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$^{13}CO_2$-labelling and Sampling in Algae for Flux Analysis of Photosynthetic and Central Carbon Metabolism

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Abstract

The flux in photosynthesis can be studied by performing $^{13}CO_2$ pulse labelling and analysing the temporal labelling kinetics of metabolic intermediates using gas or liquid chromatography linked to mass spectrometry. Metabolic flux analysis (MFA) is the primary approach for analysing metabolic network function and quantifying intracellular metabolic fluxes. Different MFA approaches differ based on the metabolic state (steady vs. non-steady state) and the use of stable isotope tracers. The main methodology used to investigate metabolic systems is metabolite steady state associated with stable isotope labelling experiments. Specifically, in biological systems like photoautotrophic organisms, isotopic non-stationary ^{13}C metabolic flux analysis at metabolic steady state with transient isotopic labelling (^{13}C-INST-MFA) is required. The common requirement for metabolic steady state, alongside its very short half-timed reactions, complicates robust MFA of photosynthetic metabolism. While custom gas chambers design has addressed these challenges in various model plants, no similar tools were developed for liquid photosynthetic cultures (e.g., algae, cyanobacteria), where diffusion and equilibration of inorganic carbon species in the medium entails a new dimension of complexity. Recently, a novel tailor-made microfluidics labelling system has been introduced, supplying short $^{13}CO_2$ pulses at steady state, and resolving fluxes across most photosynthetic metabolic pathways in algae. The system involves injecting algal cultures and medium containing pre-equilibrated inorganic ^{13}C into a microfluidic mixer, followed by rapid metabolic quenching, enabling precise seconds-level label pulses. This was complemented by a $^{13}CO_2$-bubbling-based open labelling system (photobioreactor), allowing long pulses (minutes–hours) required for investigating fluxes into central C metabolism and major products. This combined labelling procedure provides a comprehensive fluxome cover for most algal photosynthetic and central C metabolism pathways, thus allowing comparative flux analyses across algae and plants.

Keywords: Photosynthesis, Algae, Isotope labelling, Non-stationary ^{13}C-metabolic flux analysis, Metabolomics

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Background

Over the last two decades, numerous analytical and computational approaches have emerged for deciphering metabolic networks. Understanding metabolic pathways and their regulation is crucial for metabolic engineering, biotechnology, or pharmacology, which can be explored through metabolic flux analysis (MFA) studies. Metabolite levels do not provide reliable information about pathway flux, due to its complex relationship with protein activity and substrate level (Blank and Sauer, 2004). Major progress has been made through the use of isotopic labelling, allowing a better and more accurate assessment of the concentration of metabolites by NMR, gas chromatography (GC), or liquid chromatography (LC) mass spectrometry (MS) and combinations thereof (Niedenführ et al., 2015). Various tools have been developed alongside computational and mathematical modelling methods (e.g., INCA, METRAN, SUMOFLUX), aiming to fit a theoretical model to estimate metabolic fluxes (Heise et al., 2015).

Isotopic non-stationary ^{13}C metabolic flux analysis (^{13}C-INST-MFA) offers a robust framework for flux estimation in single carbon substrate–based systems (e.g., autotrophic, methanotrophic) (Jazmin and Young, 2013). Aiming to resolve differential balance equations for the time-dependent labelling of intermediate metabolites, ^{13}C-INST-MFA is ideally suited to slowly labelled systems (e.g., due to large intermediate pools or pathway bottlenecks). Thus, as the entry point for the single-carbon substrate system of most photoautotrophs, photosynthetic metabolism represents a unique challenge for ^{13}C-INST-MFA, due to its relatively small intermediate pool size and, consequently, very rapid turnover rates (Szecowka et al., 2013; Heise et al., 2014; Abernathy et al., 2017; Allen and Young, 2020). Despite this challenge, this approach has been widely used in the past decade to estimate fluxes in photoautotrophic systems, ranging from cyanobacteria to land plants (Ma et al., 2014; Adebiyi et al., 2015; Cheah and Young, 2018; Wieloch, 2021; Xu et al., 2022).

Supported by the development of targeted analytical methodology (Arrivault et al., 2009 and 2015) and tailored labelling setups (Szecowka et al., 2013; Arrivault et al., 2017), these studies provided robust quantitative data on fluxes through central C metabolism and photosynthesis. The pioneering study of Szecowka and colleagues (Szecowka et al., 2013) has obtained the fluxes in canonical pathways of photosynthetic carbon metabolism in *Arabidopsis* rosette based on a combined $^{13}CO_2$-labelling/GC- and LC-tandem MS/MS approach. Similar achievements have been made in *Arabidopsis* (under different conditions) (Ma et al., 2014), maize (Arrivault et al., 2017), tobacco (Chu et al., 2022), and *Camelina sativa* (Xu et al., 2021), but only partly in algae (Xiong et al., 2010; Wu et al., 2015) due to the experimentally challenging supply of rapidly labelled inorganic carbon pulses.

Using a tailor-made microfluidics labelling system to supply $^{13}CO_2$ at steady state, we investigated in vivo labelling kinetics in intermediates of the Calvin Benson cycle and sugar, starch, organic acid, and amino acid synthesis pathways, and flux into protein and lipids, in several model and non-model green algae (Treves et al., 2022). Our system allows sampling at the 0–40 s pulse timescale in a highly reproducible manner, yielding high quality data. Furthermore, when combined with traditional labelling setups such as open systems with bubbling of $^{13}CO_2$ for longer pulse times, our protocol, which is applicable to other unicellular photoautotrophic systems, largely improves the precision of flux estimates through the application of ^{13}C-INST-MFA.

Materials and reagents

1. Inorganic ^{13}C (Sigma-Aldrich, catalog number: 364592)
2. N_2 gas (Oxygen and Argon Works Ltd.)
3. O_2 gas (Oxygen and Argon Works Ltd.)
4. $^{12}CO_2$ gas (Oxygen and Argon Works Ltd.)
5. $^{13}CO_2$ (isotopic purity 99-atom percentage) gas (Sigma-Aldrich, catalog number: 364592-10L-EU)
6. Lime soda (Merck Millipore, catalog number: 1068395000)
7. *Chlamydomonas reinhardtii* strain CC-124 (UTEX Culture Collection of Algae)
8. *Chlorella sorokiniana* UTEX 1663 (UTEX Culture Collection of Algae)
9. *Chlorella ohadii*
10. HEPES (Sigma-Aldrich, catalog number: 54457)
11. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 655104)

12. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C4901)
13. Ammonium chloride (NH_4Cl) (Sigma-Aldrich, catalog number: 213330)
14. Magnesium sulphate heptahydrate ($MgSO_4·7H_2O$) (Sigma-Aldrich, catalog number: 230391)
15. Dipotassium phosphate (K_2HPO_4) (Sigma-Aldrich, catalog number: P3786)
16. Monopotassium phosphate (KH_2PO_4) (Sigma-Aldrich, catalog number: PX1562)
17. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
18. Boric acid (BO_3H_3) (Sigma-Aldrich, catalog number: B0394)
19. Zinc sulphate heptahydrate ($ZnSO_4·7H_2O$) (Sigma-Aldrich, catalog number: 221376)
20. Manganese(II) chloride tetrahydrate ($MnCl_2·4H_2O$) (Sigma-Aldrich, catalog number: 221279)
21. Iron(II) sulphate heptahydrate ($FeSO_4·7H_2O$) (Sigma-Aldrich, catalog number: 215422)
22. Cobalt(II) chloride hexahydrate ($CoCl_2·6H_2O$) (Sigma-Aldrich, catalog number: 255599)
23. Copper sulphate pentahydrate ($CuSO_4·5H_2O$) (Sigma-Aldrich, catalog number: 209198)
24. Ammonium molybdate tetrahydrate ($Mn_7O_{24}(NH_4)_6·4H_2$) (Sigma-Aldrich, catalog number: 09878)
25. Methanol (Sigma-Aldrich, catalog number: 34860)
26. Chloroform (Sigma-Aldrich, catalog number: 650498)
27. tert-Butyl methyl ether (Sigma-Aldrich, catalog number: 650560)
28. Liquid nitrogen (N_2)
29. Dry ice
30. Ethanol, technical
31. HP (HEPES-phosphate) medium (pH 7.2) (see Recipes)
    a. Nutrient stock (see Recipes)
    b. Phosphate buffer 1,000× (see Recipes)
    c. Hutner's solution (see Recipes)
32. MTBE (methyl tert-butyl ether) solution (see Recipes)

Equipment

1. Gas (mass-flow) controllers calibrated for N_2, O_2, and CO_2 (Brooks Instruments, model: 5850S) and smart control software (Brooks Instruments)
2. 20 mL syringes
3. Syringe pump (KF Technology, model: NE-1600 Multi-Channel Syringe Pump)
4. Transparent tubing (Vici Jour, catalog number: R-T-4007-M10)
5. Luer adapters (Upchurch Scientific, Union Polypropylene, UPP series)
    a. Flangeless ferrule (Upchurch Scientific, catalog number: UPP-200N)
    b. Union assembly (Upchurch Scientific, catalog number: UPP-360)
    c. Luer adapter (Upchurch Scientific, catalog number: UPP-658)
6. Gas washing bottle (Robu Glasfilter-Geräte, catalog number: 41101)
7. Flat glass photobioreactors (PSI, model: FMT-150)
8. Cool-white LED array (PSI, model: 3500-D)
9. Centrifuge (Eppendorf, model: 5418 R)
10. LC-MS (Thermo Fisher Scientific, model: TSQ Quantum Ultra with Excalibur 2.07 SP1 and TSQ Quantum 1.4 software)
11. GC-MS (LECO Instruments GmbH, model: Pegasus III TOF-MS)
12. Gas chromatograph (Agilent Technologies, model: 6890N24)
13. Humidifier:
    a. Laboratory bottles (DURAN, Sigma-Aldrich, catalog number: Z305197)
    b. Head with filter-disc for GL 45 (ROBU Glasfilter-Geraete GmbH, catalog number: ISO 4793-80)
14. 50 mL centrifuge tubes (Sarstedt, catalog number: 62.547.004)
15. Lyophilizer (Martin Christ, model: Alpha 2-4 LSCbasic)
16. Microfluidic mixer (Manufactured based on original design from Sivashankar et al., 2016)
17. Light meter (LI-COR, model: LI-250A)

18. Air pump (Sera precision, model: air 275 R plus, catalog number: 08814)
19. Autoclave (Witeg, model: WAC-60 230 V)
20. Filter 0.22 µm (Sigma-Aldrich, catalog number: SLMP025SS)
21. Water bath (GFL, model: 1002)
22. Oxygen sensor (Mettler Toledo, model: InPro 6800)
23. pH sensor (Mettler Toledo, model: InPro 3250)
24. Upright microscope (Nikon, model: Eclipse E200)
25. SpeedVac concentrator (Thermo Fisher Scientific, model: SPD210)
26. Gas analyser (LI-COR, model: LI850)

Procedure

A. Algal cultures

Grow algal cultures in flat glass bioreactor vessels, as specified in the instructions below. Further details associated with this technique are described in Treves et al. (2017).

1. Prepare HP medium (see Recipes) for the bioreactors.
2. Autoclave (121 °C, saturated steam, 30 min) each bioreactor cuvette (see https://photo-bio-reactors.com/products/photobioreactors/#details) with 1 L of HP medium and both oxygen and pH sensors connected.
3. On a sterile bench, connect 0.22 µm filters to all air inlets/outlets of the system and add 10 mL of axenic inoculum into the bioreactor medium, to reach an initial cell density of OD_{735nm} = 0.02. The optical density of the culture is measured by the bioreactor following activation (see https://photo-bio-reactors.com/products/photobioreactors/#details).
4. Initiate the bioreactors at the optimal or target temperature defined for each strain and irradiate the cells at light regimes (in µmol photons/m^2/s) optimised and designed for the study goals. For reliable estimation of in vivo fluxes, illumination levels used for labelling should be set to reflect the penetrating light levels within the bioreactor, as measured using an integrating sphere inside the running culture in the bioreactors.
5. Supply air for bubbling using an air-pump at approximately 1 L/min and monitor pH, dissolved oxygen concentration, and optical densities (OD) at 680 and 735 nm every 1–5 min, through integral bioreactor sensors (https://photo-bio-reactors.com/products/photobioreactors/#details).
6. Perform independent biological replicates of three separate bioreactor runs for each alga or condition.
7. Determine the weight of organic material within the samples through the measurement of ash-free dry weight per volume for each culture, as previously described in Klassen et al. (2015).
   Note: Assess and validate the axenicity of the cultures with light microscopy and Luria-Bertani plating/incubation.

B. $^{13}CO_2$-labelling and sampling procedures

Two setups are used to provide inorganic ^{13}C to algal liquid cultures (Figure 1).

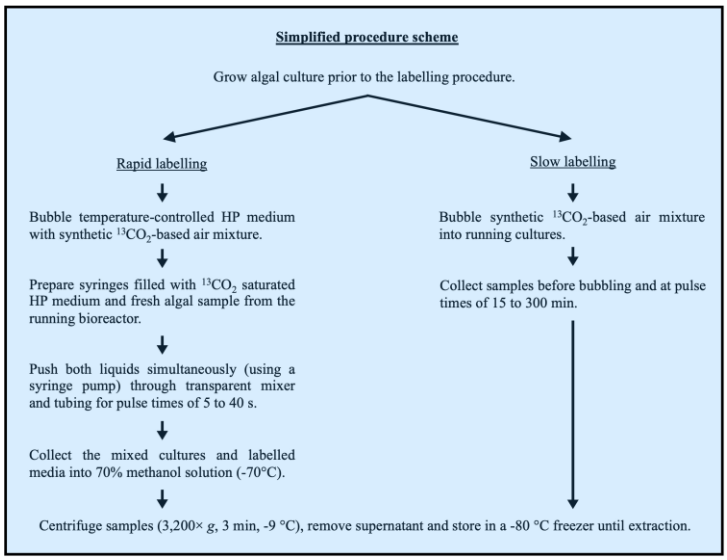

Figure 1. Simplified labelling procedure scheme. Flowchart presenting the main steps of rapid (left) and slow (right) labelling procedures.

1. Very short pulses (up to 40 s) and rapid labelling (time points 5, 10, 20, and 40 s):
   a. Fill a glass bottle with 400 mL of fresh HP medium (pH 7.2).
   b. Place the glass bottle in a temperature-controlled water bath kept at the growth temperature of each algal culture, preferably in a separate room to avoid $^{13}CO_2$ contamination in the bioreactors' running cultures prior to mixing.
   c. Bubble with the synthetic gas mixture (78% N_2, 21% O_2, and 400 ppm $^{13}CO_2$) for 30 min to reach < 1% residual $^{12}CO_2$ levels (see Figure 2B). Run exhaust flow through lime soda pellets via gas washing bottle to avoid $^{13}CO_2$ cross contamination of running cultures.

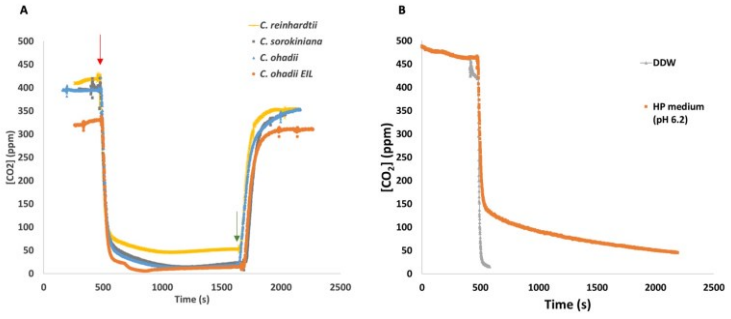

Figure 2. Kinetics of $^{13}CO_2$ supply via bubbling (A) through algal cultures or (B) HP medium or double-distilled water (DDW). Monitoring of $^{12}CO_2$ levels at the pulse, as measured on the exhaust

of algal cultures/medium/DDW using a gas analyser. (A) Different algal cultures, grown to a density corresponding to T0 for each alga and condition, were bubbled with natural air. Thereafter, the natural air was rapidly changed (red arrow) to ^{13}C-based synthetic labelled air mixture (see Procedure). Reciprocally, approximately 20 min after the first change, bubbling is rapidly replaced again for natural air (green arrow).

d. Wash a 20 mL syringe three times and fill it with the bubbled solution (keep contact with air to a minimum).
e. Load the syringe to a syringe pump.
f. Connect the syringe with transparent tubing by screwing the luer to the screw tip of the syringe.
g. Turn on the light source above the syringe pump and bioreactor and adjust to illumination levels measured in step A4.
h. Connect transparent tubing (with length adjusted to pulse time, see Notes) between the mixer and the cooled 50 mL tubes (see Figure 3A).

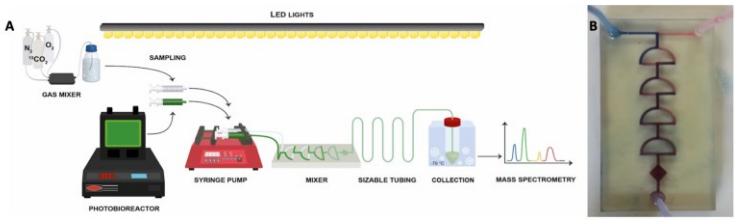

Figure 3. Rapid algal labelling system scheme. (A) Fresh medium bubbled with $^{13}CO_2$-based synthetic air mixture (see Procedure) is mixed with fresh algal sample withdrawn from the bioreactor under controlled LED illumination. (B) Solutions are rapidly (< 1 s) mixed in a tailor-made transparent chip and injected through transparent tubing into 70% methanol solution kept at -70 °C. Pulse length (5–40 s) is controlled via tubing length.

i. Withdraw a fresh algal sample into a 20 mL syringe using a transparent tube under the light. Load the syringe to the pump while avoiding any shading throughout the process.
j. Connect the syringe to transparent tubing by screwing the luer to the screw tip of the syringe (Figure 3A).
k. Operate the pump manually to push the liquids in both syringes and remove residual air from the tubes to the microfluidic mixer. Program the syringe pump to the required volume in each of the four tube lengths (see Section B, step 1h, above). Push the cultures and fresh media through the transparent mixer and tubing and collect the 1:1 mixture to a 70% methanol solution in 50 mL tubes cooled to -70 °C. Tubes are cooled in an ethanol bath precooled with dry ice (see scheme in Figure 3A). Inject the mixed sample into the cooled 70% methanol to reach a 1:2 ratio (e.g., 15 mL of sample into 30 mL of 70% methanol) (see Notes).
l. Keep the cooled 50 mL tubes at -70 °C until centrifugation (3,200× *g*, 3 min, -9 °C).
m. Discard the supernatant (see Notes) and freeze the pellet immediately in liquid nitrogen.
n. Repeat the same procedure for the non-labelled samples for each pulse time series replica by mixing the same algal culture with fresh HP medium pre-bubbled with ambient air at the same temperature as in Section B, step 1b.
o. Following rapid freezing, samples may be stored in a -80 °C freezer until extraction.
p. Resuspend the frozen pellets in ice-cold methanol/chloroform (5:1, v/v).
q. Perform four freeze-thaw cycles (see Notes) of resuspended cells and perform metabolite extraction following the methanol/chloroform procedure (see Mettler et al., 2014).

Notes:

i. Synthetic gas mixture is mixed using mass flow controllers as described previously (Szecowka et al., 2013).
ii. Light is provided by an upper-positioned cool-white LED array through the entire route from sampling at the bioreactor to the quenching tube, with intensities corresponding to the penetrating light for each culture and avoiding shading.
iii. Pulse duration is implemented by fitting the length of the transparent tubing downstream to the mixer to spray the culture/$^{13}CO_2$ solution mixture into the quenching tube at desired timings.
iv. Metabolites level within the supernatant (see section B, step 1m) should be assessed by LC-MS/MS and should ideally be up to approximately 1% that of equivalent pellets.
v. Depending on the cell wall rigidity of the algal strains, the number of freeze-thaw cycles should be adjusted to the point where all chlorophyll migrates to the chloroform phase.
vi. Microfluidic mixer (Figure 3B) may be produced by 3D printing, according to the detailed design provided in Sivashankar et al., 2016.

2. Long ^{13}C incubation times (15–300 min) and slow labelling:
Introduce inorganic ^{13}C via direct bubbling of the cultures with the gas mixture used for rapid labelling.
   a Bubble algal cultures with $^{13}CO_2$ gas mixture (approximately 1 L/min) through a humidifier as done for the rapid labelling. Switch the gas mixture from natural air to the same synthetic ^{13}C-labelled air mixture (T0). (Residual $^{12}CO_2$ in the cultures should be below 2% within a few minutes of pulse. See Figure 2A). Run exhaust flow from labelled culture through lime soda pellets via gas washing bottle to avoid $^{13}CO_2$ cross contamination of other running cultures.
   b Collect samples into 50 mL Falcon tubes before switching (T0) and at 15, 30, 60, 120, 180, and 300 min of bubbling with ^{13}C synthetic gas mixture.
   c Centrifuge immediately (3,200× *g*, 3 min, -9 °C), remove supernatant, and freeze pellets in liquid nitrogen.
   d Resuspend frozen pellets in precooled (-20 °C) MTBE solution.
   e Perform four freeze-thaw cycles of resuspended pellets, followed by a two-phase MTBE extraction to analyse metabolites, protein, and starch, as described in Jüppner et al. (2017).
   i. Dry the upper MTBE phase, containing lipids, in a SpeedVac concentrator and store at -80 °C to analyse lipid content and ^{13}C labelling.
   ii. Dry the lower phase, containing the polar and semipolar metabolites, in a SpeedVac concentrator and store at -80 °C for metabolite profiling.
   iii. Store the solid pellets containing the precipitated protein and starch at -20 °C for further analysis of labelling into starch and protein as previously described (Bradford, 1976; Arrivault et al., 2009; Ishihara et al., 2015; Fernandez et al., 2017).
   Notes:
   1) Slow labelling is required, since longer incubation times would lead to Ci depletion in the closed microfluidic system.
   2) Measured residual $^{12}CO_2$ in the cultures reaches below 2% within a few minutes of pulse (Figure 2A) and $H^{12}CO_3^-$ is rapidly equilibrated by the cells as previously reported for low CO_2-acclimated algae (Tchernov et al., 2003) and demonstrated here by the absence of a slow $^{12}CO_2$ decay phase in the cultures (Figure 2A and 3B).
   3) In step 2c, centrifugation is necessary as some metabolites can accumulate in the medium over time, thus leading to labelling patterns in the complete suspension not reflecting that in the cells.

Data analysis

Data analysis, peak annotation, and quality control (QC) procedures are thoroughly described in Arrivault et al. (2009) and Ma et al. (2014). Briefly, data analysis includes metabolite peak annotation and correction, followed by correction of raw data for the abundance of stable isotopes using the Corrector software tool (https://www.mpimp-golm.mpg.de/19405/Corrector_package_V1_91.zip), analysis of calibration curves for each metabolite and correction for signal decay along each run using periodic injection of standard mix solution; relative isotopomer

abundance (m + n) for each metabolite is calculated as in Szecowka et al. (2013).

Recipes

(Harris, 1989)

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1. **HP medium, pH 7.2 (for 1 L)**
   HEPES 1.19 g
   Nutrient stock 25 mL
   Phosphate buffer 1,000× 1 mL
   Hutner's solution 1 mL
   Make up to 1 L with DDW, set pH at 7.2 using NaOH, and autoclave.
   Store in a glass bottle at room temperature (solution is not light sensitive).
   Note: Following autoclave, add 25 mL of $CaCl_2$ 18 mM.

2. **Nutrient stock (for 1 L)**
   NH_4Cl 8.57 g
   $MgSO_4 \cdot 7H_2O$ 4 g
   Make up to 1 L with DDW and autoclave.
   Store in a glass bottle at room temperature (solution is not light sensitive).

3. **Phosphate buffer 1,000× (pH = 7) (for 1 L)**
   K_2HPO_4 106 g
   KH_2PO_4 53 g
   Make up to 1 L with DDW and autoclave.
   Store in a glass bottle at room temperature (solution is not light sensitive).

4. **Hutner's solution, pH = 6.5–6.8 (for 1 L)**
   EDTA 50 g
   Bo_3H_3 11.4 g
   $ZnSO_4 \cdot 7H_2O$ 22 g
   $MnCl_2 \cdot 4H_2O$ 5.06 g
   $FeSO_4 \cdot 7H_2O$ 4.99 g
   $CoCl_2 \cdot 6H_2O$ 1.61 g
   $CuSO_4 \cdot 5H_2O$ 1.57 g
   $Mn_7O_{24}(NH_4) \cdot 4H_2O$ 1.1 g
   Store in a glass bottle at 4 °C and wrap the glass bottle in aluminium foil (solution is light sensitive).

5. **MTBE solution**
   Methanol/methyl tert-butyl-ether (1/3).
   Store in a glass bottle at 4 °C (solution is not light sensitive).

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Competing interests

The authors declare no competing interests.

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