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Accelerating the development of a sustainable bioenergy portfolio through stable isotopes

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Abstract

Bioenergy could help limit global warming to 2°C above pre-industrial levels while supplying almost a fourth of the world's renewable energy needs by 2050. However, the deployment of bioenergy raises concerns that adoption at meaningful scales may lead to unintended negative environmental consequences. Meanwhile, the full consolidation of a bioenergy industry is currently challenged by a sufficient, resilient, and resource-efficient biomass supply and an effective conversion process. Here, we provide a comprehensive analysis of how stable isotope approaches have accelerated the development of a sustainable bioeconomy by advancing knowledge about environmental sustainability, feedstock development and biological conversion. We show that advances in stable isotope research have generated crucial information to: 1) gain mechanistic insight into the potential of bioenergy crops to mitigate climate change as well as their impact on water and nutrient cycling; 2) develop high-yielding, resilient feedstocks that produce high-value bioproducts *in planta*; and, 3) engineer microbes to enhance feedstock conversion to bioenergy products. Further, we highlight knowledge gaps that could benefit from future research facilitated by stable isotope approaches. We conclude that advances in mechanistic knowledge and innovations within the field of stable isotopes in cross-disciplinary research actions will greatly contribute to breaking down the barriers to establishing a sustainable bioeconomy.

Introduction

Bioenergy is central to all socioeconomic pathways compatible with limiting global warming to 2°C above pre-industrial levels (Fuss et al., 2014). However, concerns persist over large deployment of biofuels for climate mitigation planning on the grounds of uncertain environmental impacts, the effective delivery of C savings and potential to meet energy production targets (Calvin et al., 2021; Field et al., 2020; Reid et al., 2020). Therefore, the development of a sustainable bioeconomy at rates compatible with mitigation ambitions requires a deep understanding of the interactions between biofuels and the environment. Further, reaching this sustainability goal requires the engineering of robust, resilient, high-yielding feedstocks that use natural resources – water and nutrients – more efficiently. Moreover, a resilient bioeconomy leans heavily on the optimization of the energy conversion process through rapid engineering of microbial strains that can efficiently produce diverse, high-value bioenergy molecules and specialty bioproducts.

As with most scientific disciplines, our ability to address critical gaps in knowledge in the field of bioenergy has often been constrained by tools available (Dawson et al., 2002; Ehleringer & Osmond, 2000). Arguably, stable isotopes have recently emerged as one of the most powerful techniques for breaking the barriers to measure otherwise elusive processes *in situ*. In this context, the leverage of stable isotopes lies on a relatively well understood behavior within biogeochemical, physiological, genetic and chemical frameworks, providing crucial knowledge to accelerate the development of a sustainable bioeconomy.

Here, we review recent scientific advances that have used stable isotopes to address questions relevant to the development of a sustainable bioeconomy. Using an integrative cross-disciplinary approach, we explore this through the lens of the inherent challenges associated to sustainable bioenergy systems within the areas of environmental sustainability, feedstock development, and microbial conversion. We also analyze how stable isotopes can help ascribe causality and elucidate regulating mechanisms and interactions to target research for the improvement of the economic and environmental value of bioenergy feedstocks. Further, we highlight the knowledge gaps for attaining a sustainable bioeconomy that could benefit from future research led by innovative stable isotopes approaches. Finally, we aim to open cross-disciplinary discussions regarding the advantages of an integrative framework across environmental sustainability, feedstock development and microbial conversion for advances in bioenergy research.

Environmental Sustainability

While designed to lower the pressure on climate, the large-scale deployment of bioenergy crops has been challenged over a potential acceleration of soil C losses and non-CO_2 greenhouse gas (GHG) emissions associated to direct and indirect changes in land use, as well as increased stresses on our already scarce water and nutrient resources. Failing to bring the bioenergy sector to deliver a climate benefit that does not rely on the intensification of the use of our natural resources could unintendedly push the Earth system closer to the environmental limits within which humanity can safely operate (Fuss et al., 2014; Hanssen et al., 2020; Heck et al., 2018; Smith et al., 2016).

Urgent challenges hampering advancements towards a sustainable bioeconomy include robust understanding of the potential role that bioenergy plays in climate stabilization scenarios and their capacity to optimize the use of our natural resources (Fig. 1). Bioenergy systems should: (1) be net sinks of C (i.e. emit less CO_2 than they capture from the atmosphere); (2) sequester C in stable and persistent soil C pools; (3) deliver enhanced GHG savings (i.e. CO_2 and non-CO_2 GHGs); and, (4) minimize the use of water and nutrients as we strive to claim more marginal land for bioenergy crop production (Fig. 1). In the following sections, we take a closer look at how the application of stable isotopes in the plant-soil-atmosphere continuum provides key insights into achieving enhanced environmental sustainability of bioenergy cropping systems. We also identify areas in need of a deeper understanding that could benefit from the application of stable isotopes methods.

Assessing C capture, stabilization and persistence processes

With proven potential for soil organic C (SOC) sequestration, bioenergy systems, particularly perennial lignocellulosic feedstocks, can significantly contribute to negative emissions and deliver significant GHG savings adding to the climate benefit of fossil fuel displacement and potential geologic carbon capture and storage (Harris et al., 2015; Ledo et al., 2020; Whitaker et al., 2018). However, soil type, climate and previous land use and management can significantly influence SOC sequestration and the net GHG intensity of these systems, suggesting that not all bioenergy systems will likely deliver the savings targeted in some renewable fuel policies (Fuss et al., 2014; Kato & Yamagata, 2014). Implied in this observation is an urgent need of a deeper understanding of the mechanisms enhancing the environmental sustainability of bioenergy crops to predict confidently under which circumstances bioenergy systems will be most effective in decarbonizing the atmosphere.

At the ecosystem scale, a large fraction of C fixed by photosynthesis can be (i) allocated to above- and belowground plant biomass or respired back to the atmosphere by the canopy and roots (i.e. autotrophic respiration); (ii) released via rhizodeposition (root sloughing, mucilage and exudates) to the rhizosphere; (iii) incorporated into soil organic matter (SOM), including particulate organic matter (POM) and mineral-associated organic matter (MAOM) fractions; and

(iv) assimilated into microbial biomass or respired by the microbial community (i.e. heterotrophic respiration) (Fig 2). Accurately quantifying CO_2 capture, and C stabilization processes and persistence into biogenic pools, and identifying the mechanisms driving responses to a changing environment (e.g. crop species, soil type, climate and management) is challenging. The challenge lies on the fact that the C metabolism across the soil-plant-atmosphere continuum is defined by synergies and thresholds of mechanisms operating simultaneously and at different time-scales in a highly dynamic system. The natural abundance of C isotopes (^{12}C and ^{13}C) or enriched ^{13}C labeling techniques allows tracing C dynamics through the atmosphere-plant-soil continuum in real time (Box 1). The movement of elements through the ecosystem may be tracked *in situ* and nondestructively, with minimal or no disturbance, with stable isotope methods, providing a unique opportunity to assess the C budget of bioenergy feedstocks (Box 1; Fig. 2).

Photosynthesis and respiration are key determinants of the overall ecosystem C balance of bioenergy cropping systems. Therefore, understanding the drivers of these processes is essential for improving predictions of the C balance of bioenergy systems across the landscape. Combined with micrometeorological techniques and soil gas exchange measurements, ^{13}C provides a powerful tool for partitioning ecosystem CO_2 fluxes into source components (Griffis, 2013; Siebers et al., 2021; Voglar et al., 2019) (Table 1). While not yet widely implemented in bioenergy cropping systems, ^{13}C-CO_2 exchange measurements can be used to directly partition net ecosystem exchange (NEE) into gross primary productivity (GPP) inputs and ecosystem respiration (Re) outputs (Wehr & Saleska, 2015). For example, under controlled conditions, a recent study demonstrated that peak daily GPP was about 50% greater in maize than soybean, but peak daily Re was similar between species, resulting in greater NEE in maize than soybean

(Fassbinder et al., 2012). Compared with isotopic partitioning of NEE, partitioning of soil respiration into autotrophic (root and rhizosphere) and heterotrophic (microbe and fauna) components via ^{13}C has been more widely used (Table 1; Box 1). A study (Rochette et al., 1999) used natural abundance ^{13}C in a maize (C4) system planted on a C3-derived soil to show that autotrophic respiration was driven primarily by changes in plant activity whereas heterotrophic respiration varied with soil temperature. Although technical limitations exist, recent improvements in situ ^{13}C-CO_2 instrumentation coupled with decreasing costs (Griffis, 2013; Voglar et al., 2019) will help facilitate future studies of ecosystem CO_2 fluxes in bioenergy systems.

Agricultural activities have caused the loss of up to 133 Pg C from soils globally (Sanderman et al., 2017), and thus building soil organic matter (SOM) provides a key step toward climate stabilization (Blanc-Betes et al., In review; P. Smith et al., 2016). While ecosystem scale measurements can paint a broad picture of C inputs, outputs, and balance, understanding the detailed dynamics of SOM storage requires more targeted approaches. Stable isotopes can help to identify predominant SOM inputs, assess the relative persistence of SOM, and trace the interactions between plant and microbial components (Table 1). For instance, Berhongaray et al. (2019) used natural abundance ^{13}C to show that belowground inputs dominated the SOM pool of a poplar plantation, while Austin et al. (2017) used ^{13}C-CO_2 pulse labeling to demonstrate that rhizodeposition from a rye cover crop accounted for a third of total belowground inputs in a maize system. Because SOM is known to be comprised of different pools representing varying levels of persistence (e.g., particulate organic matter vs. mineral-associated organic matter), it is also critical to understand the effect of bioenergy crops on different SOM pools. In this light, Fulton-Smith & Cotrufo (2019) found that root litter was less effective at forming more stable mineral-associated SOM compared to aboveground litter in a sorghum system, highlighting the need to consider aboveground biomass removal when targeting SOC storage. The priming effect, which represents the change in microbial SOC decomposition in response to fresh C inputs, is an important interaction between plants and soil relevant to the vulnerability of recently fixed and old organic C pools in changing conditions and can be elucidated using ^{13}C. For example, Weng et al. (2020) showed that incorporated residue caused higher positive SOC priming than surface applied residue in a sugarcane system, but that incorporated biochar led to negative SOC priming. The results of some ^{13}C SOM studies have already been implemented into modern bioenergy cropping systems models, but additional empirical work is direly needed to further constrain SOM dynamics across a wide array of scenarios (Juice et al., 2022).

Growing evidence reveals a prominent role of the microbiome on the regulation of the SOC budget. The contribution of microbial necromass to the total SOC pool is greater than previously anticipated, and microbial ecology (i.e. identification and function) has been proven critical to the stabilization and persistence of C in soils (Li et al., 2021). Stable isotopes are powerful tools for tracking C fluxes within the soil microbiota *in situ* ascribing functionality to microbial taxa and unveiling impacts on C sequestration and stabilization in soils. For instance, Elias et al. (2017) found that *Miscanthus* led to greater ^{13}C enrichment in bacterial phospholipid fatty acids (PLFA) whereas willow allocated greater C to ectomycorrhizal fungi and more shallow roots. These observations accompanied significantly less CO_2 released as ^{13}C-CO_2 in *Miscanthus*, casting some light into the role of fungal and bacterial communities in the microbial C assimilation and SOC stabilization pathways of bioenergy systems (Table 1). In another study using stable isotope probing of PLFAs (Box 2), Roosendaal et al. (2016) showed that switchgrass root architecture can influence fungal versus bacterial activity, which in turn affects SOC sequestration, whereas Sher et al. (2020) used a ^{13}C-CO_2 tracer to show that switchgrass stimulated microbes producing more extracellular polymeric substances, which have been linked to more soil aggregation and thereby increases in SOC. Therefore, ^{13}C-CO_2 labeling experiments can help elucidate the interdependencies between crops species, microbial C turnover and soil C stabilization, paving the path to identifying desired traits for the development of a sustainable bioenergy portfolio (Table 1).

Use of stable isotopes to evaluate the emission of non-CO_2 GHG (i.e. CH_4 and N_2O emissions)

Amplified global warming potential, high sensitivity to both biotic and abiotic factors, and a strong dependence on local parameters make unintended increases of non-CO_2 GHG from bioenergy cropping systems central to evaluation of sustainability. Curbing methane (CH_4) emissions is central to short-term actions for climate stabilization (Ocko et al., 2021). Bioenergy crops in temperate, semi-arid and arid climates are usually net sinks of CH_4 (Drewer et al., 2012; Gauder et al., 2012; Oertel et al., 2016; Toma et al., 2011). However, increasing trends in the frequency of flash flood events and the expansion of bioenergy crops into marginal land and tropical and subtropical regions could decrease or reverse this sink potential (Box 3).

The net emission of CH_4 to the atmosphere results from a dynamic equilibrium among CH_4 production (methanogenesis), CH_4 oxidation (methanotrophy), plant-mediated CH_4 transport and physical processes (i.e., ebullition and diffusion) (Bridgham et al., 2013). Further, methanogens produce CH_4 by two major pathways, acetate fermentation and CO_2 reduction. All these processes operate simultaneously, and their tight interdependencies and distinct sensitivities to both biotic and abiotic factors make the identification and quantification of individual contributors to net CH_4 fluxes crucial, particularly in changing environmental conditions. For example, fertilizers and high soil pH can either increase or decrease CH_4 emissions in response to changes in water management, fertilizer type, and the baseline N status of ecosystems (Blanc-Betes et al., 2016; Gomez-Casanovas et al., 2016, 2020). Further, methanogenic pathways have distinct kinetic efficiencies and sensitivities to soil redox, temperature and substrate availability (Zhang et al., 2021). Therefore, *in situ* assessments of these individual processes quantified with minimal or no disturbance by means of stable isotopes are critical to determine the net CH_4 source or sink strength (Banger et al., 2012) (Fig. 2A).

Studies using natural abundance of ^{13}C-CH_4 in bioenergy crops to partition net CH_4 emissions into gross fluxes (oxidation vs methanogenesis) are rare, particularly in marginal lands and in tropical and subtropical regions (Supplemental Information, Table S1). The stable isotope trace gas pool dilution technique, an iterative model that solves for gross production rates based on the isotopic dilution of the isotopically enriched chamber headspace pool of CH_4 by natural abundance CH_4 emitted by the soil, is an underutilized approach to the partitioning gross CH_4 fluxes. Using this technique, Yang & Silver (2016) revealed substantial gross CH_4 production and oxidation occurring within the soils of corn fields with negligible CH_4 emissions. Evaluations of the predominance of the CH_4 metabolic pathways (i.e. acetate fermentation and CO_2 reduction) in bioenergy systems, typically determined using natural abundance C isotopic composition of CH_4 and CO_2 in surface emissions and soil water pores, are also scarce (Supplemental Information, Table S1). Albeit scarce, some studies have used the natural abundance of ^{13}C-CH_4 to elucidate predominant CH_4 transport mechanisms in bioenergy systems (Supplemental Information, Table S1). A recent study in *Populus* spp., a biomass crop particularly well-suited for bioenergy generation, found substantial CH_4 transport through *Populus* stems, whereas CH_4 transport through leaves was negligible, improving the accuracy of predictions of CH_4 emissions from this crop (Kutschera et al., 2016). Studies focusing on gross process rates and CH_4 pathways could be extremely valuable to inform climate smart decisions on the deployment and management of bioenergy systems to reduce the CH_4 footprint of bioenergy systems in a climate change context.

Agriculture accounts for over half of anthropogenic N_2O emissions to the atmosphere (Tian et al., 2020). Understanding of mechanisms regulating soil-atmosphere N_2O fluxes is therefore, critical to policy making and management decisions aimed to minimize a collateral forcing on climate that may significantly reduce the efficacy of biofuels to abate global warming (Reay et al., 2012). With lower fertilizer requirements, prioritizing biomass- over grain-based feedstocks could significantly reduce the N_2O feedback from the bioenergy sector (Oates et al., 2016; Ruan et al., 2016), but further emission reductions could potentially be achieved (Table 2). Designing high-yielding bioenergy landscapes with minimal soil N_2O emissions requires resolving the partitioning of N_2O production and reduction metabolic pathways and regulatory mechanisms (Shan et al., 2021; Yu et al., 2020; Zhu-Barker et al., 2015). This can be achieved using both labeling and natural abundance stable isotope approaches (Fig. 2A).

Unlike more qualitative source partitioning approaches, stable isotope techniques can avoid the uncertainty inherent in direct measurements to accurately identify the dominant processes driving N_2O production. For example, N_2O producing metabolism may be partitioned into nitrification and denitrification, by tracking labeled ^{15}N recovery in $^{15}N_2O$ from ^{15}N-labeled ammonium, a dominant substrate for nitrification, relative to ^{15}N-labeled nitrate, the main substrate for denitrification (Krichels et al., 2019; Morse & Bernhardt, 2013). While labeling experiments are useful for identifying predominant pathways and potential activities, trace substrate additions may alter microbial function and limit experimentation in uncontrolled environments (Yang et al., 2014). Natural abundance stable isotope approaches avoid disturbance allowing for the direct determination of *in situ* activities under field conditions. Most promising is the use of N_2O isotopomers, the difference between $\delta^{15}N$ of the alpha and beta N atoms in N_2O (i.e., the central or terminal position on the asymmetrical N_2O molecule), to differentiate between bacterial denitrification versus nitrification and fungal denitrification (Ostrom & Ostrom, 2017). This approach has revealed that denitrification prevails over nitrification as the N_2O source in switchgrass soils (Ostrom et al., 2021), directing mitigation efforts to manipulating conditions for the anaerobic, heterotrophic process of denitrification rather than the aerobic, chemolithoautotrophic process of nitrification. Molecular approaches, such as functional gene transcript analysis, used in conjunction with stable isotope approaches can potentially reveal the importance of other microbial N_2O source processes, such as dissimilatory nitrate reduction to ammonium, that cannot be differentiated from denitrification and nitrification based on stable isotopes alone.

Soil N_2O reduction rates are notoriously challenging to quantify (Groffman et al., 2006) but recent advances in stable isotope approaches are beginning to provide much-needed field estimates of these rates that are lacking across all upland terrestrial ecosystems (Almaraz et al., 2020). The stable isotope trace gas pool dilution technique has been used for *in situ* surface flux measurements of gross N_2O production and consumption (Yang et al., 2011), showing that on average, roughly one-third of N_2O produced in a well-drained agricultural field can be reduced to N_2 before emitted to the atmosphere (Yang & Silver, 2016). Natural abundance stable isotope approaches for measuring *in situ* N_2O reduction rates have more recently been developed, for instance using clumped isotopes of $^{15}N_2$ along soil profiles (Yeung et al. 2019). Increased access to the instrumentation and training to use these advanced approaches will broaden their use across ecosystems, including in bioenergy cropping systems (Almaraz et al., 2020).

Use of stable isotopes to study the use of water resources

Rising water demand and reduced supply is increasing global water stress, which can be further aggravated by climate change. At present, agriculture consumption represents 87% of global freshwater use (D'Odorico et al., 2020; Wu et al., 2022). Even limiting deployment to unirrigated bioenergy would increase evapotranspiration (ET; i.e. water loss at the ecosystem scale) by ~5% over the average short vegetation, leading to an additional water use of ~3% of global agricultural water appropriation for a targeted minimum deployment of biomass production compatible with < 2ºC scenarios (Smith et al., 2016; Wu et al., 2022). Moreover, high biomass targets would require the expansion into irrigation zones more than doubling agricultural water withdrawals (Heck et al., 2018; Stenzel et al., 2021).

ET is defined by two fluxes, plant transpiration (T) and evaporation (E) of water from plant surfaces and the soil (Bernacchi & VanLoocke, 2015). A key metric of the sustainable use of water resources is the water use efficiency (WUE; i.e. amount of C assimilated per unit of water lost) (Table 3). Among the diverse WUE metrics, a crucial parameter is the T to ET ratio (Table 3), that can be enhanced by genetic improvement or by adopting practices that reduce E and divert more water into T (Table 4). Given the strong link between T and plant productivity and the role of ET in determining water loss and availability, achieving a sustainable bioenergy portfolio requires understanding how a variety of bioenergy crops and management strategies affect both the T and E components of ET, and how these components respond to changes in climate (Kool et al., 2014; Xiao et al., 2018).

Water isotopes provide invaluable information for a more efficient use of freshwater resources. They can be used to directly partition ET *in situ* without disturbing the normal functioning of an ecosystem given the difference in isotopic fractionation between E and T (Rothfuss et al., 2021; Xiao et al., 2018) (Fig. 2A; Annex 1). While many studies have isolated the T flux in ET using water isotopes in cropping systems and grasslands, only a few focused on bioenergy crops including maize, *Populus* and sorghum (Rothfuss et al., 2021), suggesting that more studies are needed to improve the sustainable use of water by bioenergy crops. Using this isotopic approach, Lu et al. (2017) partitioned ET in an irrigated *Sorghum bicolor* plantation for bioenergy production. They found that of all the irrigated water, only 28-39% was used by T, and that E accounted for as much as 54% of ecosystem ET. These results suggest that management and bioengineering strategies to improve WUE in sorghum will play a major role in the sustainable use of water by this lignocellulosic biofuel system.

In addition to partitioning ET, stable isotopes can be used to better understand the source of the water consumed by plants as recently reviewed (von Freyberg et al., 2020), guiding both genetic engineering efforts and management decisions to optimize bioenergy use of water resources. Under the assumption that the isotopic composition of xylem water reflects the mixture of water sources used by roots (Oerter et al., 2019; Penna et al., 2018), studies on this topic could resolve interactions between root architecture and drought tolerance, leading efforts towards the development of a sustainable bioenergy portfolio that builds resilience in the face of climate change (Joo et al., 2016).

Use of isotopes to investigate the use of nutrient resources

Bioenergy crop production is plagued by the same tradeoff as food crop production. Routine biomass removal results in the depletion of soil nutrients and has a toll on soil health and future productivity. Fertilizer inputs to maximize yields leads to nitrogen (N) losses that contribute to both air and water pollution (Tilman et al., 2011). Perennial bioenergy crops require less N inputs than annual bioenergy or food crops due to nutrient retranslocation before harvest, resulting in lower N and phosphorus (P) losses (Lawrence et al., 2021; Oates et al., 2016). However, the N mass balance of perennial bioenergy crops suggest greater N removals with harvest than inputs (Smith et al., 2013), indicating the need of additional fertilizer N inputs to sustain yields in the long-term (Sharma et al., 2022).Therefore, sustainable production of bioenergy crops requires strategies that minimize anthropogenic nutrient inputs.

Increased reliance on biological N fixation (BNF), internal nutrient cycling such as mineralization and translocation, and minimizing N losses due to leaching or gaseous fluxes can improve the sustainability of plant nutrient provisioning. There is a tradeoff between mineralization and BNF as potential N sources for bioenergy crops. BNF introduces new N into the agroecosystem, replacing N lost through biomass removal or biogeochemical processes, but the process is inhibited by other available N (e.g. fertilizer or mineralized N). BNF can be considered a “renewable” source of N, whereas mineralization is a “non-renewable” source that depletes soil N over time if relied upon for nutrient provisioning. Increasing plant nutrient provisioning from BNF should be prioritized for long-term sustainability. However, the relative importance of fertilizer N, N fixation, and N mineralization as sources of N to support bioenergy crop production is poorly characterized. Nitrogen stable isotopes can be used for quantifying process rates to address these major gaps in understanding of N mass balances that underlie the N sustainability of bioenergy cropping systems (Wewalwela et al., 2020).

Abundant indirect evidence suggests that BNF supports the productivity of perennial bioenergy grasses (Davis et al., 2010; Dohleman & Long, 2009; Heaton et al., 2004), but quantifying BNF at relevant time and spatial scales has proved challenging. The isotope dilution approach has been used in annual cropping systems to quantify BNF rates and monitor plant N assimilation dynamics over entire growing seasons following an initial isotope (^{15}N) enrichment (Boddey et al., 1990; Iniguez et al., 2004; Rennie, 1982) (Fig. 2A). Plants that benefit from N_2 fixation will incorporate unlabeled atmospheric $^{14}N_2$, resulting in a dilution of the ^{15}N–^{14}N ratio of the enriched soil nitrogen pool, compared to plants without N_2 fixation. However, homogenous incorporation of ^{15}N-enriched fertilizer to the volume of soil occupied by perennial grass systems is infeasible due to their extensive and heterogenous roots and rhizomes, leading to uncertainties in BNF estimates (Chalk, 2016; Keymer & Kent, 2014; Miranda et al., 1990; Miranda & Boddey, 1987). Approaches that take advantage of ^{15}N natural abundance in soil (Fuertes-Mendizábal et al., 2018; Houngnandan et al., 2008; Urquiaga et al., 2012; Wewalwela et al., 2020) avoid the pitfalls of isotope tracer incorporation, but these methods have other limitations (Table 5). The identification of reference plants that do not benefit from N_2 fixation is particularly challenging in non-legume systems, as many grasses benefit from associative or free-living diazotrophs. Yield-independent isotope dilution models (Chalk, 2016; Witty, 1983) have been used to address this limitation in *Miscanthus* (Keymer & Kent, 2014), but this is not a panacea. Alternatively, BNF can be quantified by measuring incorporation of ^{15}N into plant tissues or soil incubated with ^{15}N-enriched dinitrogen gas (Chalk, 2016). However, the high cost and logistical constraints of this approach (Table 5) limits its use for field-scale measurements and for capturing intra- and inter-annual variability in BNF, as observed in switchgrass (Roley et al., 2018, 2019; Wewalwela et al., 2020). Despite the challenges of using these stable isotope approaches for quantifying BNF (Chalk & Craswell, 2018), these remain the best approaches available and should be used more broadly to address the uncertainty in the current role of BNF in bioenergy cropping systems and to advance strategies to enhance BNF for sustainability benefits (Chalk, 2016).

Feedstock development

The great challenge that lies ahead of feedstock development is the need to design crops to support an environmentally and economically sustainable bioeconomy. This entails selecting for and/or adding traits that improve yields while modifying the cell wall to be more easily converted to bioproducts and introducing *in planta* production of high-value, energy-dense molecules including lipids (Amthor et al., 2019; Baligar et al., 2001; Gomez-Casanovas et al., 2007; Ort et al., 2015; Zhu et al., 2010). The development of optimal crops and specialty bioproducts requires developing state-of-the-art tools to trace photosynthetically fixed C into end-products (Allen & Young, 2020). In the next section, we focus on how stable isotopes can help us explore the plant primary metabolism, a crucial first step to develop robust high-yielding bioenergy crops, and how they can be used to better inform the production of important bioproducts as well as the enhancement of water and nutrient use at the plant level.

Use of isotopes to explore plant primary metabolism

Mapping the flow of C through primary metabolism, including the Calvin-Benson-Bassham (CBB) cycle is essential to determine allocation to bioenergy products. A suitable experimental approach, together with a powerful computational algorithm, have been developed in what is known as Isotopically Nonstationary ^{13}C-Metabolic Flux Analysis (INST-MFA) (Jazmin et al., 2014; Young et al., 2011). INST-MFA is typically performed on photosynthetically active leaves under light. The experiment starts by changing the C source of a leaf from unlabeled CO_2 to $^{13}CO_2$. The ensuing transient in label signatures of CBB cycle intermediates is traced through a series of time points at which tissues are sampled and isotopomer signatures are measured in central metabolism intermediates. Since different pathways lead to different atomic rearrangements in the C chains of CBB cycle intermediates, flux-dependent transients in isotopomer signatures can be computationally resolved into a flux map representing the primary metabolism (Jazmin et al., 2014). As an example, using INST-MFA, Ma et al. (2014) revealed a tight regulation of photorespiration through acclimation to light levels on 4-week-old *Arabidopsis thaliana* labeled plants. Another study using INST-MFA on *Camelina sativa* leaves attributed additional respiratory C losses to operation of the 6-phosphogluconate shunt (Xu et al., 2021) – a recently described metabolic cycle where photo-assimilated CO_2 is released by the oxidative pentose phosphate pathway (Sharkey & Weise, 2016). While the INST-MFA approach can produce a fairly detailed flux map of the central metabolism, it may also be the case that co-occurring fluxes can only be resolved with large statistical uncertainties in their values. Therefore, kinetic flux profiling (KFP) was developed as an alternative approach that is more explorative and makes use of the isotope tracer data to derive metabolic rates for individual reactions (Szecowka et al., 2013; Yuan et al., 2008).

Both INST-MFA and KFP approaches are well suited to monitor the primary metabolism under various physiological conditions on the premise that the cell population of the test sample is representative of one uniform metabolic state. For plants, which are complex multicellular organisms, this assumption may often not hold true. The tissue under study may present marked cellular and subcellular metabolic heterogeneity. If labelled transients from a labeling experiment result from the operation of distinct metabolic processes in different cell types, the analysis of tissue-extracted metabolites represents an averaged analytical readout and the flux modeling process might give a distorted or unreliable picture of cellular metabolism (Chu et al., 2020;

Szecowka et al., 2013). Thus, the effects of metabolic heterogeneity must be diagnosed and considered by the metabolic modeling process in order to avoid misinterpretations. Cellular heterogeneity in metabolism can be resolved if the different cell types are separated prior to extraction and analysis of metabolites. For example, quite tedious non-aqueous fractionation techniques have been applied to separate fractions enriched in mesophyll and bundle sheath cells of C_4 plants prior to extraction to capture the pronounced metabolic differences between the two cell types (Arrivault et al., 2017). Alternatively, spatial heterogeneity of metabolism can be explored by mass spectrometry imaging (MSI) techniques (Boughton et al., 2016). If combined with isotope tracer techniques, MSI might allow developing a dynamic approach to analyze metabolic flux *in situ* with spatial resolution. Towards that goal, Romsdahl et al. (2021) presented a workflow combining isotope labeling of developing embryos of oilseed crops with MSI analysis of the major membrane lipid component phosphatidylcholine. Their analysis suggests different flux patterns of fatty acid elongation and desaturation in cotyledon *vs.* embryo axis tissue. In the future, the integration of $^{13}CO_2$ labeling protocols of photoautotrophic tissue and MSI techniques should improve our ability to spatially resolve metabolism in leaves and other photosynthetic plant tissues. Efforts to improve bioenergy crops should benefit from the resulting refined understanding of multicellular plant systems.

Use of stable isotopes to assess the production of high-value biofuel products

Much of the C fixed by photo-assimilation is programmed for storage in stems as low-value lignocellulose. There is a significant interest to re-route some of this C into high-value end-products. This is the case of energy-dense lipid-based biofuels that promote triacylglycerol (TAG) accumulation in plant biomass by the upregulation of lipid synthesis in vegetative tissues (Vanhercke et al., 2019; C. Xu & Shanklin, 2016). It has been estimated that if plants could be engineered to accumulate TAG in all the above-ground plant biomass at a 10% w/dw level, oil yields per acre could be substantially higher than achievable for any conventional oilseed crop (Ohlrogge & Chapman, 2011). However, vegetative plant tissues like leaves tend to have low intrinsic capacity to produce and store TAG at high levels (Chapman et al., 2013; Xu & Shanklin, 2016). Engineering strategies in the leaf must therefore aim at complex metabolic reprogramming. In fully grown mature leaves, CO_2 is assimilated mainly into sucrose and starch (Fig. 3). While sucrose is exported from the leaf to other tissues, most of the starch accumulated during the day is remobilized during the night to sustain energy demands in the dark (Fig. 3). This transient diurnal accumulation pattern is often pronounced. Therefore, the engineering strategy could be to divert the C flow away from sucrose export towards oil synthesis (Fig. 3). A recent theoretical modeling study on *Sorghum bicolor* found that, based on daily rates of net CO_2 photo-assimilation observed for mature leaves under field conditions, TAG could accumulate to a 20% (w/dw) level in less than a month (Clark & Schwender, 2022). However, this goal could only be accomplished if 5 % of the photo-assimilated C was diverted towards oil accumulation, and futile cycles of lipid biosynthesis and degradation operated on a massive scale, which would significantly impede the overall photo-assimilation (Clark & Schwender, 2022). In many cases, reported TAG levels are substantially below a 20% w/dw level, although some plants such as *Nicotiana tabacum* have been engineered to produce TAG levels above 30% w/dw level (Box 4). It can be expected that genetic manipulation of metabolic enzymes involved in TAG biosynthesis in leaves regularly results in unforeseen effects like lipid futile cycles. Nevertheless, we envision that isotopic tracer experiments will play a major role in diagnosing such roadblocks, allowing for further refinements of metabolic engineering strategies.

Use of stable isotopes to investigate water and nutrient use efficiency related processes

As described above, water stress is increasing worldwide as a result of the large water footprint of agriculture – and other sectors – and shrinking water resources due to climate change. Increasing WUE at the plant level is therefore critical for the development of a sustainable bioeconomy. From a physiological standpoint, high WUE means that high rates of photosynthetic C fixation can be achieved with minimal water loss. Quantifying WUE requires direct measurements of transpiration rates with gas exchange methods. This approach, however, is not easily applicable on a high-throughput scale, which is crucial for fast identification of best-performing engineered crop species (Ellsworth & Cousins, 2016; Vadez et al., 2014). Isotopes can help us with rapid quantification of plant WUE as shifts in the ^{13}C /^{12}C isotope ratio in leaf biomass relative to atmospheric CO_2 reflect the plant water state serving as a proxy for plant WUE estimates (Ellsworth & Cousins, 2016; Seibt et al., 2008). While rapid WUE quantification method is yet to be applied in bioenergy research, it has been successfully used in conjunction with quantitative trait loci analysis to identify genes that promote enhanced WUE in cereals (Chen et al., 2011). The application of these emerging methods along with standardized approaches that facilitate the process of estimating WUE (Mathias & Hudiburg, 2022) could substantially expedite the identification of transgenic bioenergy crops for high WUE traits.

A major component of plant biomass and a key yield limiting factor is N. The engineering of dedicated crops with a more efficient uptake and utilization of N would reduce fertilization requirements and limit environmental pollution from nitrous oxides and nitrate leaching (Vitousek et al., 2009). Improving N use efficiency requires detailed understanding of the complexity of N metabolism at both cellular and plant scales (Beatty et al., 2016). To that end, ^{15}N tracers can be used to trace N uptake and its movement among plant organs (Malagoli et al., 2005), and when coupled with ^{13}C analyses they can provide a tool for metabolic profiling of distinct engineered phenotypes. For instance, Dersch et al. (2016) combined ^{13}C and ^{15}N tracers with subsequent labeling analysis of entire plants, plant tissues and metabolites to quantify assimilate movements between leaf and root. In this study, the design of the labeling chambers allowed tracing both elements *in situ* under controlled environmental conditions, providing a technology that allows for fast screening and metabolic profiling. Although this technology has not been applied to bioenergy crops yet, this approach could provide a tool for high-throughput plant metabolic phenotyping.

Biological Conversion

A major challenge in the field of conversion is to develop robust microorganisms for the efficient bioconversion of plant biomass into biofuels and bioproducts at large scale with improved energy yields and titer of products (Jullesson et al., 2015). In this light, enhancing the efficiency of the conversion process requires exploitation of novel microorganisms with less-understood metabolism as well as rewiring their native metabolism towards producing non-natural compounds. This often proceeds in iteration, where diagnosing metabolic bottlenecks in engineered cells informs next-round design. Further, bioconversion will also benefit from the efficient utilization of alternative C sources for the high-performance generation of value-added compounds (An et al., 2021). Stable isotopes are powerful tools that can contribute to achieving these two goals.

By rewiring the metabolism of yeast and other microorganisms, metabolic engineering facilitates the production of a greater diversity of high-value products. Engineering designs often build on a thorough knowledge of the microorganism's native metabolism, which makes model organisms successful microbial factories (e.g. *Saccharomyces cerevisiae* and *Escherichia coli*). However, wide diversity of less studied organisms may be better platforms for the production of certain chemicals. These microorganisms are increasingly accessible for metabolic engineering as genomic sequence and genetic editing toolboxes become available (Fatma et al., 2020). Understanding metabolic activity or flux (i.e. rate of substrate conversion to product per unit time) is the necessary first step in pathway design (Keasling et al., 2021). Metabolic activity, however, is not a physical entity which can be captured in a test tube and measured. Rather, it must be inferred, and isotope tracers combined with a suitable detection method for the labeled metabolites (e.g. Nuclear Magnetic Resonance or mass spectrometry) provide the most powerful tool for such inference (Fig. 4A). We dedicate the following sections to explain how stable isotopes can help us understand metabolism of microorganisms, measure activity in C co-utilization pathways, and diagnose metabolic bottlenecks in engineered cells.

Use of stable isotopes to infer metabolic microbial flux

Isotope tracing allows metabolic flux to be resolved accurately. The most common tracer to assess metabolic flux is ^{13}C, in the form of ^{13}C Metabolic Flux Analysis (^{13}C MFA) (Box 5) (McAtee et al., 2015; Wiechert, 2001). A typical experiment involves culturing the microbes in ^{13}C labeled C source(s) to a steady state, and then measuring isotope labeling in metabolites or proteinogenic amino acids by NMR or mass spectrometry. With proper tracer choice, valuable information on pathway usage can be obtained (Fig. 4A) and used to constrain flux (Antoniewicz et al., 2007; Blank et al., 2005) either for a subset of metabolism of particular interest -typically central metabolic reactions- or at the genome scale (Gopalakrishnan & Maranas, 2015; Martín et al., 2015; Suthers et al., 2007).

The flux distribution in wild type cells provides the initial point for optimization algorithms for engineering design. These algorithms test deletion or activation of reactions (or combinations thereof) *in silico* and identify the most promising strategy for optimal production. Strategies for optimal production often eliminate competitive or redundant reactions but may occasionally involve non-intuitive mechanisms or suggest coupling target production to biomass generation thereby enabling growth-based lab selection and adaptation (Burgard et al., 2003; Patil et al., 2005; Ranganathan et al., 2010; Rocha et al., 2010). A notable example is severing gluconeogenesis by deleting phosphoglycerate mutase and thereby forcing CO_2 fixation as essential reaction for gluconeogenesis in *E. coli* (Antonovsky et al., 2016). The effectiveness of all these approaches is greatest when starting from a rigorous knowledge of the endogenous metabolism, enabled by isotope tracing.

Usually after testing the initial metabolic design, metabolic flux analysis can be a useful part of “learning” in the classical design-build-test-learn (DBTL) cycle of metabolic engineering to overcome the obstacles associated with driving biological systems to produce non-natural compounds. The DBTL cycle represents a framework that helps systematize metabolic engineering and increase its efficacy and generalizability. Potential next-round engineering targets can be identified by looking for reactions which flux is positively correlated with the improved production performance (McAtee et al., 2015). Following this principle, the oxidative pentose phosphate pathway was identified as a target for improving lipogenesis in an oleaginous yeast; pyruvate carboxylase for an engineered lysine producer; pyruvate kinase in cyanobacterial isobutyraldehyde production; and TCA cycle flux for protein production in *Schizosaccharomyces pombe* (Jazmin et al., 2014; Klein et al., 2014; Koffas & Stephanopoulos, 2005; Wasylenko et al., 2015).

Use of stable isotopes to enable the utilization of alternative C sources

An important engineering objective, crucial to a sustainable bioeconomy, is enabling the utilization of C sources, other than glucose, that are less preferred, to enhance the microbial metabolic conversion process. With this context, stable isotope tracing can help us validate the assimilation of alternative C sources independent of benefits in growth. These less preferred substrates include abundant components in plant tissues such as lignocellulose and xylose. ^{13}C isotope tracing provides a powerful tool to monitor the *in vivo* activity of the alternative substrate utilization relative to glucose (Table 6). Lignocellulosic hydrolysate is produced by enzymatic or thermochemical pretreatment of plant mass and contains a complex mixture of hexose (C6 sugars) and pentose (C5 sugars). Xylose is the second most abundant sugar in lignocellulosic hydrolysates besides glucose, thus efficient xylose (co-)utilization is highly demanded (Stephanopoulos, 2007). Pentose requires different enzyme series from hexose catabolism, thus understanding how some native pentose pathways operate inspires pathway design in non-native host.

Another important engineering objective is the development of conversion processes that assimilate CO_2, motivated by the goal of developing C-neutral microbes. Tracing $^{13}CO_2$ can be used to assess how much fixed CO_2 is used for bioproduction compared to glucose (Fig. 4B). Non-photosynthetic organisms cannot live on CO_2 as the only C source (autotrophy), hence CO_2 is often co-utilized with simple sugars (mixotrophy). In *Clostridium ljungdahlii*, a non-photosynthetic mixotrophy, CO_2 can be assimilated through the native Wood-Ljungdahl pathway (Jones et al., 2016). This occurs in parallel with glucose catabolism, and both processes make acetate as the final product. In this case, $^{13}CO_2$ tracing was used to confirm active CO_2 assimilation, and reveal that CO_2 has major contribution to acetate. It was further shown that co-utilization of CO_2 improves the mass yield compared to glucose alone. Besides monitoring a native pathway in mixotrophs, CO_2 assimilation has also been achieved through a non-native Calvin-Benson cycle (CBC) in *E.coli*, as revealed by ^{13}C tracing into biomass components (Antonovsky et al., 2016; Gleizer et al., 2019). Similar to CO_2, 1C chemicals such as methanol and formate are also promising renewable C sources as they can be effectively obtained by electrochemical reduction of CO_2. Therefore, monitoring 1C assimilation with isotope tracing can guide pathway design and optimization (Meyer et al., 2018; Yishai et al., 2017).

Use of stable isotopes to overcome metabolic bottlenecks

Stable isotopes can also be used to reveal pathway mechanisms and overcome metabolic bottlenecks (i.e., key reactions that limit conversion efficiency) (Fig. 4C). Production of the desired engineered chemical often involves many metabolic or transport reactions, and pathway flux is often limited by suboptimal operation of one or a few enzymatic steps (Fig. 4C). Pre-steady-state isotope tracing, such as kinetic profiling or nonstationary metabolic flux analysis, is often applied to resolve metabolic flux when steady-state labeling is not informative, such as CO_2 tracing in autotrophic organisms (McAtee et al., 2015; Young, 2014). The core concept is simple: all else being equal, fast labeling means high flux. By following labeling kinetics through a pathway, one could potentially identify where flux is blocked. An important caveat is that labeling into large pools is slow, and therefore it is important to figure out if apparent bottlenecks are due to actual flux impairment or just large metabolite pool size. Despite this complication, the approach has proven valuable for metabolic engineering. For example, kinetic $^{13}CO_2$ labeling in wild type cyanobacteria reveals an incomplete tricarboxylic acid (TCA) cycle, with a gap between oxoglutarate and succinate. The gap could be bridged by heterologous expression of an ethylene forming enzyme that also converts oxoglutarate to succinate, effectively redirecting the native metabolic flux (Xiong, Morgan, et al., 2015). Kinetic ^{13}C labeling has also been applied to reveal potential metabolic channeling, where metabolites are transferred between enzymes without mixing with the bulk pool, and pulse ^{13}C labeling can also be used to reveal shift in pathway activity in the course of fermentation (Abernathy et al., 2019). For example, in an engineered strain that produces α-Ionone, the production is driven by glucose in the early phase of fermentation and shifts to re-assimilation of an overflow metabolite, mevalonate, in the later phase (Czajka et al., 2020).

We have focused our conversion efficiency section on C stable isotopes. However, isotopes other than C have also been used to enhance the conversion efficiency of microbes. For example, deuterium isotopes are useful to reveal the metabolic sources of the key reductive cofactors, NADH and NADPH (Chen et al., 2019; Fan et al., 2014). Deuterium tracers are also well suited to quantify reaction reversibility, and thereby thermodynamics. For metabolic pathways to run efficiently, it is desirable for most steps to be moderately (e.g. 2 – 10 kJ/mol) thermodynamically forward driven. Smaller driving force may translate into high enzyme requirements and hence the cost of sustaining the desired forward flux. Canonical glycolysis contains several steps operating near thermodynamic equilibrium, but this is tolerated because those steps involve intrinsically very fast enzymes, while the Entner–Doudoroff pathway is a more thermodynamically favored glucose catabolism branch (Jacobson et al., 2019; Park et al., 2019). Larger driving force disadvantageously wastes energy, and isotope tracers can be used to diagnose engineered pathway energetics and thereby optimize them.

Cross-disciplinary research across environmental sustainability, feedstock development and biological conversion

The grand challenge of achieving a sustainable bioenergy economy will only be possible with a shift towards an integrative framework that encompasses simultaneous advances to enhance the environmental sustainability of bioenergy crops while developing highly productive, resilient and resource-efficient feedstock crops and optimizing biological conversion processes (Fig. 1). We have shown that stable isotopes contribute feedstock development in the provision of plant-generated products, whereas new systems biology and synthetic biology approaches draw from isotopic tracing to inform developments in conversion efficiency. Further, stable isotope methods in biological conversion guide feedstock engineering targeting high-value molecules and inform land requirements for bioenergy deployment based on the assessments of bioconversion efficiencies of plant biomass into biofuels and bioproducts, and subsequent environmental evaluation (Fig. 1). Finally, stable isotopes provide invaluable insight into the bioenergy interactions with the environment, identify critical tradeoffs and guide new avenues of research in feedstock development and conversion designs (Fig. 1).

Outlook and future directions

Stable isotopes have generated crucial discoveries urgently needed for a sustainable bioeconomy. They have been used to generate mechanistic understanding to evaluate the role of bioenergy crops as climate mitigation strategies, to assess the potential impacts of a large-scale deployment in the use of our already scarce natural resources (i.e., nutrients, water) and to identify research priorities that guide the development of a sustainable bioenergy and bioproducts portfolio. These powerful tools have also facilitated the development of optimized crops and specialty bio-products, for instance by engineering C allocation patterns to produce energy-dense lipids or increasing resource use efficiency at the plant level. Stable isotope tracing has also found broad applications in numerous non-model organisms and has inspired pathway design to enhance the microbial metabolic conversion process.

Challenges remain in the path toward a sustainable bioeconomy. There is an urgent need for *in situ* and undisturbed determination of the regulatory mechanisms behind the impacts of changes in land use (feedstock and management) and climate on process rates (e.g. gross production, BNF), flux partition (e.g. evaporation vs transpiration, nitrification vs denitrification) and the fate of key elements in sustainability research (C, N and water). With continuous high-frequency technology and improved instrumentation becoming more available, stable isotopes are deemed to continue playing a prominent role in the development of a sustainable bioenergy portfolio. Our current interpretation of the primary metabolism of plants is still constrained by technical difficulties and future advances using a combination of stable isotopes and other techniques will expedite the development of higher-yielding bioenergy crops. Future research using stable isotopes will allow rapidly refining engineering strategies to re-route C in bioenergy crops to produce higher-value bioenergy products (e.g. TAG) and higher-nutrient and water use efficient crops. As more high-throughput engineering tools are made available, we envision a rapid development for isotope tracing techniques adapted for high-throughput analysis, thus accelerating the DBTL cycle. Finally, the advances that can be made for a sustainable bioeconomy by integrating the use of stable isotopes within a cross-disciplinary environmental sustainability, feedstock development and microbial conversion framework are auspicious and deserve further attention.

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Table 1. Bioenergy studies where stable C isotopes were used to address how changes in land cover and/or management practices affect C cycling, stabilization and persistence in soils and ecosystems. Soil priming refers to a change in decomposition of soil organic carbon (SOC) in response to fresh C inputs; soil microbial carbon use efficiency (CUE) refers to the amount of organic C allocated to microbial biomass production relative to C consumed and reflects the balance of anabolic and catabolic reactions.

Crop	**Gained knowledge in bioenergy related with the use of stable C isotopes combined with other methods**	**References**
Miscanthus and switchgrass	Switchgrass contributed a greater proportion of SOC in the root zone than *Miscanthus*.	(Xu et al., 2022)
Miscanthus	Microbial CUE decreased with fertilization and was negatively correlated with root C concentration.	(Kane et al., 2022)
Switchgrass	Increased bulk SOC over ten years was positively associated with switchgrass-derived C across a marginally-productive landscape.	(Zegada-Lizarazu et al., 2022)
Sorghum	Sorghum increased total SOC stocks in two years under both dry and wet conditions under free-air CO_2 enrichment (FACE).	(Leavitt et al., 2022)
Miscanthus	*Miscanthus*-derived SOC stocks increased as a function of time for at least 20 years following establishment.	(Leifeld et al., 2021)
Miscanthus	Micro-scale SOC dynamics beneath *Miscanthus* are controlled by slow aggregate turnover, but aggregate turnover is faster on the soil surface compared to deeper soil.	(Vergara Sosa et al., 2021)
Corn, cover crops, fertilized and unfertilized prairie	Adding cover crops to cropping systems or partial conversion of annual grain crops to perennial systems partially promote slow-C pool sequestration relative to conventional annual bioenergy systems but decrease fast-cycling SOC pools.	(Ye & Hall, 2020)
Switchgrass	Deep-rooted grass cultivars can increase SOC relative to conventional crops in as little as 10 years, while expanding energy biomass production on marginal lands.	(Slessarev et al., 2020)
Switchgrass	Extracellular polysaccharides played a prominent role in SOC stabilization.	(Sher et al., 2020)

Pennisetum purpureum (Elephant grass)	Biochar increased SOC stocks relative to control in coarse textured soils.	(Silveira et al., 2020)
NA	Biochar amendments enhance soil microbial CUE compared to straw-amended soils.	(Liu et al., 2020)
Sugarcane	Priming of soil organic carbon induced by sugarcane residues and sugarcane biochar regulated the source of N for plant uptake.	(Weng et al., 2020)
Sorghum	The belowground interaction of amended substrate and sorghum genotype influenced sorghum rhizosphere-associated exudates.	(Miller et al., 2019)
Miscanthus and willow	Crop type determined the abundance and structure of soil communities as well as which soil resources (root exudates, dead organic matter or microbial derived compounds) were preferentially consumed.	(Briones et al., 2019)
Sorghum bicolor	Sorghum root-derived C had higher biochemical recalcitrance which led to more C retention in soil than leaf residues. Root residues resulted in higher particulate organic matter and lower C persistent mineral associated organic matter than leaf residues.	(Fulton-Smith & Cotrufo, 2019)
Populus	Belowground C inputs contribute the most to soil C accumulation compared to aboveground C inputs. These findings are important in the context of bioenergy crops as aboveground C inputs are often removed for bioenergy production.	(Berhongaray et al., 2019b)
Miscanthus, willow	Following conversion, the sequestration of soil C under genotypes of willow and Miscanthus is substantial, and fresh C derived from cropping also enhances deep soil C accumulation.	(Gregory et al., 2018)
Cover crop in a maize plantation	Cover crop belowground C inputs contributed the most to soil C accumulation compared to aboveground C inputs, and C derived cover crop root was most abundant in the mineral-associated fraction, deemed to be the most persistent fraction in soil.	(Austin et al., 2017b)
Miscanthus and willow	Crop type determined the dominance of ectomycorrhizal fungi vs bacteria, which in turn affected soil CO_2 fluxes.	(Elias et al., 2017)

Miscanthus	Fresh C inputs do not enhance SOC accumulation after 7 years of *Miscanthus* growth, and this phenomenon is not explained by soil priming accelerating the decomposition of old SOC.	(Robertson et al., 2017)
Switchgrass ecotypes	Variation between switchgrass ecotypes could alter microbial communities (i.e. bacteria vs fungi) and impact C sequestration and storage as well as potentially other belowground processes.	(Roosendaal et al., 2016)
Miscanthus, switchgrass, semi-perennial crops (fescue and alfalfa), annual crop (sorghum and tricale)	New SOC accumulation was higher under semi-perennials than under perennial crops due to variations in C input from crops rather than decreases in mineralization rates. N fertilization applied during the establishment and maturity phases did not impact SOC accumulation.	(Ferchaud et al., 2016)
Switchgrass cultivars	Initial (< 3 years) soil C losses due to land conversion were quickly offset by the accumulation of newly C from switchgrass. Cultivars with the highest SOC stocks tended to have the highest C concentrations in the persistent mineral soil fraction rather than in the particulate organic matter fraction.	(Adkins et al., 2016)
Switchgrass	The ratio of root exudate C inputs to total soil C influences priming effect. The impact of changes in exudate C inputs on the priming of SOC differs in shallow versus deep soil.	(de Graaff et al., 2014)
Land conversion to several *Miscanthus* hybrids and *Miscanthus x giganteus*	SOC stocks do not change significantly after 6 years of Miscanthus growth, but greater initial SOC decreases were observed in genotypes with higher belowground productivity likely due to enhanced priming effect.	(Zatta et al., 2014)
Maize and soybean	Maize and soybean showed different daily patterns of net ecosystem exchange, gross photosynthesis, and respiration using isotope flux partitioning.	(Fassbinder et al., 2012)
Miscanthus × *giganteus, Cryptomeria japonica*	The C input and recalcitrance of *Miscanthus* × *giganteus* (a fast-growing grass) litter was higher relative to *Cryptomeria japonica* (a fast-growing conifer) suggesting longer persistence of C under the studied grass system compared to the forest plantation.	(Toma et al., 2012)

Miscanthus x giganteus	A study of the priming effect; decomposition of old SOC was induced only when fresh C exceeded C content in soil microbes	(Blagodatskaya et al., 2011)
Switchgrass	After 5 years of cropping, SOC stocks in low C soils increased substantially due to newly C derived from switchgrass	(Collins et al., 2010)
Maize	Plant activity regulated root rhizosphere respiration.	(Rochette et al., 1999)

Table 2. Strategies to reduce soil N_2O emissions. Both tracer and natural abundance stable isotopes are used to partition the many microbial and abiotic processes involved in N_2O production and consumption in soil, which is necessary due to the differing controls on these processes.

	Strategies to reduce soil N_2O emissions	**References**
Management practices	Using alternative fertilizers, designed to release N gradually during periods of plant growth	(Shoji et al., 2001)
	Enhancing the synchrony between N applications and crop N demands	(Venterea et al., 2012)
	Optimizing the physical placement of fertilizer	(Nkebiwe et al., 2016)
	Inhibiting soil N transformations with chemical additions or with precision application of fertilizer	(Paustian et al., 2016)
	Using soil amendments including biochar and basalt	(Blanc-Betes et al., 2020; Gomez-Casanovas et al., 2021)
	Using species with root exudates containing biological nitrification inhibition compounds (BNIs)	(Luo et al., 2018)
Bioengineering and microbial inoculants	Manipulating plant-microbe interactions including BNI enabled species	(Calvo et al., 2016; Subbarao et al., 2021; Usyskin-Tonne et al., 2019)

Table 3. Summary of water use efficiency (efficiency in the use of water; WUE) metrics. The definition, methodology and description of each metric are also shown. Methodological approaches to measure water cycle related processes include biometric (i.e., productivity and yield), gas exchange and eddy covariance (EC) methods, and water stable isotopes (Annex 1).

Metric	Definition	Methodology	Description
Productivity or Yield, WUE_T	Aboveground productivity or yield: T	Biometric; stable isotopes	Emphasizes agronomic efficiency Reflects the consumption of water through plants
Canopy or Ecosystem, WUE_T	C exchange: T	Gas exchange; EC; stable isotopes	Emphasizes environmental efficiency Reflects the consumption of water through plants
Ecosystem WUE_{ET}	Gross Primary Productivity or Net Ecosystem Production: ET	EC	Emphasizes environmental efficiency Reflects the consumption of water through both plants and soil evaporation
Productivity or Yield, WUE_{ET}	Aboveground productivity or yield: ET	Biometric; EC	Emphasizes agronomic efficiency Reflects the consumption of water through both plants and soil evaporation
Biome WUE_{ET} (BWUE)	Net Ecosystem Production – Harvest: ET	Biometric; EC	Emphasizes environmental efficiency Reflects the consumption of water through both plants and soil evaporation

Table 4. Strategies to enhance the transpiration (T) to evaporation (ET) ratio by genetic crop improvement and by adopting practices that reduce E and divert more water into T. These strategies are not mutually exclusive, and their implementation to enhance water use efficiency (WUE) could be combined. T fluxes reflect the consumption of water through plants, and it is associated with productivity, whereas E does not contribute to it. Agrivoltaics refers to the novel strategy of collocating solar panels in agricultural land for the simultaneous production of energy and food.

	Strategy	**Potential desired impact**	**References**
Management practices	Crop residue management, mulching, row spacing	Reduce the amount of E	(Farooq et al., 2019; Hatfield & Dold, 2019)
	Soil fertility	Enhance the resilience of ecosystems to drought	(Farooq et al., 2019; Kantola et al., 2022)
	Transformative strategies such as Agrivoltaics	Enhance WUE and decrease ET Tighter regulation of water if rainfall collected from the panels is routed to irrigated areas under or adjacent to the panels	(Gomez-Casanovas et al., 2021)
Genetic crop improvement of plant traits	Canopy size, leaf orientation morphology	Reduce the amount of E	(Condon, 2020)
	Root architecture and deeper root length	With deeper roots, plants can extract subsurface water available. With certain root architectures, the resistance to water movement decreases from soil to root (e.g. longer root hairs) and within the roots (e.g. larger xylem diameter).	(Condon, 2020; York et al., 2022)

Table 5. Stable isotope methods for assessing biological N fixation (BNF) in bioenergy crops, rationale, benefits and limitations

Method	Benefits	Limitations in bioenergy crops
^{15}N isotope dilution Alters the isotopic signature of soil inorganic N (enriching ^{15}N) to allow source partitioning. N derived from the atmosphere (as $^{14}N_2$) will "dilute" out the ^{15}N signal from the soil derived N.	Allows soil N sources to be distinguished from atmospheric N sources Allows long-term studies	Difficult to evenly incorporate label Label must be incorporated into all soil sampled by roots Isotope incorporation is especially challenging for established deep rooting perennial crops Challenging to identify reference plants that do not benefit from N_2 fixation Variance is large
^{15}N natural abundance Uses the different isotopic signatures of soil N sources (naturally ^{15}N enriched) and atmospheric N sources to allow source partitioning. N derived from the atmosphere (as $^{14}N_2$) will "dilute" out the ^{15}N signal from the soil derived N.	Allows soil N sources to be distinguished from atmospheric N sources Does not require incorporation of ^{15}N label. Allows long-term studies	Challenging to identify reference plants that do not benefit from N_2 fixation. Variance is large, making it challenging to identify and measure N fixation
^{15}N-enriched dinitrogen gas Alters the isotopic signature of atmospheric N_2 (enriching ^{15}N) to allow source partitioning. Plants or soil benefiting from N fixation will be enriched in ^{15}N.	Direct measurement of N inputs from atmospheric N allow ready assessment of BNF inputs	Limited to short term incubations Challenging to deploy in the field Expense of $^{15}N_2$-enriched gas Limitations to plant size Requires use of a gas-tight chamber

Table 6. Use of stable isotopes to establish native or engineered pathways for alternative C source utilization.

Microbe	Method	Discovery	Relevance in advancing science in the area of conversion	Reference
Clostridium acetobutylicum and cyanobacteria	Tracing [1-^{13}C] xylose or [1,2-$^{13}C_2$] xylose	Phosphoketolase pathway has major contribution to pentose sugar catabolism in addition to pentose phosphate pathway	Facilitated understanding of the native sugar catabolic pathway	(Aristilde et al., 2015; Xiong, Lee, et al., 2015)
Engineered *Saccharomyces cerevisiae*	Tracing ^{13}C-labeled acetate into ethanol	Engineered acetate reduction pathway is active	Confirmed flux through (engineered) pathway during substrate co-feeding	(Wei et al., 2013)
Clostridium ljungdahlii	Tracing $^{13}CO_2$ into acetate	CO_2 assimilation occurs in mixotrophy	Confirmed flux through (engineered) pathway during substrate co-feeding	(Jones et al., 2016)
Escherichia coli	Tracing $^{13}CO_2$, ^{13}C-methanol or ^{13}C-formate into biomass or central metabolic pathway intermediates	Engineered CO_2, methanol or formate assimilation pathway operates in a non-C-fixation host	Confirmed flux through (engineered) pathway during substrate co-feeding	(Antonovsky et al., 2016; Gleizer et al., 2019; Meyer et al., 2018; Yishai et al., 2017)

Figure 1. Challenges and cross-disciplinary research actions across environmental sustainability, feedstock development and microbial conversion for advances in bioenergy research. Numbers link to specific challenges across environmental sustainability, feedstock development and microbial conversion; and, arrows indicate cross-disciplinary research actions that integrate the use of stable isotopes.

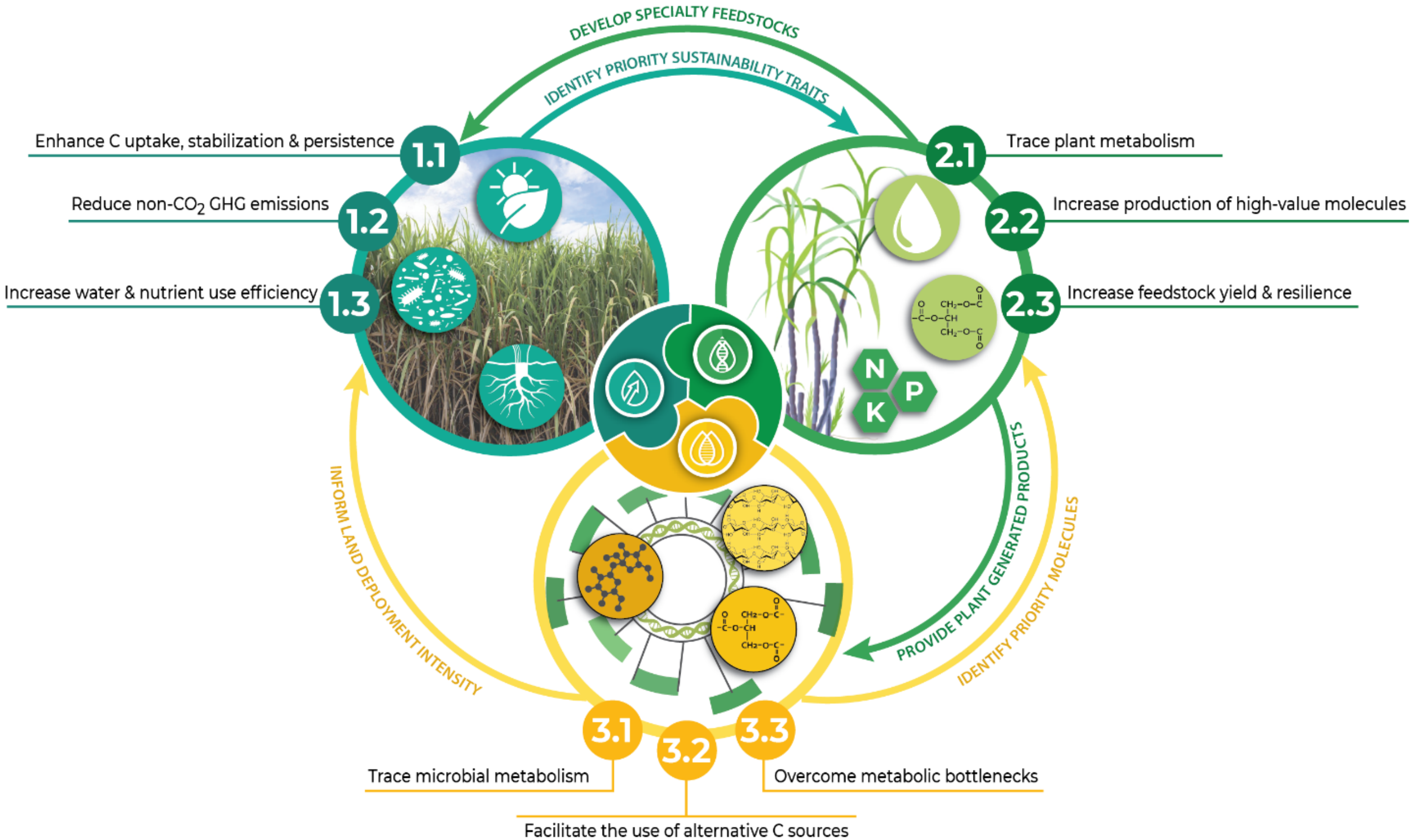
Figure 2. Use of stable isotopes to track the fate of elements (C, N, and water) across the soil-plant-atmosphere continuum, and to determine process rates, flux partition and function. Both natural abundance isotope (green) and enriched isotope (purple) methods used to identify, estimate and determine key processes and properties are shown. Numbers link to specific key processes and properties that determine the environmental sustainability of ecosystems. Rh refers to heterotrophic respiration, and Ra,s and Ra,m to autotrophic structural and maintenance respiration, respectively.

Figure 3. Assimilation of CO_2 via the Calvin-Benson-Bassham (CBB) cycle into sucrose, starch or TAG. Core reactions for these processes are outlined and the metabolic shunt for bypassing pyruvate kinase is shown in blue. Abbreviations: 3PGA, 3-phosphoglycerate; G6P, D-glucose-6-phosphate; ME, malic enzyme; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; RuBP, ribulose 1,5-bisphosphate; TP, triose phosphates.

Figure 4. Isotope tracing in microbial metabolic engineering. (a) Isotope tracing reveals internal fluxes. A classic example is that [1,2-$^{13}C_2$] glucose produces different isotope labeling in pyruvate, depending on whether it is metabolized through glycolysis or pentose phosphate pathway. (b) Isotope tracing reveals alternative carbon source utilization. For example, in mixotrophs, $^{13}CO_2$ tracing can reveal relatively how much CO_2 is fixed into a product like acetate. (c) Dynamic isotope tracing reveals metabolic bottlenecks. Abbreviations: G6P, glucose-6-phosphate; Ru5P,

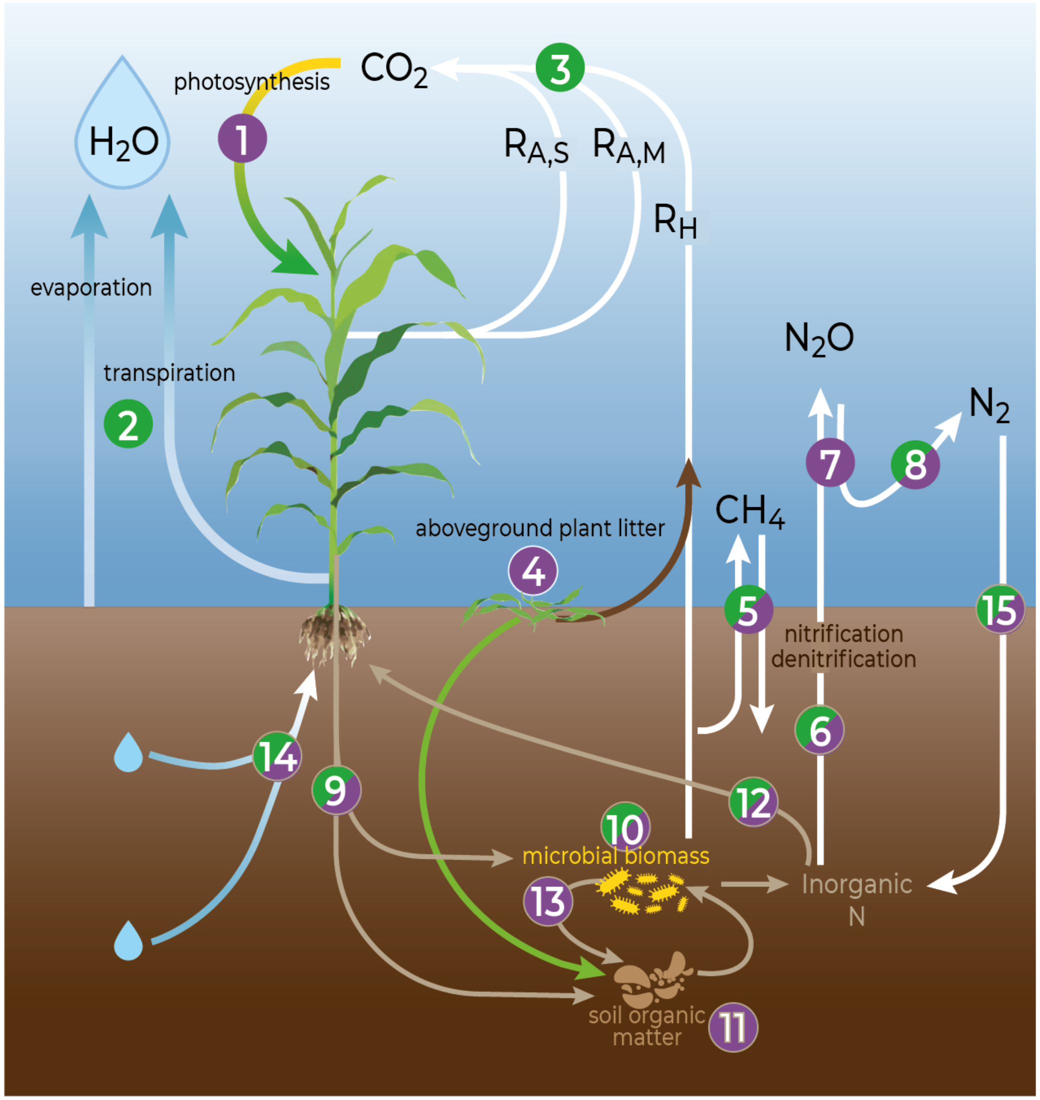
ribulose-5-phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; JPPP, pentose phosphate pathway metabolic flux; Jglycolisis, glycolysis metabolic flux; J glucose, glucose metabolic flux; JCO_2, CO_2 metabolic flux; WLP, Wood-Ljungdahl pathway.

.**Figure 1.**



DEVELOP SPECIALTY FEEDSTOCKS
IDENTIFY PRIORITY SUSTAINABILITY TRAITS
1.1
Enhance C uptake, stabilization & persistence
1.2
Reduce non-CO_2 GHG emissions
1.3
Increase water & nutrient use efficiency
2.1
Trace plant metabolism
2.2
Increase production of high-value molecules
2.3
Increase feedstock yield & resilience
N
P
K
PROVIDE PLANT GENERATED PRODUCTS
IDENTIFY PRIORITY MOLECULES
INFORM LAND DEPLOYMENT INTENSITY
3.1
Trace microbial metabolism
3.2
Facilitate the use of alternative C sources
3.3
Overcome metabolic bottlenecks

Figure 2



Natural abundance   Enriched

1. Determine fate of photosynthate
2. Partition evapotranspiration
3. Partition respiration sources
4. Determine fate of aboveground plant litter inputs
5. Estimate gross production and oxidation of CH_4, partition methanogenic pathways and determine CH_4 emission pathways
6. Partition N_2O production pathways
7. Estimate gross N_2O production and consumption rates
8. Estimate N_2O reduction rates
9. Determine fate of belowground soil C and N inputs
10. Identify microbial composition and assess function
11. Estimate soil C turnover rates
12. Determine fate of soil N
13. Determine fate of microbial necromass
14. Determine soil water sources
15. Estimate biological N fixation rates and partition soil versus atmospheric N sources

Figure 3

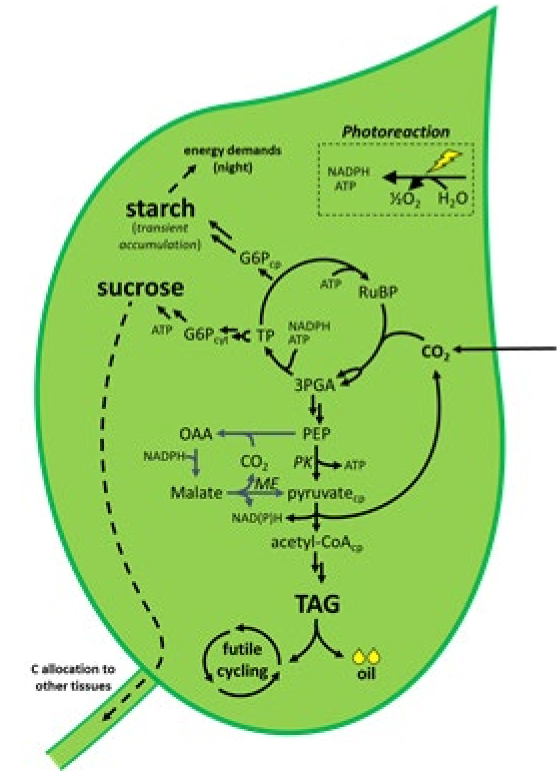
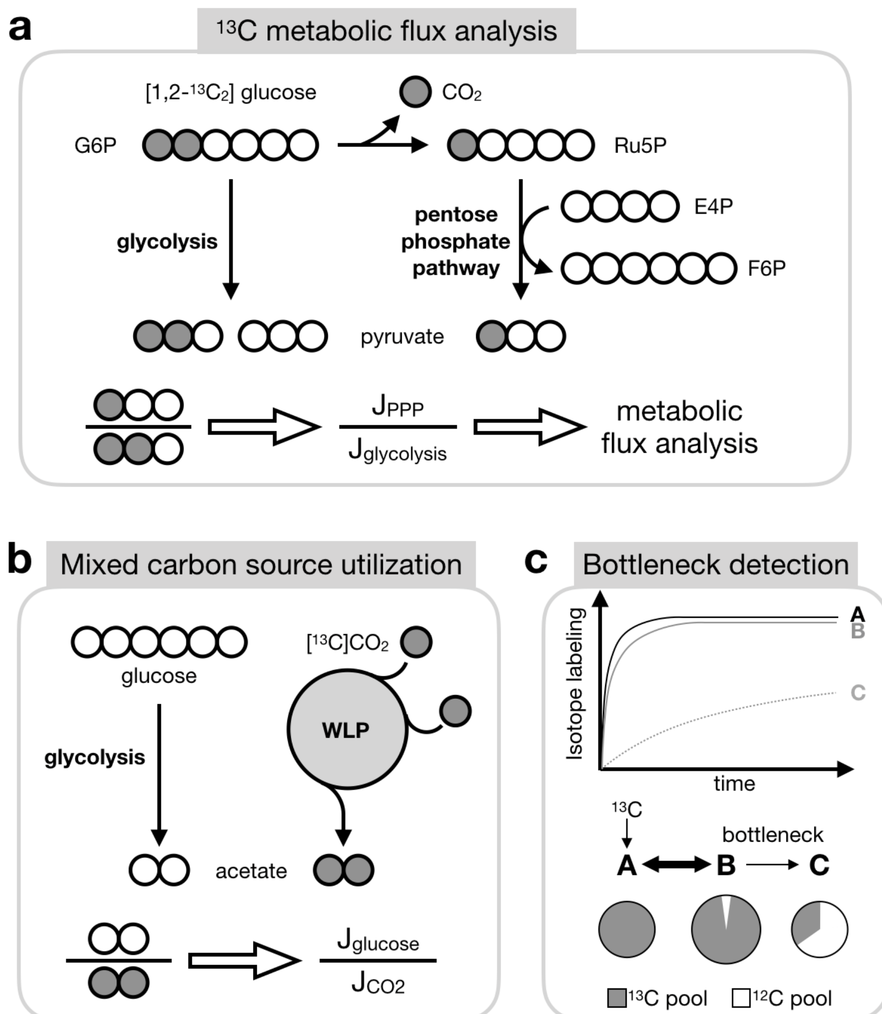

Photoreaction
NADPH
ATP
½O_2
H_2O
energy demands
(night)
starch
(transient
accumulation)
$G6P_{cp}$
sucrose
ATP
RuBP
ATP
$G6P_{cyt}$
TP
NADPH
ATP
CO_2
3PGA
OAA
PEP
NADPH
CO_2
PK
ATP
Malate
ME
$pyruvate_{cp}$
NAD(P)H
$acetyl\text{-}CoA_{cp}$
TAG
futile
cycling
oil
C allocation to
other tissues

Figure 4



a
13C metabolic flux analysis
[1,2-13C2] glucose
CO2
G6P
Ru5P
E4P
F6P
glycolysis
pentose phosphate pathway
pyruvate
JPPP
Jglycolysis
metabolic flux analysis
b
Mixed carbon source utilization
[13C]CO2
glucose
WLP
glycolysis
acetate
Jglucose
JCO2
c
Bottleneck detection
Isotope labeling
time
A
B
C
13C
bottleneck
A
B
C
13C pool
12C pool

Box 1. Differences in atomic mass, physical and chemical properties of substances containing different stable isotopes (e.g. $^{12}CO_2$ and $^{13}CO_2$) result in different reaction kinetics and thermodynamic properties. Changes in the partitioning of heavy versus light isotopes result in a process called fractionation, which varies the isotopic composition of compounds involved in such processes. The heavy-to-light stable isotope abundance ratio of different elements vary naturally within the plant, and across the plant-soil-atmosphere continuum (i.e. natural abundance experiments); or, alternatively, a labeled substrate can be traced through organisms (enrichment experiments) or a labeled product pool can be diluted by ongoing reactions at natural isotopic abundances (pool dilution experiments). Measured isotope ratios are reported in δ notation (‰) relative to the international standard Vienna Pee Dee Belemnite from known international standards (Brüggemann et al. 2011).

Box 2. Stable isotope probing (SIP) is a method where isotopically labeled C, N, or water substrates are traced into microbial biomarkers, such as phospholipid fatty acids (PLFAs) or nucleic acids (DNA and RNA), to provide targeted information on the active microbial community (Radajewski et al. 2000). However, the issue of cross-feeding, where isotope-labeled microbes or their exometabolites are consumed by other microbes, is a substantial challenge in SIP studies (Manefield et al. 2007). This limitation has recently been overcome by flow-through SIP, where microbes are continuously fed an isotopically labeled substrate in a flowing medium (Mooshammer et al. 2021). In ^{13}C-CO_2 pulse labeling experiments, ^{13}C-PLFA profiles can be used to distinguish bacterial and fungal functional groups, providing broad taxonomic characterization of the microbial community. Nucleic acid SIP provides more detailed taxonomic resolution, particularly in $H_2{}^{18}O$-SIP where heavy water is assimilated by the entire microbial community. Quantitative stable isotope probing (qSIP) is an advance in ^{18}O-water SIP where the sample is divided into multiple density fractions and compared to the density gradient of an unlabeled control to directly link density to isotope tracer assimilation, and to account for the effects of guanine-cytosine content on density (Hungate et al. 2015). qSIP also allows for estimation of microbial growth and death rates (Schwartz et al. 2016). Another quantitative improvement in nucleic acid SIP is to divide the sample into multiple density fractions. Then, samples are compared to the density gradient of an unlabeled control to account for the effects of guanine-cytosine content on density. (Pett-Ridge and Firestone 2017) outlined other integrations of SIP with omics and mass spectrometry imaging techniques to characterize plant-soil-microbial interactions.

Box 3. While the livestock sector and the cultivation of rice are major sources of anthropogenic CH_4, bioenergy crop production may have a substantial impact on the CH_4 budget. The expansion of bioenergy crops in temperate, semi-arid and arid climates will likely help offset global CH_4 emissions as agricultural land in mesic and xeric systems are usually net sinks of CH_4, particularly as soils are fertilized (Toma et al. 2011; Drewer et al. 2012; Gauder et al. 2012; Oertel et al. 2016; Mi et al. 2018). However, with growing interest in using marginal land, which also includes temporarily and sporadic waterlogged areas, for growing dedicated bioenergy crops to avoid land competition with food crops, CH_4 emissions from these crops could increase substantially as these fluxes are strongly regulated by soil wetness (Pancaldi and Trindade 2020; Khanna et al. 2021; Bates et al. 2021). Another important source of CH_4 may come from the cultivation of bioenergy crops in the Subtropics and Tropics, which is on the rise (Gomez-Casanovas et al. 2018). Despite a currently modest contribution to US bioethanol production (~6.4%), the subtropical Southeastern US has the capacity to produce more than 10 billion Ga. of advanced biofuels annually, nearly a third of the 36 billion Ga. target established by the Renewable Fuel Standard, Energy Independence and Security Act. Subtropical and tropical bioenergy crops such as canes can be strong CH_4 sources during wetter times of the year (Weier 1999; Denmead et al. 2010; Lisboa et al. 2011), indicating that the expansion of canes in this region will likely reshape the subtropical US bioenergy landscape with uncertain consequences on the CH_4 budget.

Box 4. As an outstanding case, TAG levels above 30 % (w/dw) were achieved in leaves of transgenic lines of tobacco (*Nicotiana tabacum*), which resulted from an effort of iterative improvement of lines by testing combinations of factors to be expressed (Vanhercke et al. 2017). Upon further characterization of one of the best performing lines, it was found that a substantial shift from starch biosynthesis to triacylglycerol (TAG) accumulation took place (Chu et al. 2020; 2021). Of importance for the ability of tobacco leaves to accumulate TAG seems to be that, despite the often-observed pronounced transient diurnal starch accumulation, wild-type tobacco leaves accumulate starch non-transiently at substantial levels (Chu et al. 2020; 2021). Furthermore, INST-MFA showed that, in the transgenic line, about 75 % of chloroplast pyruvate destined to TAG synthesis is generated by malic enzyme and 25 % by pyruvate kinase (Chu et al. 2021). This means that, as shown in Figure 3, a metabolic shunt bypasses pyruvate kinase via malic enzyme. Whether this bypass is of more general significance for the engineering of high TAG levels in other species remains to be further determined.

Box 5. Genome-scale metabolic models, which map genes to metabolic reactions and specify metabolite stoichiometry, have been reconstructed now for many microbes (O'Brien et al. 2015). These models can be constrained by "flux balance" (i.e., the sum of production fluxes have to equal the sum of consumption fluxes for every metabolite), a natural consequence of the law of conservation of matter combined with steady-state assumption. Together with model stoichiometry, such Flux Balance Analysis (FBA) can identify flux distributions that are optimal for meeting goals, which could include growth or production of a desired engineered products (Orth et al. 2010; O'Brien et al. 2015). However, FBA *per se*, does not reliably measure actual fluxes. This requires additional experimental constraints, beyond the stoichiometry of the metabolic network. Some of the most valuable constraints are overall nutrient uptake and product excretion rates. These often substantially constrain the feasible fluxes, but do not reliably reveal metabolic partitioning at internal branch points, like between glycolysis and the pentose phosphate pathway. To reliably obtain actual flux distributions, isotope tracers are needed. The most common tracer is C-13, in the form of ^{13}C Metabolic Flux Analysis (^{13}C MFA) (Wiechert 2001; McAtee et al. 2015).

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