against ^{18}O in CO_2 , $\Delta^{18}O$is estimated in a similar way [9]:

$$\Delta^{18}O = \frac{\bar{a} + \varepsilon\Delta_{ea}}{1 - \varepsilon\Delta_{ea}/1000} \tag{3}$$

where $\Delta_{ea} = 1000 \cdot [(\delta_e/1000 + 1)/(\delta_a/1000 + 1) - 1]$, $\varepsilon = c_c/(c_a - c_c)$; δ_a, δ_e represent the $\delta^{18}O$ of CO_2 in the overlying air and in full isotopic equilibrium with water in the chloroplast, and c_a, c_c the respective CO_2 concentrations (see Fig. 1); is the weighted-mean diffusional fractionation through boundary layer, 5.8‰, stomata, 8.8‰, and aqueous leaf media, 0.8‰. The oxygen isotopic composition of CO_2 in equilibrium with chloroplast water, δ_c, was determined by solving equation 3* for δ_c, so that

$$\delta_c = 1000.\left(\frac{c_a - c_c}{c_c}\left(\frac{\Delta^{18}O}{\Delta^{18}O/1000 + 1} - \bar{a}\right) + 1\right).\delta_a \tag{4}$$

Thus, δ_c was estimated from equation 3 using direct measurements of discrimination and the appropriate estimates of CO_2 concentrations.

The $\delta^{18}O$ of transpired water vapour, δ_t, was calculated as

$$\delta_t = \xi_L(\delta_{out} - \delta_{in}) + \delta_{in} \quad (5)$$

where $\xi_L = e_o/(e_o-e_e)$, e_e, e_o and δ_e, δ_o referring to the H_2O vapor pressure and isotopic composition of air entering and leaving the cuvette, respectively [10]. Following their example, δ_t was then substituted into the Craig and Gordon model [11] of surface water enrichment (as modifed by Flanagan et al. [12]) to predict the $\delta^{18}O$ at the sites of evaporation, δ_e. This method has two benefits over using the source water value based on the assumption of isotopic steady state, because a) water arriving at the leaf may differ from source water applied to the roots (due to evaporation from the soil or from unsuberised stems) and b) it does not restrict estimation of δ_e to isotopic steady state (ISS) conditions.

3. ISOTOPIC DISCRIMINATION DURING CO_2 EXCHANGE OF LEAVES: FIELD MEASUREMENTS

3.1. Introduction

Although laboratory conditions offer the best conditions to investigate leaf scale processes, these studies must eventually be extended to field conditions. Only under these conditions different plant species can be investigated in their natural habitat and under natural conditions. At present, IRMS is not a transportable, field instrument and samples must be collected in the field and transported to the lab. However, on-line field sampling systems have been used in the field in several studies such as [9], [13]. In these cases, the gas exchange and gas sampling systems is similar to that described above but simplified and made portable. The gas samples are purified in the field and sample of, say, CO_2 ready for analysis are transported to the lab. The term "on-line" applies in this case to the experimental and sampling system only and not to the analytical system.

3.2. Gas-exchange measurements and plant sampling

Rates of photosynthesis and transpiration, stomatal conductance, as well as micro-meteorological conditions (relative humidity, photon flux density and air and leaf temperatures), were measured using a portable leaf gas exchange system (ADC-3 with PLC-3 leaf cuvette, ADC, Hoddesdon, Herts, England) and calculated according to [14]. Boundary layer conductance in the leaf cuvette was estimated to be about 2 mol m^{-2} s^{-1}. Leaf area was determined from traces made for each leaf in the field. Subsequent to each gas exchange measurement, a similar, nearby leaf and a woody (non-green) stem sample were sealed, separately, in test tubes (Vacutainers, Rutherford, NJ, USA). Notably, in several cases, sampling was repeated twice for the same plant to evaluate within-plant variation. These tests, and previous experience indicated that a careful sampling strategy keeps such variations to less than 1‰. Atmospheric moisture samples were simultaneously collected by flowing air (250 ml min^{-1}) through a cryogenic trap (–80°C) at a field site exposed to turbulent air away from nearby canopy.

The above mentioned leaf cuvette was used for measurements of leaf discrimination against $^{13}CO_2$ and $C^{18}O^{16}O$ by attaching it to a 1/4" stainless steel vacuum line (better than 1.0×10^{-3} torr) used for cryogenic drying (–80°C) and trapping of CO_2 at liquid nitrogen temperature (Fig. 2). CO_2 and water vapor in the air flow (ca. 250 ml min^{-1}) entering and exiting the cuvette was measured with an infra-red gas analyzer (LiCor-6262, Lincoln, NE, USA). CO_2 samples were trapped for 2 min, after which the vacuum line was evacuated and the CO_2 transferred and sealed in a glass ampoule. After each measurement, the leaf was cut and sealed in a test tube. Ambient environmental conditions at the time of the measurements were also recorded to allow estimation of leaf water $\delta^{18}O$ values.

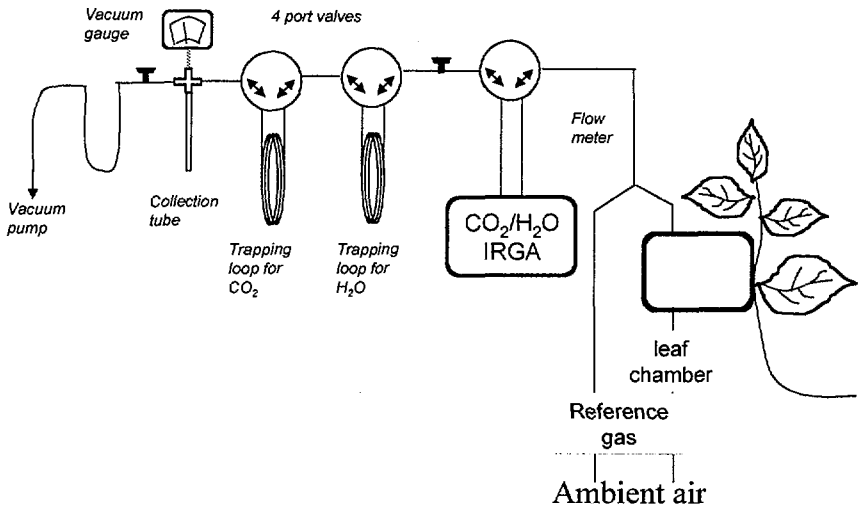

Fig. 2. On-line sampling system for the field sampling of gases exchangeed between leaves and atmosphere. An attached leaf is enclosed in a leaf cuvette and an air stream is samples before and after passing above the leaf. Water vapor and CO_2 concentrations are measured by an infra-red-gas analyzer, water is traped at –8°C and CO_2 at –170°C. Samples are cryogenically transferred to a collection tube for the mass spectrometric analysis.

3.3. Isotopic analysis

Leaf and stem water was extracted by vacuum distillation at 60°C in the lab. $\delta^{18}O$ values of the water samples were determined by equilibration with CO_2 at 25°C over night followed by cryogenic separation of CO_2 for the mass spectrometric analysis (Finnigan MAT-250). Precision for the water analysis was ±0.1‰. Samples of CO_2 from the on-line discrimination measurements were directly analyzed on the mass spectrometer with an external precision of ±0.05‰ for $\delta^{13}C$ and ±0.1‰ for $\delta^{18}O$. Estimating $\delta^{18}O$ values of chloroplast water and other calculations are similar to those described in the previous section.

4. ISOTOPIC DISCRIMINATION DURING SOIL CO_2 EXCHANGE

4.1. Introduction

Soil CO_2 exchange carries a unique isotopic signature and influence the isotopic composition of atmospheric CO_2 at the local, regional and global scales. Using ^{13}C, soil respiration can trace the source of the CO_2 and help distinguish between root respiration and organic matter decomposition, as well as in estimating raters of its turnover in the soil. Using ^{18}O in CO_2, soil respired CO_2 provides another useful link between the land biosphere and the atmospheric ^{18}O budget. In this case, the principal process in the soil is similar to that in leaves, namely the isotopic exchange of oxygen between water and CO_2. However, other factors such as the rate of exchange, competition between diffusion and exchange, atmospheric invasion into soils and spatial and temporal variations in the $\delta^{18}O$ of soil water require addressing the soil system as an independent experimental system. Here again, both on-line sampling and on-line analysis were carried out in the lab., but on-line sampling methods were also adapted to field work. Both systems are briefly described below.

4.2. Direct soil CO_2 measurements

A newly designed system using a gas-chromatograph-isotope-ratio-mass-spectrometer (GC-IRMS) was built for sampling small quantities of soil air at 1 cm vertical resolution from near the soil surface

for direct, on-line, isotopic analysis [15], (Fig. 3). 200 µL aliquots of soil air were sucked by vacuum from different soil depths within the collar described above through a fused silica capillary (0.32 mm i.d., 0.45 mm o.d.) and a stainless steel sample loop fitted to a two position, six-port Valco valve (Fig. 4). After switching the valve position, the air sample trapped within the sample loop was carried with a He carrier gas through a Nafion membrane drying trap (0.4 mm I.D. x 15 cm), and onto a capillary GC column (0.32 mm × 25 m, J&W GS-Q at 35°C). The column effectively separated CO_2 from air and N_2O without any cryo-focusing. The GC eluant passed through an open-split and changeover valve into the mass-spectrometer (Micromass Optima). The changeover valve allowed the air peaks to be pumped to waste and the remainder of the sample to be directed to the ion source of the mass spectrometer. The CO_2 peak eluted within two minutes of the six-port valve switch with a peak width of 5 s (FWHM), and peak height between 1.5 and 15 nA, depending on sample loop size and sampling depth. Peaks for the different isotopic masses (44, 45, 46) were integrated and their ratios normalized to a CO_2 standard injected one minute after each sample from the bellows of the dual inlet of the mass spectrometer.

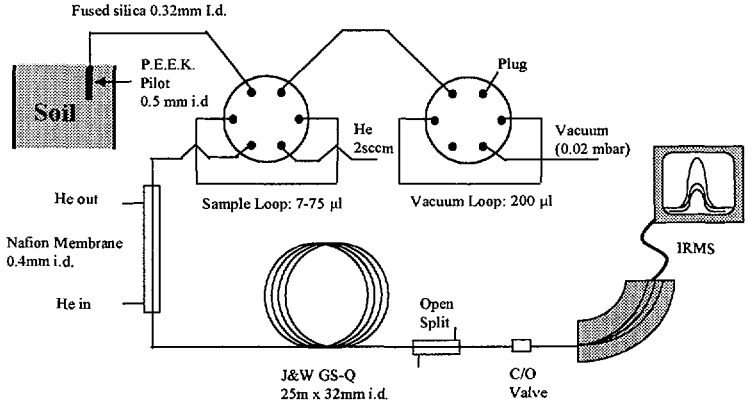

Fig. 3. On-line sampling and analysis system for soil CO_2.

Insertion of the sampling capillary into the soil must insure that the capillary did not become clogged with soil and that room air did not mix with soil air. A plastic pilot tube (PEEK, 1/16" o.d. × 0.5 mm i.d.) was first inserted to the appropriate depth and immediately plugged with a dummy sampling capillary. The dummy capillary was also used to clear any soil present in the pilot tube. The snug fit of the fused silica inside the PEEK tubing prevented room air from mixing down to the sampling depth. Small soil fractures around the outside of the PEEK pilot, resulting from its insertion, were immediately pressed down by hand. After the fused silica sampling capillary had been flushed with dry He, the dummy capillary was removed and quickly replaced by the sampling capillary. Soil air profiles were always measured from the top down to avoid mixing of deeper soil air into the sampling region as the capillaries were withdrawn. The 200 µL air samples sucked out of the soil corresponded to a volume of soil with a radius of 4 mm, assuming a soil air fraction, θ_a, of 0.25 and isotropic removal of soil air. 200 µl of air was sufficient to flush our largest sample loop (75 µl) and sampling capillary more than twice. Suction of 200 µL air was achieved by connecting the vent of the Valco valve to a second valve containing a 200 µL vacuum loop that could be, according to the valve position, connected to the vacuum system of the mass spectrometer (Fig. 3). After evacuation, the 200 µL loop was switched on-line to the sampling system described above, filling up with soil air while flushing and filling the sample loop on the first valve.

Precision of the ^{18}O analysis of repeated sampling of tank air (360 ppm) was better than 0.1‰. However, soil CO_2 mole fractions often varied from atmospheric levels near the surface to more than 10,000 ppm 8 cm below the surface. It was therefore critical to establish any correlation between the isotopic ratio and the sample size. This "linearity" was checked by measuring isotopic ratios of standard tank air with sample loops ranging in volume from 7 to 75 μL. Any possible correlation over this range was smaller than the measured precision. Our approach of using increasingly smaller sample loops with increasing depth allowed us to measure $\delta^{18}O$ in soil CO_2 with mole fractions varying by a factor of more than twenty with no apparent non-linearity. Although this approach is somewhat limited in its dynamic range, experiments with on-line dilution methods exhibited poor reproducibility.

Drying sample air prior to analysis is a prerequisite for obtaining accurate results [16, 17], especially since soil moisture can exceed 30%. Water vapor extracted from the soil may re-condense in the sampling or measurement apparatus and exchange oxygen atoms with the sampled CO_2, modifying the original $\delta^{18}O$ value of the sampled CO_2. The installation of the Nafion drying tube immediately downstream of the sample loop ensured measurement of dry air while retaining excellent peak shapes. The small sample size insured that disturbance of soil air as a result of sampling was negligible, and the five minute analysis time minimized potential changes in environmental parameters during the course of an experiment.

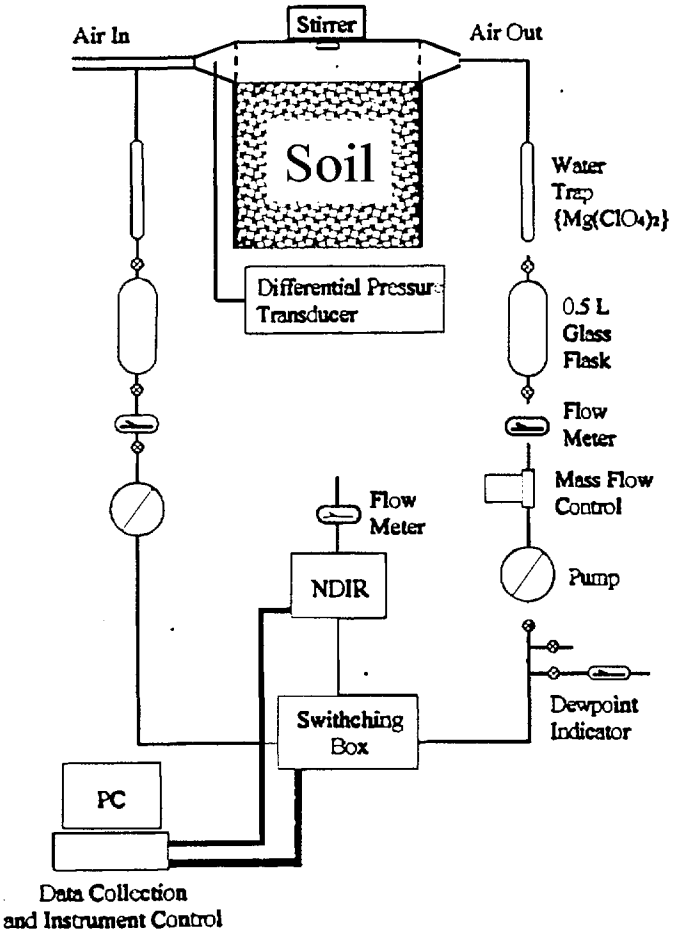

Fig. 4. On-line sampling system for the isotopic analysis of soil respired CO_2.

4.3. Dynamic chamber measurements

An open-bottomed flow-through chamber [15]; Fig. 4 based on the design of [18] was placed into a groove on top of the steel collar and sealed with putty (Apiezon Q). Both the collar and the chamber were leak-tested using an infra-red gas analyzer (IRGA, Li-Cor 6251) and pure CO_2. Before sealing the chamber, we placed a magnetic stir-bar on the ceiling of the chamber with a standard lab magnetic stirrer placed on top of the chamber. Using the stirrer at the minimum speed prevented the development of laminar flow inside the chamber. The chamber intake air was pumped (KNF diaphragm pump) from a 120 L plastic reservoir located inside the laboratory but was fed by a separate pump with outside air. Air exiting the chamber passed through a 8" × 0.5" I.D. $Mg(ClO_4)_2$ drying trap and a 0.5 l glass flask (J. Young stopcocks with Teflon o-rings.) The airflow was then split with a constant flow of 100 ml min^{-1} into an IRGA via a switching box. Flow was regulated with a mass flow controller (Edwards 825) with a range of 0–4 L min^{-1}. The intake air was sampled through a tee in the intake hose immediately prior to the chamber into a parallel drying and sampling system, except that flow was controlled by a needle valve and monitored by a ball flow meter. The switching box alternated the intake and outlet streams that passed on to the IRGA at a computer controlled frequency of 0.0083 Hz. Data from the IRGA was recorded only after the appropriate flushing time between cycles. During experiments, the system was allowed to reach steady state, indicated by a constant difference between the CO_2 mole fractions of the intake and outlet flows. After steady state was reached and mean CO_2 mole fractions were recorded, the stopcocks of the 0.5 L flasks were closed, and samples of the intake and outlet air were taken for isotopic analysis.

The pressure difference between the chamber and atmosphere was continuously monitored by a differential pressure transducer (Edwards 1018) fitted to the wall of the chamber. At all times, measured pressure differences were smaller than the 0.2 Pa needed to avoid significant advective contributions to CO_2 diffusion in and out of the soil [18]. Dewpoints of the dried air streams were measured to insure that the flask air samples were dry enough to eliminate the possibility of isotopic exchange between CO_2 and liquid water on the walls of the flasks or the tubing [17]. Dewpoints were kept below −15°C by changing the $Mg(ClO_4)_2$ drying traps as needed.

5. ISOTOPIC DISCRIMINATION IN O_2 DURING LEAF RESPIRATION

5.1. Introduction

The first measurements of ^{18}O discrimination in atmospheric O_2 were made in 1956 when Dole [19] reported several single point measurements of respiratory discrimination by a variety of organisms ranging from bacteria to human beings. Thirty years later stable isotope methodology was applied to the terminal oxidases of the mitochondrial electron chain and a substantial difference was found in the discrimination of the alternative and cytochrome oxidases [20]. This difference forms the basis of a new technique which can be used to estimate steady state partitioning of electron flow between the two mitochondrial pathways. This method has since been developed to enable rapid on-line measurements of the dynamics in oxygen isotope discrimination during respiration [21, 22].

At least two samples are necessary to calculate discrimination, a reference sample (with $^{18}O/^{16}O$ ratio R_O) and a subsequent sample with isotopic ratio R taken after a substantial proportion of the available O_2 has been consumed in respiration. For statistical purposes several such comparisons are made over a broad range of O_2 concentrations. D is calculated as the slope of a linear regression of ln $(R/R_O)\cdot 1000$ plotted against -ln f (where f is the fraction of oxygen remaining in the reaction vessel). Two types of "environments" have been used. First, liquid-phase environment in which marine organisms, cells in suspension or isolated mitochondria can be analyzed [23–25], and second, air-phase environment in which whole tissues of higher plants can be analyzed [22]. Note that in all cases it is necessary to follow both the changes in isotopic composition and in the partial pressure of oxygen in the system's atmosphere (head-space). The elemental analysis can be accomplished with a gas chromatograph, corrected for the co-elution of oxygen and argon. Alternatively, both the isotopic

composition and the elemental concentration can be determined from the mass spectrometric analysis. As shown here, the change in oxygen partial pressure over time can also be used to estimate the rate of respiration. Care must be taken, hoever, to account for the unique conditions in the chamber due to decrease in barometric pressure with sampling and increase in CO_2 partial pressure.

5.2. On-line ^{18}O measurements during leaf respiration

Respiring tissue is enclosed in a leak tight vessel from which samples can be withdrawn (Fig. 5). In the liquid phase system, samples of the solution is withdrawn and the air is separated by sparging [23] or gravimetrically [24] and dried. In the air-phase system a sample of the "head-space" is withdrawn and dried. In both cases isotopic analysis of the O_2 in the air is accomplished by direct measurement in a mass spectrometer [21, 22, 24] or by first converting the O_2 to CO_2 on hot graphite [23]. An accurate determination of the O_2 concentration is also required for the calculation of D. A complication arises because O_2 pressure in the closed system is reduced both by respiration and by the gas sampling. To account for this effect, the amount of oxygen in the samples is normalized to that of a conservative gas in the air, such as nitrogen (which is not consumed in respiration). Determinations of the two gases can be achieved by separating them in a GC [21, 22] or in the mass spectrometric analysis of the air mixture [24], Fig. 5. In both cases, CO_2 is removed from the air prior to analysis. Argon does not interfere with the mass spectrometric analysis, and can be corrected for in the GC determination. Recently, both an air-phase and liquid-phase systems have been developed which are connected on-line to a MS. These systems significantly increase the speed of overall analysis, minimize contamination and reduce sample size [21]. Further, it now seems that the combined isotopic and elemental oxygen analysis on the MS allows also estimation of the rate of respiration [26]. The operation of a gas-phase system [26] is briefly described below.

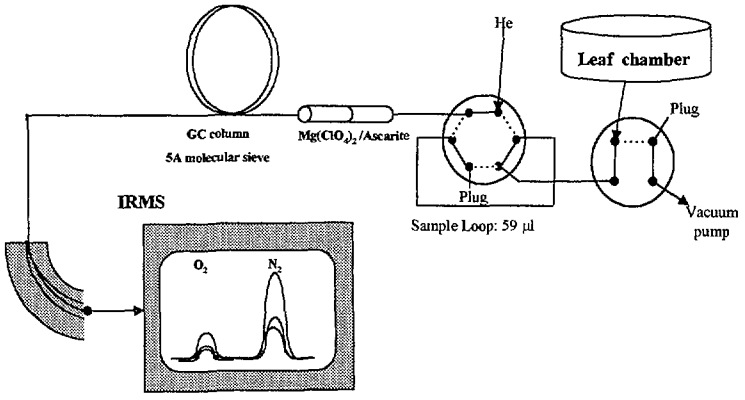

Fig. 5. On line sampling and analysis system for O_2 isotopic composition during respiration.

To measure *D* of plant materials we used freshly cut tissue samples (leaves, stems, and whole seedlings) from the selected plants. Samples were sealed in chamber (Hansatech Instruments Ltd, England) (Fig. 5). The chamber was vented and the tissue sample allowed to equilibrate with ambient air. The inlet vent was then closed and the tissue was allowed to respire in the dark. Samples of the head space were withdrawn at regular time intervals (5–20 minutes depending on rate of respiration) through a capillary (5.2 µL) into a pre-evacuated loop (59 µL) on one of two six-port, two positions Valco valve (Valco Instruments Co. Inc.). The valve was then switched to a carrier helium flow at flow rate of ~75ml/min. Carbon dioxide and water vapor were removed from the He and sample stream by using Ascarite and $Mg(ClO_4)_2$ traps, respectively. Separation of N_2 and O_2+Ar gases was done by 5A Molecular sieve GC column (Elemental Microanalysis Limited) kept at 40°C. The GC

peaks were transferred to the MS via a 1/16" stainless steel capillary. The MS detected and O_2 and N_2 by a peak jump procedure (magnet jump using Hall probe). Ratios of masses 32/28 (O_2/N_2) were used to correct oxygen uptake in respiration for sampling effects, and of 34/32 for estimating changes in ^{18}O. External precision was estimated by repeated measurements of $\delta^{18}O$ of lab air sampled through the system, yielding a precision of ±0.2‰.

5.3. Calculating ^{18}O discrimination in respiration

To calculate the discrimination factor two measurements are required for each sampling time the concentration of O_2 in the reaction vessel and the $^{18}O/^{16}O$ ratio of the sample. Oxygen must be separated from the other gases in air (N_2, CO_2, H_2O and Ar) using traps and gas chromatography (GC) before the isotopic ratio can be measured by mass spectrometry (MS). In Guy's system respiration occurred in a liquid phase reaction vessel from which samples were removed at regular intervals via a syringe. Oxygen was then purged from these samples using He, separated from the other gases and converted to CO_2, from which the isotopic ratio of oxygen could be determined [23, 27]. This method is both technically demanding and time consuming. A similar method for liquid phase determinations has been used by [24] to measure discrimination in various marine organisms. This method is simpler because the O_2 isotopic ratio is determined directly thus eliminating the steps associated with conversion of O_2 to CO_2. An on-line system developed at Duke University [21] allows direct measurements of respiratory oxygen isotope discrimination in the gas phase. Samples are removed directly from the gas phase surrounding the tissue and fed into a GC/MS system which provides measurements of both the concentration and the isotopic ratio of oxygen in the chamber. The on-line system is considerably faster than the previous method enabling measurements of discrimination in less than an hour. It also dispenses with the sample preparation steps which are the main source of contamination or error associated with the vacuum line system [27]. The volume requirement is reduced a 100-fold and some of the diffusional problems encountered previously are overcome in the gas phase system. Although many of the limitations to the stable isotope method identified by [27] have been overcome by the on-line system, the equipment remains expensive.

Traditionally, discrimination (D) is determined by comparing the isotope ratios (R, e.g. $R=^{18}O/^{16}O$) of the substrate (Rs) and of the product Rp:

$$D= (1-Rp/Rs)1000 \quad (6)$$

Note that $D = (1-\alpha)1000$; and $\Delta = (D/1000)/(1-D/1000)$, where α and Δ are alternative ways for defining isotopic discrimination [28, 29]. Obviously, in respiration isotopic analysis of the product (H_2O) is difficult. This problem can be overcome by considering that in a close system changes in Rs can be related to D by a Rayliegh type equation:

$$D=[(\ln R/R_0)/(-\ln f)]1000, \quad (7)$$

where R is the isotope ratio of the substrate at sampling time, R_0 is its initial isotope ratio of the substrate and f is the fraction of unconsumed substrate. In practice, D is obtained from the slope of the linear regression line of $\ln R/ R_0$ against $-\ln f$ of a series (ca. 5) of samples. In this approach, although only the substrate is analyzed, it is important to accurately determine its concentration, f, (O_2 concentration when respiration is considered).

The partitioning of electrons to the alternative pathway in the absence of inhibitors (P) was calculated as outlined by [27]:

$$P = [(D_n - D_c)/ (D_a - D_c)] \cdot 100 \quad (8)$$

where D_n is the net uninhibited discrimination on a linear scale between D_a (discrimination by the alternative oxidase) and D_c (discrimination by the Cyt oxidase). The flux of electrons through the alternative pathway was calculated by multiplying the total respiratory rate in the absence of inhibitors by the partitioning factor, *P*.

5.4. Calculation of respiration rates

Rates of respiration were estimated directly from the MS measurements (Fig. 5). Assuming atmospheric composition of 20.95% O_2, 78.08% N_2, 0.93% Ar plus trace gases, we denoted:

$$\theta_{air} = P_O/P_N \tag{9}$$

where P_O and P_N are the partial pressures of oxygen and nitrogen respectively and during measurements θ_{air} at time zero ($\theta_{air(0)}$) is equal to θ_{atm} in the open atmosphere (0.26, standard conditions). However, due to different instrument sensitivity to N_2 and O_2, $\theta_{air(0)}$ as measured by the MS is slightly different from θ_{atm} and must be corrected:

$$\theta_{air} = k \cdot O_t/N_t \tag{10}$$

where O_t and N_t are the measured peak areas at time t of oxygen and nitrogen. The sensitivity correction factor, *k*, was found to be a constant over variable conditions in continuous measurements performed in a closed and empty chamber.

We follow the decrease in O_2 concentration due to respiration only, and remove the affect of sampling by first considering the change in the non reacting nitrogen:

$$P_N = 0.78N_t/N_0 \tag{11}$$

We then estimated P_O at any time step due to sampling changes only (P_O^S) by assuming this change to be similar to that in N_2:

$$P_O^S = P_N \cdot \theta_{atm} \tag{12}$$

The O_2 partial pressure at a time step is then given by:

$$P_O = P_N \cdot \theta_{air} = (0.78N_t/N_0)\, \theta_{air} \tag{13}$$

and due to respiration only (P_O^R) by subtraction:

$$P_O^R = P_O - P_O^S = P_N \cdot \theta_{air} - P_N \cdot \theta_{atm} = P_N(\theta_{air} - \theta_{atm}) \tag{14}$$

values of P_O^R were converted to mole units with the ideal gas equation where V, the volume of the chamber ($5.34*10^{-3}$L) was corrected for the volume of the tissue sample by assumed for convenience it is mostly water and using fresh weight. Finally, the respiration rate was expressed on the leaf dry weight basis and the time of the relevant time interval (μmol O_2 min^{-1} g^{-1}). Precision for respiration rates was estimated from the calculated "rates" obtained in an empty chamber (0.001 μmol O_2 min^{-1}).

Notably, respiration rates often decreased during the time of the measurement. Several reasons were suggested as possible cause for a decrease in dark respiration in chamber measurements. The CO_2 concentration buildup in the closed chamber during the measurement might inhibit the respiration, especially via the Cty pathway [31, 33]. Since elevated CO_2 inhibit the cytochrome respiration,

the discrimination against ^{18}O could increase during the measurement. In our experiments, even when respiration rates decrease substantially, the *D* values were relatively constant during the time course of the measurements. Moreover, adding CO_2 absorber (Soda lime, Fisher Scientific) to the chamber did not affect the decrease in the respiration rate or the *D* value.

The dark respiration may also decrease due to changes in the carbohydrates status in the leaves. In our measurements, dark respiration decreased immediately upon darkening which might be consistent with carbohydrates status. It is also possible that the sampling and the resulting decrease in pressure in the chamber caused decrease in respiration. The range of respiration rate measured for alfalfa sprouts (control plant) at the beginning of the measurements was 0.11–0.13 (μmol O_2 $min^{-1}g^{-1}$), consistent with the respiration rate reported by the accepted method [23]. Therefore, the respiration rates, which were determined at the beginning of each sampling set, were considered reliable.

6. ON-LINE MEASUREMENTS OF ^{18}O IN WATER VAPOR: PYROLYSIS OF μL SIZE WATER SAMPLES

6.1. Introduction

Methodology for isotopic analysis of large water samples is well documented [30]. Recent attempts to apply stable isotopes to the study of evapotranspiration at the canopy scale required, however, modifications associated primarily with the analysis of small quantities of atmospheric moisture. Although for hydrogen isotopic analysis the conventional methods using Uranium [32] or Zinc [34] were designed for small water samples of a few μL, oxygen isotopic analysis was usually carried out on samples of a few mL (but see section on small sample equilibration above). The availability of an efficient method for the ^{18}O analysis of μL size water vapor sample has been a major limiting factor in the incorporation of stable isotopes to eco-hydrology studies. Here I briefly describe an on-line pyrolysis-IRMS method for the ^{18}O analysis of very small water samples that has been successfully used in field studies of atmospheric moisture.

6.2. Air moisture sampling

A general method was recently employed by [35] for simple short term sampling of ambient air moisture at different locations within and above a canopy. In this approach, air is sucked, at different levels, by a small diaphragm pump through low-adsorption plastic tubes (e.g. Teflon, Bev-a-Line) and a small, low cost cryogenic trap at –80°C, at a rate of about 250 ml min^{-1} for about 30 min. Pump and traps are located on the ground down wind of the sampling site and the tubings are flushed with sample air before the actual trapping. After sampling, traps are sealed and transported to the lab for analysis. Small, simple and inexpensive glass traps were used both for the moisture trapping and sample storage.

The trap design was based on that of [36] used for trapping reduced sulfur compounds and consisted of a 6 mm o.d. Pyrex tube looped six times over a central arm (Fig. 6). The trap had two bulb type configurations with the first to prevent clogging by ice and was positioned at the top of the cold portion of the trap on the intake side; the second was at the bottom of the trap, where most of the moisture accumulated. In addition, Pyrex wool was fitted a few cm below the outlet of the trap to prevent the escape of flakes. The trap had two simple tube endings and were connected on-line with conventional connectors (e.g. ultra-torr Cajon fittings). After sampling, traps were disconnected and sealed with plastic fittings (e.g. Swagelock).

Large water samples could be decanted off the trap, while small samples were quantitatively distilled under vacuum at 60°C and collected by freezing into a short 6 mm tube on a vacuum line. After thawing, 20~30 μL aliquots were stored in 50 μL uncoated Pyrex capillaries (Monoject Scientific, USA) that were flame sealed at both ends (Blazer, Piezo micro torch, Japan). These samples could be stored for extended periods and were convenient for shipping when necessary.

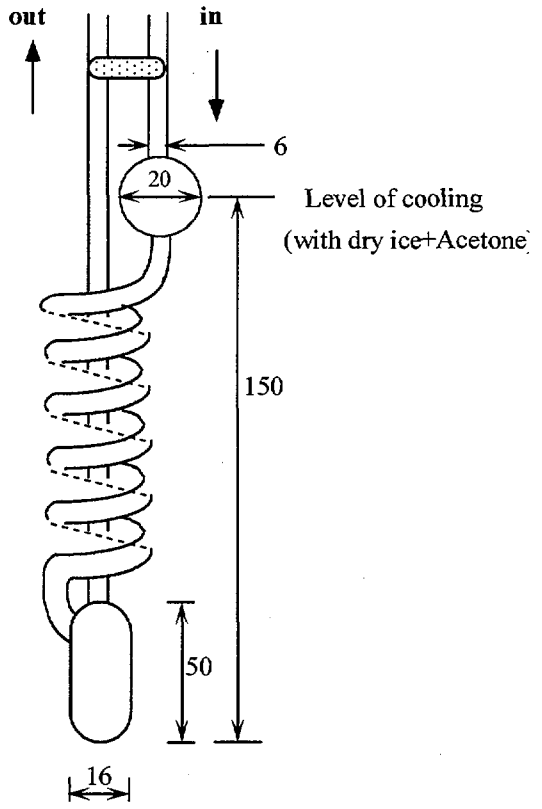

Fig. 6. A water trap for collecting small air moisture samples (b) used in the field setup. The trap is made of Pyrex tube and the dimensions shown are in mm.

6.3. ^{18}O analysis of μL water samples

The conventional water-CO_2 equilibrium approach can be used for small water samples [37], see section on equilibration above), but is labor intensive and requires corrections for equilibrium and evaporation isotope effects in the equilibration vessels. Small water samples can also be analyzed by the Guanidine Hydrochloride method in which the oxygen of water is quantitatively converted to CO_2 for the isotopic analysis [38]. This method, and a few others [30, 39] have not been widely applied, probably because of their complexity and the relatively low sample output. Here we describe a simple and rapid technique for the analysis of 0.2~2 μL water samples carried out on-line with an isotope ratio mass spectrometer. This method is based on the original approach of [40].

An elemental analyzer (e.g. EA1108, Carlo Erba Instruments, Inc., Italy) is used for the pyrolysis (1090°C) of 0.1–2 μL of water samples (the same method is used also for pyrolizing about 1 mg of organic matter) on a nickelised carbon (Elemental Microanalysis Limited, Devon, UK) column to quantitatively produce carbon monoxide. The CO sample was separated from other gases (primarily N_2) and focused on a packed GC column (9 mm, molecular sieve 5A, 80/100 mesh, 70°C) and was carried, on-line, by the He carrier gas (120 mL min^{-1}) through an open split into the isotope ratio mass spectrometer through an on-line port (e.g. Optima, Micromass, UK). The 30/28 mass ratio of the sample and a reference CO gas (injected between samples from the reference-bellow of the mass spectrometer's dual inlet) were used for determination of the $\delta^{18}O$ values of the samples. Because mass 28 used for the analysis of CO is the same as that for N_2, care must be taken to avoid air leaks in the system. The pyrolysis is also sensitive to both blank and memory effects [40]. The major blank effect was due to oxygen interactions with glass (e.g. the reaction tube, glass chips and glass wool). This was minimized by replacing the conventional quartz reaction tube with one made of ceramic (e.g. Carlo Erba, Italy; Bolt Technical, Texas). Similarly, any quartz chips required for the column packing

were replaced with ceramic chips (prepared by sacrificing a reaction column), and quartz wool was replaced with silver wool for packing purposes. The memory effects due to interactions of oxygen with the carbon in the reaction tube, was minimized by a combination of doping the helium carrier stream with 5% CCl4 in heptane, reducing by half the quantity of the carbon reactant, as compared to conventional packing for oxygen analysis, and by "flushing" the system with a "blank" sample prior to sample analysis. Doping was achieved by placing a glass capsule (ca 10 mm long, 5 mm o.d. with a 1 mm hole on its side) filled to about half with the doping solution inside ca. 200 mm long 13 mm o.d. glass tube that was fitted on the carrier gas line. A packed reaction column used in the above analysis was 46 cm long and filled, in going from bottom to top, with 3 cm quartz wool (positioned outside the furnace), 18 cm ceramic chips, 1 cm silver wool, 2 cm Nickelised Carbon (substituted in some cases by spectrographic graphite). The top of the carbon reagent was positioned at the center of the furnace. The speed of the analysis was about 4 min. per sample.

Handling of water samples was critical for the analysis, and two approaches were used. The first employed the standard multi-sample carousel of the EA. In this case ca. 1 μl water sample was placed with a gas-tight syringe (Hamilton Co., Reno, Nevada, USA) in a silver capsule and dropped directly into the first sample position with no waiting time for the EA analysis. Silver capsules were made hydrophobic by annealing at 400°C and slow cooling under vacuum. This allowed the placement of a single ca. 1 μl droplet onto the bottom of the capsule and sealing the capsule without "smearing" the water sample. Such arrangement avoided evaporative loss for the time needed for the transfer and initiation of the pyrolysis (tested as a weight drift<0.01 mg per minute, compared with about 10 sec for sample handling). The second approach employed an injector provided by the manufacturer of the analyzer (Carlo Erba Inst., Italy) that replaces the standard multi-sample carousel. This injector allowed conventional GC mode operation by injection of the small water sample directly into the reactor through a septa. A gas tight syringe with a long hypodermic needle was used to facilitate the delivery of the sample to the center of the furnace. The first approach described above was generally adopted.

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