NMR studies of metabolites and xenobiotics: From time-points to long-term metabolic regulation

Ina Ehlers
To my family
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Abstract

Chemical species carry information in two dimensions, in their concentrations and their isotopic signatures. The concentrations of metabolites or synthetic compounds describe the composition of a chemical or biological system, while isotopic signatures describe processes in the system by their reaction pathways, regulation, and responses to external stimuli. Stable isotopes are unique tracers of these processes because their natural abundances are modulated by isotope effects occurring in physical processes as well as in chemical reactions. Nuclear magnetic resonance (NMR) spectroscopy is a prime technique not only for identification and quantification of small molecules in complex systems but also for measuring intramolecular distribution of stable isotopes in metabolites and other small molecules. In this thesis, we use quantitative NMR in three fields: in food science, environmental pollutant tracing, and plant-climate science.

The phospholipid (PL) composition of food samples is of high interest because of their nutritional value and technological properties. However, the analysis of PLs is difficult as they constitute only a small fraction of the total lipid contents in foods. Here, we developed a method to identify PLs and determine their composition in food samples, by combining a liquid-liquid extraction approach for enriching PLs, with specialized $^{31}$P,${}^1$H-COSY NMR experiments to identify and quantify PLs.

Wide-spread pollution with synthetic compounds threatens the environment and human health. However, the fate of pollutants in the environment is often poorly understood. Using quantitative deuterium NMR spectroscopy, we showed for the nitrosamine NDMA and the pesticide DDT how intramolecular distributions (isotopomer patterns) of the heavy hydrogen isotope deuterium reveal mechanistic insight into transformation pathways of pollutants and organic compounds in general. Intramolecular isotope distributions can be used to trace a pollutant’s origin, to understand its environmental transformation pathways and to evaluate remediation approaches.

The atmospheric CO$_2$ concentration ([CO$_2$]) is currently rising at an unprecedented rate and plant responses to this increase in [CO$_2$] influence the global carbon cycle and will determine future plant productivity. To investigate long-term plant responses, we developed a method to elucidate metabolic fluxes from intramolecular deuterium distributions of metabolites that can be extracted from historic plant material. We show that the intramolecular deuterium distribution of plant glucose depends on growth [CO$_2$] and reflects the magnitude of photorespiration, an important side reaction of photosynthesis. In historic plant samples, we observe that photorespiration decreased in annual crop plants and natural vegetation over the past century, with no observable acclimation, implying that photosynthesis increased. In tree-ring samples from all continents covering the past 60 – 700 years, we detected a significantly
smaller decrease in photorespiration than expected. We conclude that the expected “CO₂ fertilization” has occurred but was significantly less pronounced in trees, due to opposing effects.

The presented applications show that intramolecular isotope distributions not only provide information about the origin and turnover of compounds but also about metabolic regulation. By extracting isotope distributions from archives of plant material, metabolic information can be obtained retrospectively, which allows studies over decades to millennia, timescales that are inaccessible with manipulation experiments.
List of Publications

I. Two-Dimensional \(^{31}\text{P},^{1}\text{H}\) NMR Spectroscopic Profiling of Phospholipids in Cheese and Fish.

Stefanie Kaffarnik, Ina Ehlers, Gerhard Gröbner, Jürgen Schleucher & Walter Vetter.


II. Elucidating turnover pathways of bioactive small molecules by isotopomer analysis: The Persistent Organic Pollutant DDT


III. Compound-Specific Carbon, Nitrogen, and Hydrogen Isotope Analysis of \(N\)-Nitrosodimethylamine (NDMA) in Aqueous Solutions

Stephanie Spahr, Jakov Bolotin, Jürgen Schleucher, Ina Ehlers, Urs von Gunten & Thomas B. Hofstetter.

*Submitted manuscript.*

IV. The 20\(^{th}\)-century \(\text{CO}_2\) rise has shifted metabolic fluxes in \(C_3\) plants towards increased photosynthesis.

Ina Ehlers, Angela Augusti, Tatiana R. Betson, Mats B. Nilsson & Jürgen Schleucher.

*Submitted manuscript.*

V. Limited suppression of photorespiration by 20\(^{th}\) century atmospheric \(\text{CO}_2\) increase in trees worldwide.

Ina Ehlers, Iris Köhler, Thomas Wieloch, Mart Vlam, Peter van der Sleen, Peter Groenendijk, Michael Grabner, Andrea Seim, Kathryn Allen, Liang Wei, Iain Robertson, John Marshall, Pieter A. Zuidema, Jürgen Schleucher.

*Manuscript.*
Abbreviations

A Rate of CO\(_2\) assimilation

[CO\(_2\)] CO\(_2\) concentration

C\(_a\) Atmospheric CO\(_2\) concentration

CAM Crassulacean acid metabolism

C\(_c\) CO\(_2\) concentration at the site of carboxylation

CCM CO\(_2\) concentrating mechanisms

C\(_i\) CO\(_2\) concentration in the intercellular leaf spaces

CSIA Compound-specific isotope analysis

D Deuterium

D\(_6^R/D6^S\) Abundance ratio of the D\(_6^R\) and D\(_6^S\) isotopomers – the two isotopomers of the C\(_6\)H\(_2\) group of glucose

DDD 1-Chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene

DDE 1,1-Bis-(4-chlorophenyl)-2,2-dichloroethene

DDT 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane

DiMePE Dimethylphosphatidylethanolamine

G3P Glyceraldehyde 3-phosphate

\(g_m\) Mesophyll conductance

GPP Gross primary productivity

\(g_s\) Stomatal conductance

IRMS Isotope ratio mass spectrometry

iWUE Intrinsic water use efficiency

\(J_{H,H}\) J-coupling between protons

\(J_{H,P}\) J-coupling between \(^1\)H and \(^{31}\)P

KIE Kinetic isotope effect

LLE liquid-liquid extraction

LPC Lysophosphatidylcholine

NDMA N-Nitrosodimethylamine

NMR Nuclear magnetic resonance spectroscopy

NPP Net primary productivity

PC Phosphatidylcholine

PE Phosphatidylethanolamine

Pg C Billion tons of carbon
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PGA</td>
<td>Phosphoglycerate</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>R</td>
<td>Isotope ratio</td>
</tr>
<tr>
<td>Rubisco</td>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>RuBP</td>
<td>Ribulose-1,5-bisphosphate</td>
</tr>
<tr>
<td>VSMOV</td>
<td>Vienna Standard Mean Ocean Water</td>
</tr>
<tr>
<td>Δ</td>
<td>Isotope discrimination</td>
</tr>
<tr>
<td>δ</td>
<td>Deviation of the isotope ratio from a reference in ‰</td>
</tr>
</tbody>
</table>
Introduction

Central to biological and environmental science is the question how systems function and respond to external influences. The presence, concentration and isotopic composition of metabolites in these systems reveal unique information about system processes on a molecular level. From measuring concentrations to determining fluxes and metabolic regulation, the functional understanding increases but at the same time it becomes more difficult to measure these parameters.

Today, much research focuses on fully characterizing the states of complex systems, especially in the recently emerging ‘omics’ fields, like metabolomics and fluxomics. Nuclear magnetic resonance (NMR) spectroscopy is a versatile technique used in such analytical applications, but also for studies of molecular structures, dynamics and interactions. Here, we exploit the fact that integrals of NMR signals strictly reflect the molar ratios of compounds, independent of their structure. This allows for accurate quantification of metabolites in complex systems. In publication I of this thesis, we develop a method to profile the phospholipid composition in food samples using NMR for identification and quantification of phospholipids.

Stable isotopes occur naturally in all compounds, and they can be enriched and introduced into a system as isotope-labeled tracer. For example, by monitoring the turnover of stable-isotope-labeled metabolites, metabolic fluxes can be elucidated, and metabolic pathways and their responses to external stimuli can be studied.

Besides in labeling applications, stable isotopes can be studied at natural abundance. Stable isotopes are ubiquitously incorporated into all chemical and biological systems, their abundances are modulated by isotope effects in physical processes and chemical reactions, and therefore they are unique tracers of a molecule’s origin and transformations. The advantage of natural abundance studies is the possibility to access dimensions not accessible by manipulative experiments: In long-lived metabolites, a long-term record of the physiological state of a system is stored, which allows studies on long time scales and in unperturbed systems. Observed isotope abundance changes can even be linked to specific fractionation reactions, if stable isotopes are studied on the intramolecular level. Chemical and biochemical reactions fractionate against stable isotopes in specific intramolecular positions, which creates an isotopic fingerprint of a molecule’s history. Variation in intramolecular isotope abundances reveals mechanistic information and allows deducing turnover pathways and metabolic regulation. NMR is the only practicable method for the measurement of intramolecular isotope distributions in metabolite-sized molecules. Publications II and III demonstrate how NMR-based intramolecular isotope measurements allow the elucidation of the turnover pathways of xenobiotics, which is essential to understand their fate in the environment. Finally, in manuscripts IV and V we demonstrate that intramolecular isotope distributions of archives of plant metabolites contain centennial records of metabolic
Introduction

changes in plants. Extracting these records reveals how metabolism of crops and of natural vegetation on the global scale have been affected by increasing atmospheric CO$_2$ concentrations.
Stable isotopes

Chemical elements can have several isotopes that differ in the number of neutrons in the nucleus. As a result isotopes of the same element have different atomic masses. In nature, isotopes occur with certain natural abundances, as shown for some elements in Table 1. Hydrogen for instance has two stable isotopes, $^1$H and $^2$H; $^2$H contains a neutron and a proton in the nucleus and is called deuterium (D). Many elements have isotopes that are radioactive (e.g. $^{14}$C), but here we are concerned exclusively with different stable isotopes.

The isotope abundances given in Table 1 denote average abundances, while the abundances in different materials or compartments can vary significantly because isotope fractionation occurring in chemical and physical processes alters these abundances. This is also the basis for the production of enriched stable isotopes. The isotope composition of a compound reports on the compound’s history, which is exploited both in isotope labelling studies and in natural abundance studies. In isotope labelling experiments a reactant is enriched in a stable isotope and the label is traced, e. g. through a metabolic pathway (Sauer 2006). Natural abundance studies, on the other hand, exploit that in the natural isotope composition of compounds information is continuously and non-invasively stored (Schmidt 2003) that can be extracted from unperturbed system, like intact ecosystems.

The rare heavy isotopes of hydrogen, carbon, nitrogen, and oxygen are most commonly used as tracers to investigate processes in environmental chemistry, plant ecophysiology, biogeochemistry, medicine and many other fields.

<table>
<thead>
<tr>
<th>Element</th>
<th>Abundance (%)</th>
<th>Light isotope</th>
<th>Heavy isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen</td>
<td>$^1$H 99.985</td>
<td>$^2$H 0.015</td>
<td></td>
</tr>
<tr>
<td>Carbon</td>
<td>$^{12}$C 98.89</td>
<td>$^{13}$C 1.11</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>$^{14}$N 99.63</td>
<td>$^{15}$N 0.37</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>$^{16}$O 99.76</td>
<td>$^{17}$O 0.04; $^{18}$O 0.20</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Natural abundance of stable isotopes commonly used in biogeochemistry.

For each element, the isotope abundance in a compound is described by an isotope ratio, the abundance of its heavy isotope (H) relative to the abundance of its light isotope (L):

$$R = H/L$$

However, as variations in the isotope ratios often are small, the isotopic composition of a sample is usually stated as deviation of its isotope ratio ($R_s$) from the ratio of a reference compound ($R_{ref}$), expressed as $\delta$ value in ‰ (Wolfsberg et al. 2010):

$$\delta(‰) = (R_s/R_{ref} - 1) \times 1000$$
Stable isotopes

The reference substance for carbon is “Vienna Pee Dee Belemite” (VPDB), for hydrogen and oxygen it is “Vienna Standard Mean Ocean Water” (VSMOW) and these reference materials are distributed by the International Atomic Energy Agency in Vienna. Conventionally, stable isotope ratios are measured by isotope ratio mass spectrometry (IRMS), which allows very sensitive measurements of isotope ratios of whole molecules. Laser-based techniques that have become available more recently allow isotope ratio measurements of gases like CO₂ in the field. While intramolecular isotope distributions of select molecules (e.g. N₂O) can be measured by IRMS, NMR spectroscopy is the only practicable technique to determine intramolecular ¹³C or D distributions of metabolite-sized molecules.

Isotope Fractionation

Molecules with different isotopic composition (isotopologues) differ in their physical and chemical properties, which is referred to as isotope effects and causes isotope fractionation, a partitioning of isotopes between substances. Most fractionations are mass dependent, the different masses of the isotopes affect atomic motions and thereby the strength of chemical bonds. The fractionation of a process can be described by the fractionation factor α, the ratio of the isotope ratios of the compounds (A and B) involved: \( \alpha = \frac{R_A}{R_B} \).

Isotope effects are primarily caused by differences in molecular vibrations. The vibrational frequencies depend on the masses of the atoms and on the forces in a molecule. These forces depend on the electronic structure, the nuclear charges and the molecular structure, which are all nearly independent of isotopic substitution, while the masses differ for isotopologues. The vibrational frequencies are inversely dependent on the mass and, thus, the higher mass of heavy isotopologues causes lower vibrational frequencies, which results in lower ground state energies compared to light isotopologues. This energetic difference is the fundamental basis of isotope fractionation (Bigeleisen 1965).

For light elements isotope fractionation is particularly strong because the relative mass differences of the nuclei are larger than for heavy elements. For hydrogen, the mass doubles between protium and deuterium and as a result deuterium isotope effect are much larger compared to e. g. carbon isotope effects.

Equilibrium fractionation: In thermodynamic or chemical equilibrium, fractionation can take place between different phases or substances of a system. Heavy stable isotopes become enriched in the state, in which the element is most strongly bound, and an isotope partitioning between phases or substances occurs, driven by differing ground state energies. Once an equilibrium state is reached no net reactions occur and the isotope compositions are constant but differ.

An example of equilibrium fractionations are the hydrogen and oxygen fractionations that occur if water undergoes phase transitions. HDO and H₂¹⁸O have lower vapor pressures than H₂O and therefore H₂O evaporates preferentially, while HDO and H₂¹⁸O accumulate in the remaining water. As a result, water vapor is
always depleted in $^2$H and $^{18}$O compared to water. This is observed in the global hydrological cycle: Compared to ocean water, atmospheric water vapor is D depleted. If precipitation occurs, HDO precipitates preferentially and the D depletion in the remaining vapor systematically increases. As a result, fresh water is always depleted in D and $^{18}$O compared to ocean water, and the depletion increases in higher latitudes, the lowest values are observed for snow at the South Pole with $-450$ ‰ in $\delta$D and $-50$ ‰ in $\delta^{18}$O. Thus, the isotope composition of precipitation is temperature dependent and from ice sheets, which preserve a record of the $\delta$D of precipitation, temperatures can be reconstructed for the past several hundred thousand years. For instance, ice cores show that during the last glacial maximum $\delta$D was $-50$ ‰ more negative than present precipitation, which indicates a 10 K lower temperature (Wolfsberg et al. 2010).

**Kinetic isotope effects (KIE):** In chemical reactions, kinetic isotope effects (KIE) occur if the reaction rates of light and heavy isotopologues differ. The presence of different isotopes in a reactant affects the zero point energy of the reactants and the energy of the transition state. Heavy isotopes are more strongly bound than light isotopes, but as bonds are normally weakened in the transition state, this difference becomes less pronounced. Therefore, the zero point energy difference of the transition state of light and heavy isotopologues is smaller than in the reactants and light isotopologues have lower activation energy barriers. As a result, isotopic-substitution affects the rate at which molecules react. The kinetic isotope effect is the ratio of the reaction rate constants of the light and heavy isotopologues:

$$\text{KIE} = \frac{k_L}{k_H}.$$  

In the case of a normal kinetic isotope effect (KIE > 1), molecules carrying the light isotope react faster and as long as the reaction is incomplete, the remaining substrate is enriched in the heavy isotope, while the product is depleted. In rare cases the reaction rate of the heavy isotopologue can be higher, resulting in KIEs smaller than 1 and such KIEs are called “inverse” (Melander & Saunders 1980).

Kinetic isotope effects are strongest for reactions in which a bond to a heavy isotope is formed or broken in the rate-limiting step of a reaction, a so-called primary isotope effect. However, the presence of a heavy isotope can alter the reaction rate even if it is not directly involved in the reaction but one or two bonds away from the reacting bond. Such secondary isotope effects are generally smaller than primary isotope effects.

The magnitude of KIEs depends on the mechanism of a reaction and its transition state structure and, thus, KIEs are studied to elucidate chemical and biochemical reaction mechanisms. KIEs do not always cause isotope fractionations, for example metabolites only experience fractionation if the KIEs occur in reactions that have incomplete turnover or are near a branching point in a metabolic pathway, so that isotopes can be fractionated either between substrate and product, or between different products. Most important, comparing the KIE with the actual fractionation
gives information on a biochemical reaction or on the relation between alternative pathways.

**Isotopomers**

KIEs fractionate against heavy isotopes in particular intramolecular groups, for example a reacting C-H group. Therefore, stable isotopes are not randomly distributed among the positions of a compound but each intramolecular position has an individual isotope abundance. For example, DeNiro and Epstein already discovered in 1977 that the oxidation of pyruvate yields acetyl CoA that is depleted in $^{13}$C in the carbonyl carbon compared to the methyl carbon (DeNiro & Epstein, 1977). A molecule carrying an isotope in a specific position is called an isotopomer. Isotopomers are isotopic isomers – they have the same number of each isotope but in different positions. Because of the low natural abundances of deuterium, the probability of having two deuterium atoms in a low-molecular-weight molecule is extremely low and only mono-substituted isotopomers are considered here. Glucose has seven deuterium isotopomers: each of the seven carbon-bound hydrogens can be substituted by deuterium (Fig. 1), whereas the hydrogens in the hydroxyl groups form no stable isotopomers because of their fast hydrogen exchange with the environment.

![Figure 1. D3 isotopomer of glucose: The hydrogen bound to C3 is substituted by D (marked in bold). Each of the carbon-bound hydrogens can be substituted by D. The prochiral C6H2 group has two isotopomers, because the biochemically distinct H^6R or H^6S positions can each carry a D.](image)

While physical isotope fractionations are not site-specific, but modify the isotope abundances of whole molecules, KIEs affect individual isotopomer abundances. In biosynthetic pathways isotopomer patterns are created by two mechanisms: First, KIEs affect isotopomers of reacting positions, and second, newly introduced molecular fragments differ in their isotope composition depending on their origin. Therefore isotopomers are sensitive to alternative formation pathways and metabolic regulation within these (Schleucher et al. 1999, Schmidt 2003, Tenailleau et al. 2004, Zhang et al. 2002). In whole-molecule isotope ratios these position-specific
abundance differences are diluted out and can even cancel each other out, therefore it is difficult to link δ values to individual reaction steps.

Today, isotopomer analysis is only used in few fields, for example in food science to detect food adulterations of wines, juices and flavors, and in atmospheric science to constrain atmospheric fluxes of the greenhouse gas N₂O (Yoshida & Toyoda 2000). But with the advance of analytical techniques and instrumentation, isotopomer distributions may be used to address new questions and in new fields. Generally, isotopomer analysis can be applied to study reaction mechanisms, origin and fate of compounds, and metabolic pathways.
Phospholipids

Lipids are a major class of biological molecules with diverse chemical structures that are characterized by the hydrophobic nature of their most relevant building blocks, i.e. fatty acids. Phospholipids (PLs) have an additional polar headgroup, which makes PLs amphiphilic and results in unique properties.

**Biological Role:** PLs are a major constituent of biological membranes: Because of their amphiphilic character PLs spontaneously form lipid bilayers. The hydrophobic tails of the PLs face each other in the center of the bilayers and the polar headgroups are oriented towards the aqueous phase. PLs form membranes that function as barriers that are impermeable to water and allow cells to separate the cytosol form the external environment and to form organelles. Besides being barriers, cell membranes have important functions in nutrient transport and signaling. The composition of a biological membrane determines its biophysical properties, its structure and function (Yeagle *et al.* 2005, Van Meer *et al.* 2008). Membranes are made up of a large number of diverse lipids and in addition contain proteins and carbohydrates. The PL headgroups determine the properties of the hydrophilic surface of a membrane, and thereby influence for example binding of ions, nutrients and proteins, which is important for biological interactions. The hydrophobic tails of the PLs are composed of fatty acids, whose chain length and degree of saturation or substitution determine membrane viscosity and permeability. The curvature of membranes, which is important for processes like cell division and budding, depends on the PL composition. Furthermore, specific PLs can for example act as second messengers (Yeagle *et al.* 2005) or regulate gene expression (Wymann *et al.* 2008).

Diseases, aging and nutritional supply affect the PL composition of cellular membranes, as has been observed for diabetes mellitus and chronic alcohol consumption (Bengmark 1998).

**Nutritional value and technological properties:** PL uptake by humans through nutrition or as drugs affects human health. Externally supplied PLs are incorporated into cell membranes and can for example prevent or mitigate inflammation and ulceration of the GI tract (Bengmark 1998, Treede *et al.* 2007). In the food industry PLs are used as dietary supplements and as food additives because of their nutritional value and their technological properties. Compared to neutral lipids PLs and especially marine PLs usually have a higher content of polyunsaturated fatty acids (PUFAs) (Henna Lu *et al.* 2011). Dairy products contain relatively high amounts of sphingosine PLs, which are involved in neurotransmitting activities (Rombaut *et al.* 2006). Technologically, PLs are interesting because of their unique amphiphilic properties. They are used as emulsifiers and for the production of liposomes. These liposomes are used to carry food ingredients, for protection or to
control the release of functional components, like enzymes or vitamins, and thus allow the design functional foods (Henna Lu et al. 2011). In pharmaceutical technology, PLs are used to make microemulsions, which act as drug delivery vehicles (Kogan et al. 2006).

![Phospholipid structures](image)

**Figure 2.** Phospholipid structures. A Phosphoglycerolipids: general structure with common headgroups and backbone modifications. B Sphingomyelin as an example of sphingophospholipids.

**Structure:** The two major PL classes are phosphoglycerolipids and sphingophospholipids. Phosphoglycerolipids have a sn-glycerol-3-phosphate backbone that is esterified with two fatty acids in the sn1 and sn2 position of the glycerol moiety yielding 1,2-diacyl-sn-glycero-3-phosphoric acid (Fig. 2A). In naturally occurring phosphoglycerolipids the fatty acids usually have chain lengths between C14 and C22 and the chains can be saturated or unsaturated with up to six double bonds in mostly cis configuration. Unsaturated acyl chains are often located in the sn2 position of the glycerol. Phospholipases hydrolyze fatty acids from the glycerol backbone releasing a fatty acid and a PL that carries only one acyl chain and one free hydroxyl group, referred to as lyso phospholipids. Other modifications are ether, or vinyl-ether chains instead of fatty acids, leading to ether and plasmalogen PLs, respectively. Phosphoglycerolipids are classified according to their headgroups, which consist of alcohols like choline, ethanolamine, serine,
Phospholipids

glycerol or inositol, which are esterified with the phosphate group. Phosphatidylcholine is the most common constituent in animal cell membranes, while bacterial cell membranes have a high phosphatidylethanolamine content (Yeagle et al. 2005).

Sphingophospholipids have a sphingosine backbone (2-amino-4-octadecene-1,3-diol) – an aliphatic amine carrying two hydroxyl groups – and the primary hydroxyl group is esterified with phosphoric acid. The most common PL of this class in humans is sphingomyelin in which a fatty acid is attached to C-2 in form of an amide and the phosphate moiety is carrying a choline headgroup (Fig. 2B).

The complexity of lipid structures and insufficient analytical techniques made lipid research difficult in the past and lagging behind compared to for example proteomics. However, with advances in lipid separation techniques, and lipid analysis by mass spectrometry and NMR spectroscopy, the field of ‘lipidomics’ made great progress over the past decade (Wenk 2005). In publication I we developed a method to profile the phospholipid content in food samples based on a liquid-liquid extraction technique combined with advanced two-dimensional NMR spectroscopy.
Contaminants in the Environment

Worldwide, environmental pollution with chemicals impacts vegetation, wildlife, and humans. After large contaminations in the past decades, public awareness has grown and the use of many chemicals has been banned while the regulations for use have generally increased. But pollution continues and new chemicals with unknown impact on the environment are continuously developed. Today, contaminants can be found anywhere on Earth, in the soil, water and atmosphere. Pesticides are a major source of pollutants, but their intended use for protection of crops from weeds, diseases, and pests and their wide spread application since the 1950s has resulted in great benefits in agricultural production. Annually pesticides are used on a million ton scale, which is expected to increase in the future but has severe effects on the environment (Tilman et al. 2001).

Persistent organic pollutants

Among the most critical environmental contaminants are persistent organic pollutants (POPs). These chemicals are particularly harmful because of their persistency, which together with mobility and toxicity can cause unexpected effects. Most of the POPs are polyhalogenated compounds that are very slowly degraded and as a result have long half-lives of at least 6 months and often much longer in the environment. Today POPs are ubiquitous on earth, even in remote areas, from the polar regions to the Tibetan Plateau and the open oceans – regions without past anthropogenic POP emissions (Sheng et al. 2013). From the source of pollution in urban and agricultural areas, POPs get distributed mostly through air fluxes and water. POPs are semi-volatile and evaporate after application e.g. from plants and soil into the air or adsorb on airborne particles and are transported through the atmosphere over long distances, returning to earth with precipitation or still attached to particles as dust. Even though POPs have low solubilities in water, they are transported, solved or bound to particles, through rivers, lakes, and oceans and are present in groundwater and aquatic sediments. Because of their persistence and their lipophilic nature POPs accumulate in fatty tissues and biomagnify in the top predators of marine and terrestrial food chains. Particularly high concentrations are found in high trophic level biota such as fish, predatory birds and mammals including humans (Ritter et al. 1995).

POPs have severe effects on wildlife and human health. Exposure to high POP amounts through accidents or unsafe handling can lead to acute toxic effects. But more problematic are the chronic effects caused by long-term exposure to low concentrations. Some POPs act as endocrine disrupters and exposure is especially harmful during fetal or egg development causing developmental problems and reproductive failure (Vos et al. 2000). In wildlife, this has caused population declines in many species, especially in predatory birds and marine mammals like seals, dolphins, minks, and beluga whales that have high POP uptake through their
Contaminants in the Environment

diet. Other effects often observed are immune and nervous system toxicity and the promotion of cancer.

POPs have been used in agriculture, industry, and disease vector control. The Stockholm Convention has banned the use of the “dirty dozen” in 2001. Included on this list of POPs are nine chloropesticides like aldrin, chlordane, DDT, and toxaphene; industrial chemicals like polychlorinated biphenyls (PCBs) that were widely used as dielectric fluids or as flame retardants, and substances that are unintentionally produced as by-products like polychlorinated dibenzo-p-dioxins and furans. This ban by the Stockholm Convention has been successful in reducing POP concentrations in the biosphere. Today the main sources of the banned POPs are old stocks and highly contaminated waste sites but also previous POP sinks like the oceans, sediments or glaciers that turned into sources and now return POPs into circulation (Geisz et al. 2008, Stemmler & Lammel 2009). Since their ban, POPs have been replaced with new chemicals that are better biodegradable. Changes in pesticide regulations aim to approve chemicals only if their degradability has been proven in experiments. However, even substances that are not persistent, can impact environment and health. Pharmaceuticals for example are increasingly found in natural waters: the drug oxazepam may cause behavioral changes in fish (Brodin et al. 2013) and degradation products of the drug ranitidine are found as toxic contaminants in drinking water (Krasner et al. 2013).

The insecticide DDT

The organochlorine compound DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) was the first purposely synthesized pesticide, its insecticidal properties were discovered in 1939. DDT does not occur naturally and exists as two main isomers that differ in the chlorine substitution pattern on the aromatic moieties. Technical DDT contains both main isomers, ~80% p,p’-DDT (Fig. 3) and ~15% o,p’-DDT; and also 4% DDD (the dichlorinated DDT congener 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene), and other byproducts.

Figure 3. Structure of p,p’-DDT and its degradation products p,p’-DDD and p,p’-DDE.
During World War II DDT was used in disease vector control to fight malaria and typhus transmitted by mosquitoes and leeches. Since then its successful application in disease control saved the lives of millions of people. In the 1950, several DDT programs were initiated with the goal of eradicating malaria. These were successful in parts of the world but failed in others, one reason being the development of DDT resistance in mosquitoes. While disease control required only relatively small amounts of DDT, the extensive application of DDT as pesticide in agriculture also started in the 1950s. On a large scale, crops were sprayed with DDT and agricultural use alone amounted to 2.6 million tons between 1950 and the mid-1990s (Li et al. 2005). This large scale application resulted in two major problems: successes in disease control were threatened, sometimes even reversed by the development of DDT resistance in mosquitoes, and DDT had detrimental effects on the environment.

In insects, DDT targets neuronal sodium ion channels, which causes neuronal dysfunction and leads to death. Mosquitos developed resistance to DDT through mutations in the target sodium channels or by increasing DDT detoxification to prevent DDT from reaching its target. Over-transcription of the gene Cyp6g1 of the cytochrome P450 family alone results in resistance to DDT and several other insecticides (Daborn et al. 2012). This mutation has been observed in mosquito strains world-wide.

DDT was classified as a POP because it has a half-life of 10-15 years in soil after application. Its first transformation products (Fig. 3), DDD and 1,1-bis-(4-chlorophenyl)-2,2-dichloroethene (DDE), are themselves both highly persistent in the environment. In fact, many of the environmental problems associated with DDT use are caused by p,p′-DDE. High p,p′-DDE concentrations in the environment caused wide-spread egg shell thinning (Vos et al. 2000) in North American and European birds, like the bald eagle and osprey, which resulted in severe population declines. DDT is an endocrine disruptor; in alligators at the contamination site at Lake Apopka in Florida juvenile alligators showed severe developmental problems and numbers declined by 90% in the years following a pesticide spill (Guilette et al. 1994).

In humans high serum DDE levels have been associated with diseases like Alzheimer’s (Robertson et al. 2014), and hypertension (Lind et al. 2014) and DDT and its degradation products are associated with diabetes, several types of cancer and neurodevelopmental problems in children (Ezkenazi et al. 2009; Turosov et al. 2002).

Since the first bans in the 1970’s, DDT use in agriculture is now banned worldwide. But in contrast to other POPs, it is still used in disease vector control, which is highly controversial, but recommended by the WHO in combination with other actions (WHO statement 2013). Today, annual DDT use is 3-4 thousand tons, mostly used for indoor residual spraying to fight malaria, dengue fever and leishmaniasis in India and South African countries.
**N-Nitrosodimethylamine formed during water disinfection**

Clean drinking water is a valuable resource and water contamination causes severe human health problems. Therefore, drinking water has to meet high quality standards and water taken from surface waters, springs or wells, which is often contaminated with soil particles, pathogens and chemicals – raw drinking water in industrial countries often contains 10 - 20 pollutants at higher than acceptable concentrations (Fenner *et al.* 2013) – has to be treated to meet these standards. One class of chemicals often still found in drinking water after treatment are nitrosamines. Nitrosamines in drinking water can originate from industrial processes and are found as by-product in pesticides but nitrosamines are also formed during water treatment itself (Krasner *et al.* 2013). One step in drinking and waste water treatment is disinfection, often disinfecting chemicals are added to the water to kill pathogens. During water disinfection with chloramine or chlorine, *N*-nitrosamines are often formed as by-products (Sharma 2012), e. g. from the reaction of chloramine with the pharmaceutical ranitidine, which is often present in waters (Fig. 4).

![Figure 4. NDMA formation during water disinfection with chloramine from the commonly found micropollutant ranitidine.](image)

The most commonly found *N*-nitrosodimethylamine (NDMA) is quickly degraded by light within hours. But in the absence of light as in the case of groundwater, NDMA is persistent with a half-life of up to one year. Furthermore, NDMA is toxic even at very low concentrations and NDMA removal from water is possible but expensive. In animals, NDMA taken up through drinking water is hepatotoxic, mutagenic and a carcinogen. NDMA is metabolized to form methylidiazonium ions that methylate DNA to yield O\(^{6}\)-methylguanine adducts, which are responsible for NDMA’s mutagenicity (WHO Background document 2008). NDMA can be formed from a variety of organic nitrogen containing compounds present in water, ranging from natural products to synthetic compounds like pharmaceuticals and pesticides. Depending on the precursor the yields vary and exact pathways and mechanisms of NDMA formation during water disinfection are unknown.

**Pollutant turnover in the environment and how to track it**

Understanding pollutant turnover in the environment is essential to assess continuous detrimental effects from past pollution, to predict environmental impact of chemicals applied at present and to make informed decisions in approval of new chemicals.
Fate in the environment: Once released into the environment, the only way to clear synthetic chemicals is to degrade them into naturally occurring metabolites. Generally, two types of transformation processes are distinguished: biotic and abiotic transformations. In biotic transformations, POPs are degraded by microorganisms or plants, while abiotic degradation occurs through chemical reactions, like hydrolysis, reduction, oxidation or photolytic transformations. The degradation processes that occur depend on the structure of the pollutant and the environmental conditions like pH, temperature and soil type. POPs are persistent in the environment, many are organohalogen compounds, containing carbon-halogen bonds, which are resistant to hydrolysis and an increasing degree of halogenation protects the compounds from biotic degradation. Biotic degradation is the most important pathway for POP degradation. In microbes, degradation can occur through promiscuous enzymes that catalyze the turnover of toxic chemicals. This serves either as detoxification process for toxic compounds or as nutrient source for carbon, nitrogen or phosphorus (Copley 2009). While detoxification is usually simpler requiring just some modifications of the pollutant, it yields transformation products not naturally occurring. In contrast, mineralization completely breaks down the chemicals to common metabolites but requires multistep transformation pathways. Transformation products can be of great concern as well, because they can be highly toxic themselves while they are often more mobile and thus can be transported and reach environmental compartments inaccessible to the parent compounds (Fenner et al. 2013). Therefore, transformation products need to be considered when evaluating the environmental impact of chemicals.

Tracking pollutant turnover: Degradation processes in the environment are complex as several degradation pathways often interact and contribute in varying amounts depending on environmental conditions. From laboratory studies it is thus difficult to predict environmental degradation processes.

By chemical analysis, identities and concentrations of pollutants and their degradation products can be monitored, for example using GC- or LC-MS. However, concentration measurements do not easily reveal origins, and if concentration differences in space or time are due to transport or transformation processes, careful mass-balance calculations have to be carried out.

Stable isotope signatures of pollutants carry additional information, which can be obtained by compound-specific isotope analysis (CSIA). Thus, a pollutant can be linked to its original source or producer, provided that no fractionation due to degradation reactions occurs. Furthermore, CSIA can reveal degradation pathways and their mechanisms (Fenner et al. 2013). During chemical reactions involved in degradation processes, KIEs alter the isotope ratios of the remaining pollutant, while dilution or sorption processes usually infer no or very small isotope fractionations. Therefore, changes in isotope ratios are evidence of pollutant degradation even if no degradation products can be detected. By observing changes in isotope ratios over time or space transformation processes can be elucidated, however, if multiple
fractionations affect isotope ratios, interpretation is difficult. The strength of CSIA is further improved by multi-element approaches, which combine fractionation information from several elements, like H, C, N and O (Hofstetter & Berg 2011). CSIA has for example been used to analyze methyl tert-butyl ether (MTBE), which was used as gasoline additive and is now widely found in groundwater (Zwank et al. 2005). The analysis at a contamination site revealed C and H fractionation along a spatial gradient from the contamination source and indicated that MTBE removal occurred via anaerobic biodegradation and via evaporation.

KIEs are specific for reaction pathways and affect specific intramolecular isotope abundances, therefore, monitoring the intramolecular isotope distributions of pollutants and/or degradation products could be used to simultaneously trace the origin of a pollutant, and elucidate the extent or pathway of its biodegradation, by the following strategy: Isotope ratios of structural parts of the molecule that remain unchanged during the degradation would be indicative of its origin, while isotope ratios of reacting moieties would reveal turnover. For MTBE it has been shown that isotopomer abundances can be measured by quantitative deuterium NMR spectroscopy, but the approach has not been applied to environmental samples (McKelvie et al. 2009).

In publications II and III, we show how intramolecular isotope distributions can be used to elucidate transformation pathways and to link transformation products to their parent compounds.
Vegetation responses to rising atmospheric [CO$_2$]

The atmospheric CO$_2$ concentration ([CO$_2$]) has exceeded the threshold of 400 ppm as monthly mean in April 2014 for the first time in human history. On the geological timescale, atmospheric [CO$_2$] has varied greatly, from very high concentrations of several thousand ppm in the Paleozoic 500 million years ago, to values as low as 180 ppm during the past glaciations. However, during the past 800,000 years [CO$_2$] ranged between 180 and 300 ppm, as measured from air inclusions in ice cores (Lüthi et al. 2008, Petit et al. 1999). The current rise occurs at an alarming rate (2 ppm/year) (IPCC 2013) and has resulted in 40% higher atmospheric [CO$_2$] today compared to pre-industrial times (Fig. 5A).

![Figure 5. A. Atmospheric [CO$_2$] over the past millennium; blue - concentrations reconstructed from ice cores (Etheridge et al. 1996), red - direct measurements from Mauna Loa Observatory (http://scrippsco2.ucsd.edu/data/atmospheric_co2.html). B. Global temperature between 1880 and 2013 shown as anomalies based on 20$^{th}$ century average (http://www.ncdc.noaa.gov/cag/time-series/global).](image)

Svante Arrhenius predicted in 1896 (Arrhenius 1896) that increasing atmospheric [CO$_2$] causes global warming, but in contrast to the globally uniform [CO$_2$] rise, the increase in surface temperature is regionally diverse, on average it has increased by ~0.8 °C since 1880 (Fig. 5B) with the largest increases during the past decades (IPCC 2013). Consequences of these climate changes are enhanced desertification and increases in extreme climate events like heat waves and flooding. To 95% certainty is global warming caused by human activities (IPCC 2013) and the strongest forcing factor is the increase in atmospheric greenhouse gases, mostly in [CO$_2$]. Anthropogenic CO$_2$ emissions result primarily from fossil fuel burning and land use change and are estimated to 375 Pg C (billion tons of carbon emitted in the form of CO$_2$) and 180 Pg C, respectively, between 1750 and 2011. While the land use change contribution is slightly decreasing – due to reduced deforestation and due to regrowth – fossil fuel emissions during the last decade are higher than ever with a growth rate of 3.2%/yr. Approximately 40% of the emitted carbon has accumulated in the atmosphere, while the rest has been absorbed by two carbon sinks: the oceans
Vegetation responses to rising atmospheric \([\text{CO}_2]\) and the terrestrial ecosystems. This \(\text{CO}_2\) uptake causes a “sawtooth” seasonal cycle in atmospheric \([\text{CO}_2]\) (Keeling 1960): over the growing season in the Northern Hemisphere atmospheric \([\text{CO}_2]\) decreases while it increases during Northern Hemisphere winter. Uptake of \(\text{CO}_2\) by natural terrestrial ecosystems increases as photosynthesis increases due to higher atmospheric \([\text{CO}_2]\), nitrogen deposition, and longer growing seasons in mid-to-high latitudes. However, the complete removal of anthropogenic \(\text{CO}_2\) from the atmosphere by natural processes will take several hundred thousand years, 20-60% will persist for 1000 year or longer (Archer & Brovkin 2008).

**Carbon fixation by plants**

Plants fix \(\text{CO}_2\) from the atmosphere during photosynthesis to produce carbohydrates. Through pores in the epidermis, the stomata, \(\text{CO}_2\) diffuses into the leaves of plants and is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco, the most abundant protein on Earth, catalyzes the reaction of \(\text{CO}_2\) with ribulose-1,5-bisphosphate (RuBP) in the chloroplasts (Fig. 6). This carboxylation on carbon-2 of RuBP is the first step in the Calvin cycle and produces two molecules of 3-phosphoglycerate (3-PGA). In the next step, 3-PGA is reduced to glyceraldehyde 3-phosphate (G3P), a 3-carbon intermediate that is utilized to produce other carbohydrates. To operate the Calvin cycle as a cycle, 5 of 6 generated G3P molecules are used to regenerate the \(\text{CO}_2\) acceptor RuBP (Taiz & Zeiger 2006).

**Figure 6.** Calvin and photorespiratory cycle. The PGAs are marked according to their origin from the head (red) or tail (blue) end of RuBP or from the photorespiratory cycle (green).
Besides the carboxylation of RuBP, Rubisco also catalyzes the reaction of O$_2$ with RuBP. 3.2 billion years ago, when Rubisco evolved at a time of high atmospheric [CO$_2$] and low [O$_2$] concentration, Rubisco specificity was not an issue but under today’s atmospheric [CO$_2$] the ratio of carboxylation to oxygenation is approximately 4:1, resulting in a substantial carbon loss for the plant (Fernie et al. 2013). In the active site of Rubisco CO$_2$ and O$_2$ are competing substrates, binding of O$_2$ leads to oxygenation of RuBP, which results in the formation of only one molecule 3-PGA and one molecule 2-phosphoglycolate (Fig. 6). 2-phosphoglycolate is scavenged in the photorespiratory cycle: 2-Phosphoglycolate is hydrolyzed to glycolate and transported from the chloroplast to the peroxisome where it undergoes a series of transformations, also involving the mitochondrion, to finally - from two molecules of 2-phosphoglycolate - release one molecule CO$_2$ and recover one molecule 3-PGA. The 3-PGA is returned to the Calvin cycle, and thereby 75% of the carbon initially lost due to oxygenation is recovered.

Under high [O$_2$] / [CO$_2$] conditions up to current [CO$_2$], Rubisco’s CO$_2$/O$_2$ specificity and Rubisco’s slow catalytic rate limit Rubisco-catalyzed carboxylation, making it the rate-limiting step in photosynthesis. However, attempts to engineer improved Rubiscos have failed, as increases in specificity result in a reduction of the carboxylation rate and vice versa, indicating that Rubisco is already optimized (Tcherkez et al. 2006). Besides its role in carbon scavenging, the photorespiratory cycle can also protect plants from photodamage. Under high light, low [CO$_2$] conditions the cycle is a sink for excess reducing equivalents from the light reactions of photosynthesis, and thereby protects the photosynthetic apparatus from overreduction (Taiz & Zeiger 2006).

In C$_3$ plants, the rate of photosynthesis depends on the [CO$_2$] at Rubisco, which is much smaller than the atmospheric [CO$_2$]. Uptake of CO$_2$ into leaves occurs through the stomata, through which CO$_2$ and H$_2$O can diffuse into and out of the intercellular leaf spaces. The [CO$_2$] in the intercellular spaces, C$_i$, is reduced by approximately $\frac{1}{3}$ compared to atmospheric [CO$_2$] due to diffusion resistances; the rate of diffusion is referred to as stomatal conductance ($g_s$). The opening of stomata is regulated by the plants, to balance CO$_2$ diffusion into the leaves with water loss through transpiration. To reach Rubisco, CO$_2$ has to diffuse further through the mesophyll, through cell membranes and the cytosol, into the chloroplast stroma, where Rubisco is located. The [CO$_2$] at Rubisco, C$_c$, is even lower than C$_i$ and depends on the mesophyll conductance $g_m$. Both stomatal conductance and mesophyll conductance affect carbon-limited photosynthesis.

To avoid the carbon loss associated with the oxygenation reaction, CO$_2$ concentrating mechanisms (CCMs) have evolved in some plants. Plants with C$_4$ photosynthetic pathway have a special leaf anatomy (Krantz anatomy) that allows them to fix CO$_2$ in the mesophyll cells, utilizing the enzyme phosphoenolpyruvate (PEP) carboxylase, which has a much higher carboxylation efficiency than Rubisco. PEP carboxylase catalyzes the reaction of CO$_2$ with the three-carbon substrate phosphoenolpyruvate to form the four-carbon acids malate or aspartate, therefore the
name C₄ plans. These acids are then transported to the bundle sheath cells where they are decarboxylated again to release CO₂, which is re-fixed by Rubisco. The advantage of this mechanism is that [CO₂] locally at Rubisco is much higher than in C₃ plants, which effectively suppresses oxygenation. In plants with Crassulacean acid metabolism (CAM), the formation of C₄ intermediates is also temporally separated, as these plants open their stomata during night to generate C₄ intermediates that are first stored in vacuoles and then transported to the chloroplasts during the day to release CO₂ for photosynthesis. Plants with CCMs require lower amounts of Rubisco and loose less water due to transpiration compared to C₃ plants, but the presence of a CCM comes at an energetic cost and, therefore, plants with a CCM have a lower efficiency in light utilization. As a result, CCMs are an advantage for plants at low CO₂ concentrations or in warm climates, CAM plants cope particularly well in hot dry conditions, and this is reflected by the spread of C₄ and CAM plants mainly in tropical savannahs and grasslands. Overall they contribute approximately 20 % to global gross primary productivity (GPP) (Beer et al. 2010).

Isotope fractionation associated with photosynthesis

Over the past 200 years, the δ¹³C of CO₂ in the atmosphere has decreased due to the input of ¹³C-depleted CO₂ from fossil fuel burning, and this source signal is detected in the δ¹³C of plant material (McCarroll & Loader 2004). Besides this, plant material shows δ¹³C variability caused by plant processes. The δ¹³C depends on the photosynthetic pathway a plant uses, and historic trends in δ¹³C in response to climate variability have been observed. Plants discriminate against ¹³C during diffusion processes and in biochemical reactions during and after carbon assimilation and therefore carbon isotope ratios are sensitive to changes both in plant physiology and in environmental conditions. This carbon fractionation is defined as Δ and can be derived from the difference between source δ¹³C and plant δ¹³C.

\[ \Delta = (\delta_a - \delta_p)/(1+\delta_p) \]

Where δₐ and δₚ refer to the δ¹³C of CO₂ in the atmosphere and the δ¹³C of plant material, respectively. The fractionations that occur in C₃ plants are described by the original Farquhar model and recent additions (Farquhar et al. 1982, Cernusak et al. 2013). In the original model, Δ is approximated by considering contributions from diffusional fractionation (a ≈ 4.4 ‰) and from fractionation during carboxylation at Rubisco (b ≈ 27 ‰) and depends on the ratio of intercellular leaf [CO₂] and atmospheric [CO₂]:

\[ \Delta = a+(b-a)*C_i/C_a \]

The fractionation carries information about environmental influences that affect Cᵢ and therefore allows the reconstruction of these. Cᵢ depends on CO₂ supply and on
photosynthetic CO₂ consumption: stomatal conductance is sensitive to humidity, soil water and Cₐ, while photosynthetic activity depends on temperature, light and nutrient availability. Nevertheless, Cᵢ/Cₐ is quite stable in plants, as plants respond to changes by matching photosynthetic capacity and conductance, and with leaf area adjustments. In trees, Cᵢ/Cₐ increases under low light and declines with tree height and elevation over sea level (Cernusak et al. 2013).

The rate of CO₂ assimilation (A) depends on stomatal conductance and the [CO₂] gradient between leaf and atmosphere, as stated in Fick’s law: \( A = g_{\text{CO}_2}(C_a - C_i) \). The intrinsic water use efficiency (iWUE) of plants is an important parameter for plants’ carbon-water relation. It describes how much carbon a plant can gain via assimilation per water that is lost due to transpiration. iWUE is defined as ratio of CO₂ assimilation rate (A) to stomatal conductance for water vapor (gₗ), \( iWUE = A/gₗ \). Since \( gₗ = 1.6 \ g_{\text{CO}_2} \), iWUE can be expressed as

\[
iWUE = \frac{A}{gₗ} = \frac{(C_a - C_i)}{1.6} = \frac{ca}{1.6} \ast \left(1 - \frac{\Delta - a}{b - a}\right)
\]

Using these relations, the intrinsic iWUE can be calculated from δ¹³C of plant material if CO₂ concentration and δ¹³C of the atmosphere are known. Thus, from historic plant material, iWUE and can be reconstructed and can be correlated to climate reconstructions to study plant-water relations.

**Isotopomer pattern of photosynthetic pathways**

Fractionations caused by diffusion and carboxylation at Rubisco affect the ¹³C abundance at all intramolecular carbon positions of photosynthetic glucose equally, so they affect the whole-molecule δ¹³C. In addition, enzymatic reactions in the photosynthetic and photorespiratory pathways, as well as post-photosynthetic reactions cause kinetic isotope effects discriminating against individual isotopomers (Badeck et al. 2005). These create non-statistical isotopomer distributions in plant glucose, both in ¹³C (Rossmann et al. 1991) and in D (Martin et al. 1992).

**Figure 7.** Typical isotopomer pattern for photosynthetic glucose from plants with C₃ or C₄ photosynthetic pathway. Here for sucrose from sugar beet (black) and sugar cane (white). The isotopomer abundances are expressed relative to an average abundance of 1.
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Furthermore, it has been observed that in plant glucose the D distributions are characteristic for the photosynthetic pathway of the plant, C₃, C₄ or CAM (Zhang et al. 2002) (Fig. 7) and that different carbohydrate metabolites, like starch and sucrose, differ in their isotopomer patterns, which shows the influence of plant metabolism on these patterns (Schleucher et al. 1999). The processes causing these different patterns are not fully understood, but several observations indicate that they can be a rich source of metabolic information.

During cellulose synthesis in trees, part of the C-H positions exchange with phloem water and thereby a source signal is introduced, which should appear especially in the largely exchanging D2 isotopomer. Precipitation contains a D signature reflecting temperature, which is transferred to plants’ source water and should appear in the D2 isotopomer (Augusti et al. 2006). Thus, isotopomer abundance measurements have the power to simultaneously yield physiological and climatic signals from plant glucose.

“CO₂ fertilization” effect

Rising atmospheric [CO₂] and changing temperature and precipitation patterns impact on the natural vegetation and on crop productivity. Responses can range from the level of leaf physiology to changes in species communities. Plant growth – especially for C₃ species - should be enhanced by rising [CO₂] – referred to as “CO₂ fertilization” – but the magnitude of the effect is unknown (IPCC 2013), and might be overpowered by other climatic influences, as has been observed for historic wheat yields (Lobell et al. 2011). Climate effects are likely to differ geographically: High latitude regions are expected to profit from longer growing seasons, while warmer areas increasingly suffer from drought. The combined effects are uncertain and so are models predicting future carbon cycle, climate and crop productivity. The central question is how plants have responded and will respond to changes in atmospheric [CO₂] and temperature over timescales of decades.

Based on fundamental biochemical concepts and the kinetic properties of Rubisco, changes in atmospheric [CO₂] and temperature are expected to affect CO₂ assimilation by plants. Experimentally, the balance between assimilation and respiration of plants can be studied as function of [CO₂]. At the CO₂ compensation point, photosynthesis and respiration balance each other out, so that the plant does not gain carbon and cannot grow. At experimental [CO₂] below the compensation point, a plant will be CO₂-starved and die. Photosynthesis increases with [CO₂] and above the CO₂ compensation point plants can grow. At current atmospheric [CO₂], Rubisco is not saturated by its substrate CO₂ in C₃ plants and [CO₂] increases at Rubisco result in an increased carboxylation rate (“Rubisco-limited photosynthesis”). In addition, as CO₂ and O₂ are competing substrates for Rubisco, an increase in the [CO₂] / [O₂] ratio inhibits oxygenation and enhances carboxylation. These effects together result in a higher net photosynthetic rate. At high [CO₂], carboxylation is faster than the regeneration of RuBP in the Calvin
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cycle, which is limited by electron transport (“RuBP-limited photosynthesis”). But even if photosynthesis is RuBP limited, net photosynthesis increases under rising [CO$_2$] because the oxygenation / carboxylation ratio shifts, and thus less RuBP is wasted by oxygenation. Finally, photosynthesis can be limited by accumulation of triose phosphates, if triose phosphate production in the Calvin cycle is faster than triose phosphate utilization (e.g. for sucrose or starch production), which results in a phosphate shortage.

Thus, photosynthesis in C$_3$ plants should greatly benefit from rises in atmospheric [CO$_2$] (Franks et al. 2013), while C$_4$ plants should be much less affected as they have a CCM and their Rubisco is CO$_2$-saturated. In C$_3$ plants, a rise in atmospheric [CO$_2$] from preindustrial levels at around 280 ppm to today’s concentration of 400 ppm should have resulted in an increase of approximately 35 % in net photosynthesis (Farquhar et al. 1980) and because the oxygenation / carboxylation ratio should have shifted, net photosynthesis should have increased even if other factors are limiting, unless the effect was offset by a down-regulation of the photosynthetic apparatus.

An increase in leaf temperature increases enzyme activities until it reaches a species-dependent optimum, at which the carboxylation at Rubisco is maximal. Temperatures above this optimum result in a decrease in enzymatic rates. However, temperature also affects the oxygenation / carboxylation balance at Rubisco. The kinetic constants of oxygenation and carboxylation differ and the oxygenation rate increases more with rising temperature than the carboxylation rate. Furthermore, the equilibrium concentrations of CO$_2$ and O$_2$ in solution are temperature-dependent. At high temperature, the solution concentrations decrease, more for CO$_2$ than for O$_2$ and as a result the ratio of dissolved [CO$_2$] / [O$_2$] at the Rubisco active site decreases. These effects result in increased photorespiration relative to photosynthesis at elevated temperature. However, an overall increase in reaction rates can still result in increased net photosynthesis; and the temperature optimum for net photosynthesis increases with [CO$_2$] (Farquhar et al. 1980). Models calculating the impacts of increasing atmospheric [CO$_2$] and temperature on historic crop yields show that rising temperature reduced crop yields over the past 30 years, except in high latitudes (Lobell et al. 2011), while rising [CO$_2$] had a small contribution to overall observed yield increases. The combined climate effects are slightly positive for rice in high latitudes and soybean but negative for wheat and the C$_4$ crop maize.

[CO$_2$] manipulation experiments

First studies investigating the effect of elevated [CO$_2$] on plants in greenhouse or growth chamber experiments showed high increases of approximately 30 % in photosynthesis and plant biomass production for ambient vs. 550 ppm [CO$_2$] (Long et al. 2006). However, it is now concluded that these results may not be representative for field conditions. In free air CO$_2$ enrichment (FACE) experiments,
which most closely mimic natural conditions, the atmosphere of study plots in open fields or forests is enriched with CO₂ by releasing CO₂ through valves around the perimeter of the plots. FACE set-ups allow the study of [CO₂] effects with minimized perturbation and in ecosystems, and allow investigation of effects on the interlinked carbon, water and nitrogen cycles. Elevated [CO₂] of 550 to 600 ppm are usually chosen based on estimates for atmospheric [CO₂] in 2050. FACE results vary between sites and environmental conditions but generally an increase in photosynthetic rate is observed, ranging from 13 % for crops to 46 % for trees (Leakey et al. 2009). The low results on crops are explained as down regulation of photosynthetic capacity, linked to genetic and environmental factors. Forest FACE studies showed transient growth responses to elevated [CO₂], presumably due to the step increase in [CO₂]. After initial strong responses, aboveground growth stimulation was small or zero, likely due to nutrient limitations and increases in below ground metabolism that have been observed (Körner 2006). Furthermore, gₛ decreased at elevated [CO₂], while the effect on gₘ varied (Franks et al. 2013). The lower stomatal conductance decreased leaf transpiration and reduced canopy scale evaporation, which led to increased soil water content. Overall, short-term responses in A and gₛ are observed, but responses on long time scales are less well understood.

Based on the detailed studies of plant and even ecosystem responses to elevated [CO₂] in FACE experiments, predictions on how plants will respond to future CO₂ levels are made. At the same time the limitations of FACE results have to be considered: the elaborate set-up limits the number and size of study sites – e.g. there are no FACE experiments in tropical forests, even though they are estimated to account for at least ⅓ of global terrestrial net primary production (NPP) (Körner 2009) – and artefacts can be created by the large step increase in [CO₂] in contrast to the gradual increase in the atmosphere. Finally, as with all manipulative experiments, they cannot cover time spans of decades or centuries, but these time scales are most relevant for climate change and adaptation to its consequences.

*Plant responses to increasing atmospheric [CO₂] since industrialization*

Plant species growing today evolved in a low [CO₂] world and over thousands to millions of years adapted to changes in the atmospheric composition, for example by introducing the photorespiratory pathway or by developing CCMs. Over the past 200 years, plants have been subjected to a steep increase in CO₂; an understanding of past plant responses to this increase allows predictions about future plant-climate interactions. Acclimation responses on the decadal to century timescale are decisive for climate change and have been documented, but our knowledge on decadal or longer responses is severely limited by a lack of experimental methods. The best approach to access decade to century timescales is to study historic plant material, e.g. herbarium material, or long-lived species. Trees are valuable archives as they form tree rings (or wood can be otherwise dated) and thereby store plant material
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with annual resolution, and because of their wide spatial distribution across the Earth.

In herbarium leaf samples, collected over the past 200 years, changes in stomatal density have been observed. Stomata control the gas exchange between leaves and the environment and they can respond to environmental changes (Hetherington & Woodward 2003). In temperate tree species from England, Woodward observed a 40% decrease in stomatal density from preindustrial levels (Woodward 1987). Similar results were detected for trees, scrubs and herbs from the Mediterranean (Penuelas & Matamala 1990) and for a larger data set from the UK, which revealed an overall significant decrease in stomatal density but with variation between species (Beerling & Kelly 1997). This suggests that plants have lowered transpirational water loss by reducing their number of stomata and, hence, reducing stomatal conductance.

Another characteristic that has been widely studied in historic plant material is the intrinsic water use efficiency. Over the past four decades, iWUE increased by 20% for trees in all biomes, while \(\text{C}_i/\text{C}_a\) stayed almost constant (Penuelas et al. 2011). This shows that plants have increased photosynthetic efficiency with regard to water, thus saving water, in response to rising atmospheric \[\text{CO}_2\].

Finally, the effect of increasing atmospheric \[\text{CO}_2\] on biomass production over the past decades, “\text{CO}_2\text{ fertilization}”, has been studied by comparing widths of tree rings laid down in the past and today. This is difficult as growth varies with tree age and observations vary greatly from reduced to enhanced growth. For three of the tropical forest sites that we analyzed in manuscript V, increases in \(\text{C}_i\) and iWUE were detected, while stem growth remained constant over the past 150 years (Van der Sleen 2014). This agrees with a meta-analysis covering the past 40 years showing no significant increases in tree growth globally, even though iWUE increased (Penuelas et al. 2011). This lack of “\text{CO}_2\text{ fertilization}” was also observed for annual plants in the long-term experiments at Rothamsted, where wheat and grasslands in unfertilized control plots showed no yield increases over the past 150 years (Jenkinson et al. 1994, Zhao et al. 2001). For warm arid regions, satellite observations show increases in foliage cover over the past 30 years, and this greening is attributed to increased iWUE as water is limiting photosynthesis under warm and dry conditions (Donohue et al. 2013).

The puzzling discrepancy between increasing \(\text{C}_i\) and iWUE and lack of tree stem growth (Penuelas et al. 2011, Van der Sleen 2014) that is often observed has large implications for the role of the terrestrial biosphere as \text{CO}_2 sink. The discrepancy could be caused by changing allocation of photosynthates to plant parts other than the stem (large increases in root biomass production have been observed in forest FACE experiments, Nowak et al., 2004), by acclimation of photosynthesis, or by negative temperature effects.
Vegetation responses to rising atmospheric [CO₂]

**Figure 8.** Plant archives accessed by isotopomer analysis in manuscripts IV and V. 
A. Tree segments of an English Oak (top) and a Tasmanian Celery Top Pine (bottom). B. Herbarium samples of fireweed collected in 1949 in Jukkasjärvi and spinach collected in 1905 in Umeå. C. Sugar cubes from Germany produced in the 1950s.

**In manuscripts IV and V** of this thesis, we use deuterium isotopomer analysis of plant glucose to study the effects of increasing atmospheric [CO₂] on plant metabolism. To access the decade to century time scale we used historic plant material from different archives: historic sugar cubes, herbarium samples and tree rings (Fig. 8).
Methods

Nuclear magnetic resonance (NMR) spectroscopy is widely used in natural and life sciences because it yields information at atomic resolution, i.e. NMR signals directly correspond to individual atoms in a molecule. For structure determination, this means that NMR signals can directly be translated into conformational restraints. But most important here, it means that there is a 1:1 correspondence between distinct NMR signals and intramolecular groups with distinct reactivity, which makes NMR ideal to study chemical and biochemical reactions. Finally, hardware and software have rapidly advanced over the past decades, which has increased the possibility to design a specific experiment to address a particular question.

The resonance frequency of an atomic nucleus, its chemical shift, depends on its chemical environment and yields structural information with atomic resolution, which allows structure elucidation of organic compounds, and with the advance of two-dimensional (2D) NMR techniques, even of biomolecules like proteins, RNA and DNA. The information content and resolution in two-dimensional NMR spectroscopy is much higher compared to one-dimensional experiments because each signal is defined by two chemical shifts, which are correlated to each other by interactions of spins through bonds or through space. This yields additional information about the connectivity and distance between nuclei and allows the study of complex systems. NMR spectroscopy can be used as an analytical tool to identify and quantify components, even in complex mixtures, and is now for example employed in metabolomics. Furthermore, NMR is sensitive to molecular conformations and allows the determination of intramolecular distances and can be used to study molecular dynamics and molecular interactions, e.g. in drug discovery the interaction of drugs with target proteins. The versatility is further strengthened by the range of samples that can be analyzed: NMR can be performed on liquids and solid samples, making it possible for example to analyze whole tissues or biomolecules in intact cells under physiological conditions.

In this thesis we use quantitative NMR spectroscopy for two applications: (1) We use $^{31}$P NMR for metabolite profiling and exploit the high resolution of two-dimensional spectra to characterize complex phospholipid mixtures. (2) We use NMR for stable isotope analysis; by quantitative deuterium NMR spectroscopy we determine intramolecular deuterium distributions of metabolites and synthetic compounds, taking advantage of the 1:1 correspondence between NMR signals and intramolecular moieties.

Phospholipid analysis by $^{31}$P NMR spectroscopy

Phosphorus has only one isotope, $^{31}$P, which is NMR active and since the 1980s $^{31}$P NMR spectroscopy has been proposed as an alternative method to analyze phospholipids (PLs). In $^{31}$P NMR spectroscopy each P-containing moiety, so each
PL, gives rise to one peak in the spectrum and the characteristic $^{31}$P chemical shifts of the PLs have been used for identification of the different PL species. Because the P nucleus is highly sensitive to its environment, the $^{31}$P chemical shift of the phosphate group in PLs reflects the structure of the attached headgroup, backbone and to a smaller degree even the fatty acid composition of the PL. However, $^{31}$P spectra of PLs can have broad lines and low resolution because of the presence of paramagnetic cations. This problem has been solved by measuring lipid samples in a chloroform / methanol / 0.2 M Cs-ETDA$_{aq}$ (10:4:1, v:v) two-phase solvent system that removes paramagnetic cations from the organic lipid phase (Meneses & Glonek 1988), so that narrow lines of less than 1 Hz and high resolution of individual PL peaks can be achieved. $^{31}$P NMR is especially suitable for PL profiling because it is applicable to complex PL mixtures, which reduces sample preparation steps and thereby avoids biases that may be introduced during separation and purification. Furthermore, PLs can be precisely quantified and the non-destructive nature of NMR allows recovery of lipid samples after measurement. However, the chemical shift dispersion in one-dimensional $^{31}$P NMR is very small, as PL signals are typically crowded in the chemical shift range between 1 and -1 ppm, and therefore individual PL peaks often overlap. This is especially problematic because the signals shift as function of solvent, pH, and sample matrix (Edzes et al. 1991, Estrada et al. 2008), preventing unambiguous identification in crowded regions.

Two-dimensional $^{31}$P,$^1$H-COSY NMR spectroscopy

To overcome the problems of one-dimensional $^{31}$P NMR with regard to resolution and signal identification, the semiconstant-time $^{31}$P,$^1$H-COSY experiment was developed by Petzold et al. 2009. Two-dimensional NMR spectra contain signals – so called crosspeaks – in a two-dimensional plane, which are usually displayed as contours of equal signal amplitude exactly as contours in a topographic map. In the 2D spectra (Fig. 9), the $^{31}$P chemical shift of the cross peaks is displayed on the vertical axis and the $^1$H shift on the horizontal axis. The crosspeaks show characteristic fine structures caused by the J-couplings, $^{3}J_{HP}$ and $^{3}J_{HH}$ as so-called anti-phase and in-phase signals, respectively, i.e. splitting with multiplet components of opposite or equal signs. These multiplet structures can be analyzed by extracting $^1$H traces from the 2D spectra (Fig. 10). PL signals overlapping in $^{31}$P can be resolved in the $^1$H dimension, which greatly increases resolution compared to 1D spectra. The identification of the PL signals rests on three parameters: the $^1$H shift, the $^{31}$P shift and the J-couplings. This is a big advantage because $^1$H shifts are not as susceptible to the solvent composition as the $^{31}$P shifts and all three parameters depend on the chemical environment of the phosphate group; combined they reveal much structural information about the PL headgroup and backbone. This structural information allows the unambiguous assignment of signals without the need for reference samples and it gives hints about the structure of rare or unexpected PLs that might be present in a sample.
Methods

Figure 9. Identification of PLs by 2D NMR. A. 2D $^{31}$P,$^1$H-COSY NMR spectrum of a lipid extract. The $^1$H chemical shift of the signals is shown on the horizontal axis and the $^{31}$P chemical shift on the vertical axis. B. Common structural part of phosphatidylcholine (PC), dimethylphosphatidylethanolamine (DiMePE) and phosphatidylethanolamine (PE), which only differ in the number of methyl groups on N in the headgroup. The modification site in the headgroup moiety is indicated by the X. Marked in the spectrum are peaks arising from these three PLs. The $^1$H4/5 signals of the glycerol backbone (green circles) show similar $^1$H shifts for all the three PLs, as expected, while the $^1$H6/7 signal of the headgroups (blue circles) is successively shifted high field with increasing number of methyl groups. This example shows how structural information is reflected in the spectrum.

Figure 10. $^1$H trace from a 2D $^{31}$P,$^1$H-COSY NMR spectrum showing $^1$H multiplets for PC (black) and lysophosphatidylcholine (LPC, red). Both PLs have similar multiplet structures due $^{31}$P,$^1$H and $^1$H,$^1$H couplings involving the headgroup protons $^1$H6/7, while the multiplet structures of the signal from the glycerol backbone ($^1$H4/5) allow a clear differentiation between the PC and LPC. The LPC coupling pattern of $^1$H4/5 signal is characteristic for lyso PLs and can serve to identify them.
Lysophospholipids for example can easily be identified because of their characteristic coupling patterns caused by the hydroxyl group in the \( sn-2 \) position of the glycerol backbone where a fatty acid is missing compared to the parent PL. To quantify the PLs in 2D spectra the crosspeaks can be integrated. However, the peak intensities of different PLs do not strictly correspond to the molar amounts and if a precision higher than ±10% is required transfer factors are needed to account for this.

For PL analysis in food samples, we enriched the PLs in the lipid samples by liquid-liquid extraction using an \( n \) -hexane/methanol solvent system and then we identified and quantified the PLs using one- and two-dimensional \( ^{31}P \) experiments. Experimental details concerning the spectrometers and configurations used, the acquisition parameters, and data processing are described in publication I.

**Stable isotope analysis**

Stable isotope compositions can be measured by isotope-ratio mass spectrometry (IRMS), absorption spectroscopy or NMR spectroscopy. The measurement of isotope abundances at natural abundance requires high measurement precision, because small abundance deviations have to be detected. But experiments exploiting variation of the natural abundance of stable isotopes have the advantage that no labelling is necessary. This makes experiments easier as no specially labelled compounds have to be synthesized, and most importantly it allows studies of natural compounds and in unperturbed systems. Different approaches are applied in isotope studies, measuring (1) isotope ratios of bulk material, (2) compound specific isotope ratios, (3) isotopomer abundances or (4) several isotopes simultaneously in multi-element approaches to determine changes in their relative abundance.

Currently, IRMS is the most commonly used method to measure isotope ratios. In IRMS, isotopes are separated based on their differing masses. For the analysis, samples have to be converted into a gas, \( H_2 \), \( CO_2 \), and \( N_2 \) for H, C, and N isotopes, respectively. The measured gases are usually generated by combustion or pyrolysis and the mass spectrometers are specially designed to measure these gases, reaching very high precision by comparison with reference materials for isotope ratios. Bulk material is used to determine isotope ratios of whole matrices and compound specific isotope analysis (CSIA) is performed after chromatographic separation of bulk samples, which is possible on-line using GC-IRMS and LC-IRMS systems. Because IRMS quantitatively converts the analytes into a measure gas, it yields whole-molecule \( \delta \) values, while intramolecular isotope abundances can only be determined if the analyte is fragmented prior to analysis, chemically or on-line by pyrolysis followed by GC separation of the fragments. Both approaches are only applicable to select compounds because isotope fractionation and exchange during degradation can occur, and chemical degradation is very labor intensive (Rossmann et al. 1991). New techniques aim to determine intramolecular isotope ratios of small
molecules like propane via measurement of fragment ions containing one or two carbons (Piasecki et al. 2013) but, generally, the strength of IRMS is to measure whole-molecule isotope ratios with high precision while isotopomer variation cannot be detected in routine applications.

Recently, absorption spectroscopy methods, using near and mid-infrared lasers, have been introduced to measure isotope abundances based on the specific light absorption lines of molecules differing in isotopic composition. These measurements are fast, non-destructive, and allow the detection of several isotopologues. At the same time, laser based systems are generally less expensive than IRMS systems, and are portable allowing measurements in the field, but they are also less precise than IRMS. Field measurements are of special interest for the greenhouse gases CO₂ and N₂O, but also in gas analysis of breath and traffic emissions (Sigrist et al. 2008). Compact devices for example using tunable diode laser absorption spectroscopy (TDLAS) have been developed to measure isotope ratios in real time in the field with precisions better than 1‰ (Bowling et al. 2003).

D isotopomer quantification by D NMR

By NMR spectroscopy individual isotopomer abundances can be measured, if the studied isotope is NMR active, as it is the case for D, ¹³C and ¹⁵N, while ¹⁸O is inactive. Without the need of compound fragmentation all isotopomers can be measured in parallel because each isotopomer creates an individual peak in the spectra at a specific chemical shift. The chemical shifts depend on the chemical environment of the isotope-substituted position and it is a prerequisite for quantification of the individual isotopomers that their signals are resolved. Under suitable experimental conditions the integrals of the peaks in the NMR spectrum reflect relative isotopomer abundances (Remaud et al. 1997, Betson et al. 2006, Lesot & Courtieau 2009). NMR only allows the determination of relative isotopomer abundances, but if an external reference standard with known isotope abundance is added, intramolecular δ values can be calculated. The studied compounds have to be pure to avoid overlapping signals from impurities, they have to be soluble, and their signals have to be resolved. For analytes with poor resolution, derivatives with resolved lines have to be synthesized.

In quantitative D NMR experiments, D isotopomer abundances for each CH group of an organic molecule can be determined (Martin & Martin 1981), even the two isotopomers of a prochiral methylene group can be distinguished if their NMR signals are resolved, while hydroxyl hydrogens cannot be studied due to their high exchange rate with water. First, the signals are assigned in ¹H experiments (Fig. 11B), which is possible because ¹H and D signals of a compound have identical chemical shifts. Then D experiments (Fig. 11C) are performed using a 90° excitation pulse, proton decoupling during acquisition and a ¹⁹F lock device for field-frequency locking.
**Fig. 11.** Determination of D isotopomer distribution of glucose by NMR. 

A. Structure of the glucose derivative 3,6-anhydro-1,2-O-isopropylidene-α-D-glucofuranose that is used for NMR experiments. 

B. $^1$H NMR spectrum used for signal assignment. The seven signals correspond to the seven CH groups of glucose. 

C. D NMR spectrum showing one peak for each of the seven glucose D isotopomers. 

D. Quantification of the signals using a Lorentzian lineshape fit. The numbers represent the relative abundances of the isotopomers compared to the average of 1.

To achieve best sensitivity and resolution of signals, a low-viscosity solvent like acetonitrile or C$_6$F$_6$ and elevated measurement temperatures, are used to minimize line widths. For each NMR spectrum a number of individual scans are acquired, which are added up to achieve a high enough signal-to-noise ratio. The time between scans (recycle time) is chosen to allow for complete decay of the previous excitation of the deuterium nuclei. The duration of this delay is governed by the relaxation times $T_1$, which are properties of the individual intramolecular groups of a compound under the respective measurement conditions. These relaxation times are determined in inversion-recovery experiments, then a recycle time of 7 $T_1$ of the
slowest-relaxing intramolecular group is used to ensure a relaxation of 99.9 % for all nuclei. For precise quantification, the integrals of the signals are determined by deconvolution of the spectra using a Lorentzian lineshape fit (Fig. 11D). This requires signals with pure Lorentzian lineshape, which can be realized by homogenizing the magnetic field of the spectrometer (“shimming”).

The NMR sensitivity of the D nucleus is much lower compared to the \(^1\)H nucleus, and combined with its low natural abundance the sensitivity of D spectra is relatively low, requiring large sample amounts compared to IRMS. The use of high magnetic field spectrometers (18.8 Tesla magnetic field) and of cryogenically cooled probes specially designed for deuterium detection reduces the sample amounts needed for isotopomer analysis down to 15 µmol, depending on desired precision. Typically 5 spectra of each sample are recorded to determine measurement precision and with sample amounts of 0.1 to 0.8 mmol for the glucose derivate analyzed signal-to-noise ratios of approximately 200 could be achieved using 12000 or 1500 scans, resulting in a precision of 5 ‰ or below.

**Determining site-specific δ values:** To determine site-specific δ values by NMR, a reference compound with known δ value has to be added to the NMR sample. A typically used reference is tetramethylurea (TMU), which can be obtained as certified standard, and is well suitable because of its 12 equivalent hydrogens, which give rise to one strong NMR signal. First, the exact amount of used standard relative to the analyte is determined in a \(^1\)H experiment, and then a direct comparison of the integrals of the analyte’s D signals to the integral of the standard yields the δ values of the isotopomers, if the correct stoichiometry is calculated in.

**Preparation of plant glucose samples for \(^2\)H NMR analysis:** To study the D isotopomer abundances of photosynthetic glucose, a derivative has to be synthesized because the signals of glucose overlap in the NMR spectrum. The glucose derivative 3,6-anhydro-1,2-O-isopropylidene-\(\alpha\)-D-glucofuranose (Fig. 11A) is most suitable, because it has low molecular weight, high solubility in low-viscosity solvents, can be synthesized without isotope fractionation, and shows resolved lines for all 7 isotopomers, including both isotopomers of the methylene group. The derivative synthesis is described by Betson *et al.* and is outlined in figure 12.

If plant material is analyzed, the material is first dried and ground in a mortar or ball mill. Soluble sugars, starch and structural carbohydrates can be extracted separately. Soluble sugars are extracted with 80 % ethanol, then the residual material is treated with amyloglucosidase in citrate buffer to enzymatically break down starch, and finally the remaining plant material is hydrolyzed by boiling in 4 % sulfuric acid solution. The obtained solutions contain monomeric glucose, which is dried and transformed to 1,2:5,6-di-O-isopropylidene-\(\alpha\)-D-glucofuranose (derivative 1) by reaction with acetone under sulfuric acid catalysis. This derivative is not isolated but directly hydrolyzed in aqueous solution to yield 1,2-O-isopropylidene-\(\alpha\)-D-glucofuranose (derivative 2), which can be used for \(^13\)C isotopomer analysis. However, for D NMR it is transformed in a condensation reaction to 3,6-anhydro-
1,2-\textit{O}-isopropylidene-\textalpha-D-glucofuranose (derivative 3). To achieve the necessary purity, the final product is extracted with chloroform and purified by flash column chromatography. For NMR analysis, up to 150 mg of derivative 3 are dissolved in acetonitrile and C$_6$F$_6$ (80 µL) to a final filling height of approximately 30 mm in a 5 mm NMR tube with teflon valve.

\textbf{Figure 12.} Breakdown of plant carbohydrates to 3,6-anhydro-1,2-\textit{O}-isopropylidene-\textalpha-D-glucofuranose (derivative 3), a glucose derivative that is suitable for deuterium NMR.
Summary of Publications

Publication I

Two-Dimensional $^{31}\text{P},^{1}\text{H}$ NMR Spectroscopic Profiling of Phospholipids in Cheese and Fish

**Aim:** Phospholipids (PLs) are bioactive molecules with diverse functions in biological systems and interesting properties for food and pharmaceutical applications. While phospholipids are major components in membranes, they constitute only a minor fraction in food lipids. To analyze the small but complex PL fraction in food samples a suitable method to enrich, identify, and quantify the PLs is necessary. Here we introduce two-dimensional $^{31}\text{P},^{1}\text{H}$-COSY NMR spectroscopy as an efficient tool for PL profiling in food samples, and develop a liquid-liquid extraction (LLE) method to enrich PLs prior to analysis.

**Methods and Results:** Using a solvent system consisting of $n$-hexane / methanol (2:1, v:v) with 3% water, we developed a LLE method that enriched the PL content in the sample by extracting neutral lipids with the nonpolar solvent fraction. The extraction effectively increased the PL content in the sample by 4-6 fold, while only showing a small discrimination against PLs with low polarity. This enrichment proved to be essential for NMR analysis of cheese samples, as cheese only contains approximately 1% PLs. For foods with higher PL fraction in total lipids like fish (approximately 10%) and egg, the PL content is high enough to allow direct PL analysis by NMR without prior enrichment.

NMR analysis was performed on cheese, fish, and an egg sample. $^{31}\text{P}$ NMR resolved numerous P species for all samples but identification of peaks based on their $^{31}\text{P}$ NMR shift alone was impossible. Individual PLs of the complex mixtures had varying $^{31}\text{P}$ shifts in the spectra likely due to solvent and matrix effects, as the samples still contained large amounts of neutral lipids. In addition, the fatty acids of a PL affect its $^{31}\text{P}$ shift, which can result in multiple or broadened peaks for the PL.

Two-dimensional semiconstant-time $^{31}\text{P},^{1}\text{H}$ COSY NMR experiments allowed unambiguous identification of known PLs based on $^{31}\text{P}$ shift, $^{1}\text{H}$ shift, and signal fine structure. The $^{1}\text{H}$ shifts were almost independent of matrix and solvent effects, and the characteristic signal fine structure showed $J_{\text{HH}}$ and $J_{\text{HP}}$ couplings. Taken together, these three parameters show a clear fingerprint for each detected PL, revealing structural information about headgroup and backbone. This was particularly important for PLs with overlapping $^{31}\text{P}$ shifts, as well as for rare and
unexpected PLs, which based on $^{31}\text{P}$ shifts alone would have been falsely assigned, while the 2D spectrum yielded crucial information about the PL’s headgroup and about backbone modifications. In this way we identified an alkyl-ether-linked PE and could clearly differentiate its signal from the common PE, even though their $^{31}\text{P}$ shifts were very similar.

NMR analysis allowed quantification of individual PLs in the lipid mixtures by integration of peaks. We observed characteristic PL profiles for samples originating from either cheese, fish or egg. In total we observed 14 different PLs with PC being the largest component in all samples; in the egg sample it constituted 75% of all PLs. The cheese PL profile showed a distinctive sphingomyelin contribution of over 20%, which is characteristic for dairy PLs. The most remarkable observation in the fish profiles was the high lysoPC content that may be due to postmortem lipolysis of PC during storage of the samples.

**Conclusion:** The two-dimensional $^{31}\text{P},^{1}\text{H}$ NMR experiment is an efficient method to analyze complex PL mixtures from food origins and can be used to profile the PL contents of food classes or in individual food samples. The PL content yields information about nutritional value of food products and changes in PL profiles may be used to detect food adulterations. For fish, the PL profiles can serve as an indicator of freshness as the PLs are affected by enzyme-catalyzed post-mortem lipolysis that depends on storage conditions.
Elucidating turnover pathways of bioactive small molecules by isotopomer analysis: The Persistent Organic Pollutant DDT

**Aim:** Tracking sources and turnover of pollutants is essential to understand their dynamics in the environment. Stable isotope analysis is increasingly used for this purpose but usually only average whole-molecule isotope abundances are considered. Here, we test isotopomer analysis as an approach to understand sources and turnover mechanisms of small molecules. Isotopomer abundances are affected by kinetic isotope effects in chemical reactions and therefore changes in isotopomer abundances are tracers of these processes. We analyze the persistent organic pollutant DDT (1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane) and its congener DDD to find characteristic fractionations that occur in alternative DDD formation pathways and explain these fractionations mechanistically.

**Methods and Results:** We analyzed DDT and DDD by quantitative deuterium NMR spectroscopy. The closely related compounds differ in only one intramolecular moiety; DDT carries a CCl₃ group while DDD carries a CHCl₂ group. In spite of this small structural difference, reference compounds of the para isomers p,p-DDT and p,p-DDD differed by over 70‰ in δD, suggesting different origins for both compounds. Analyzing the intramolecular deuterium distributions of these reference compounds we found that the molecular fragments common to both compounds have identical deuterium abundances. In contrast, the CHCl₂ group only present in DDD is strongly deuterium enriched, by approximately 700‰ relative to the rest of the molecule, i.e. the CDCl₂ isotopomer has very high abundance. The enrichment in this one position shifts the δD of the whole molecule, which contains 10 hydrogens, by 70‰ and, thus, fully it explains the δD difference between DDT and DDD. This suggests a common aromatic precursor for DDT and DDD and a deuterium fractionation during the formation of the CHCl₂ group.

Technical DDT contains approximately 4% DDD as by-product and analysis of this by-product without separation revealed that the CDCl₂ isotopomer was twice as abundant as the CD(chlorophenyl)₂ isotopomer. In the technical process DDT is synthesized from trichloroacetaldehyde, which is formed by progressive chlorination of acetaldehyde. During this chlorination a kinetic H/D isotope effect occurs; starting material and partly chlorinated intermediates that only carry ¹H react faster than deuterium-carrying isotopologues. As a result, residual dichloroacetaldehyde is D-enriched in the CHCl₂ group, and this D-enrichment is transmitted to the CHCl₂ group of DDD during DDT synthesis.
DDD is also formed by reductive degradation of DDT in the environment. By hydrodechlorination, a chlorine atom of the CCl₃ moiety is replaced with a hydride equivalent. To study this process, we degraded DDT in laboratory experiments with elemental iron. The newly formed DDD showed a deuterium depletion of 300 ‰ for the CDCl₂ isotopomer relative to the CD(chlorophenyl)₂ isotopomer. We explain this depletion as a result of an equilibrium isotope effect between H₂O and HDO, namely that HDO is a weaker acid than H₂O. This leads to D-depletion of hydride equivalents which are used to form the CD(chlorophenyl)₂ isotopomer of DDD.

In the environment, DDT degradation occurs in biotic processes through microorganisms or plants. In these processes hydride equivalents are transferred from cofactors like FADH₂, and their hydride equivalents are D depleted for the same biophysical reason, as evidenced by approximately 300 ‰ D depletions of FADH₂-derived C-H groups of natural products. Therefore, the Fe(0) reduction experiment mimics DDT degradation in the environment, and DDD formed by degradation of DDT in the environment can be expected to be strongly D-depleted in the CHCl₂ group.

Formation pathways of DDD – technically by chlorination or during degradation by hydrodechlorination – result in opposite fractionations and produce DDD that differs by 1000 ‰ in the abundance of the CDCl₂ isotopomer. This large abundance difference may be used to differentiate DDD sources in environmental samples. Even though the sample amounts needed for NMR analysis are difficult to obtain from environmental samples, the knowledge of the fractionations in both DDD formation pathways may be used to interpret δD values obtained by IRMS analysis to quantify DDT degradation progress.

**Conclusion:** Variation of intramolecular isotope abundances alters whole-molecule δ values considerably, therefore intramolecular isotope measurements are essential to link observed fractionations to individual reactions. Such measurements have been used in a few applications in the field of food adulteration and here we show their power to not only trace origins but also mechanistically understand transformation reactions of organic molecules. In the field of environmental pollution, isotopomer analysis of POPs may be applied to study turnover processes in the environment, to track original polluters, and to monitor remediation approaches.
Aim: N-nitrosodimethylamine (NDMA) is a toxic pollutant commonly found in drinking water. It can be formed during water disinfection and is mutagenic and carcinogenic even at very low concentrations. NDMA formation pathways are poorly understood but such knowledge is important to prevent NDMA formation during water treatment. Here, we develop a method to study NDMA formation by compound-specific isotope analysis (CSIA) and intramolecular isotope analysis.

Methods and Results: Using several N-nitrosamine reference compounds, a solid phase extraction (SPE) method was developed to extract N-nitrosamines from low concentration aqueous solutions with good yields and without isotope fractionation. Further, a GC-IRMS method suitable for N-nitrosamines was established to yield compound-specific $^2$H, $^{13}$C and $^{15}$N ratios with high precision. In a model reaction, ranitidine, a pharmaceutical commonly found in water, was subjected to chloramine in aqueous solution to study the formation of NDMA. In the process several intermediates were observed and finally NDMA was the product with quantitative yields. The $\delta^2$H and $\delta^{13}$C signatures of the remaining ranitidine were unchanged throughout the reaction, while $\delta^{15}$N increased with progressive turnover. Applying NMR spectroscopy we studied the $^2$H abundance in the N(CH$_3$)$_2$ moiety of ranitidine by comparison to an external standard. $\delta^2$H of the N(CH$_3$)$_2$ group differed from the $\delta^2$H of the rest of the molecule but was identical to the $\delta^2$H of NDMA formed from ranitidine. The matching $\delta^2$H signatures of NDMA and the N(CH$_3$)$_2$ moiety of ranitidine show that this group in NDMA originates directly from its precursor and is not involved in the reactions, while the changing $\delta^{15}$N signature results from a kinetic isotope effect during N bond breakage during the formation of the nitroso group.

Conclusion: This is the first time that NDMA formation has been studied by CSIA. In addition, the study demonstrates the unique strength of intramolecular isotope analysis to directly link original contaminants to their degradation products. The developed methods can be applied to further investigate NDMA formation mechanisms from a variety of N-containing precursor molecules, which may allow the development of strategies to prevent NDMA formation during water disinfection.
Manuscript IV

Quantification of a metabolic shift towards photosynthesis in C₃ plants driven by 20th-century CO₂ rise

Aim: The atmospheric CO₂ concentration is currently rising at an unprecedented rate and the vegetation’s ability to fix CO₂ from the atmosphere is decisive for the vegetation’s role as CO₂ sink in the global carbon cycle and for crop productivity in the future. However, plant responses on decadal to centennial time scales, which are most relevant for societal adaptation, are poorly understood because methods to study these on a metabolic level are not available, as manipulation experiments are intrinsically limited in the time span they can cover. Here, we first develop an isotopomer-based method to extract metabolic information from plant material. We then apply this method to historic material of C₃ plants to study long-term metabolic shifts.

Results and Discussion: Plants of several C₃ species were grown in greenhouses in low to very high (180-1500 ppm) [CO₂] and the deuterium isotopomer distribution in photosynthetic glucose of the plants was analyzed by deuterium NMR. We detected that the D₆⁸/D₆⁵ isotopomer ratio – the abundance ratio of the two deuterium isotopomers on C-6 of glucose – shows a linear dependence on the inverse of the growth [CO₂]. This inverse dependence reflects the competition of O₂ and CO₂ as Rubisco substrates, and the isotopomer ratio therefore reflects the flux ratio of oxygenation to carboxylation at Rubisco. The oxygenation is a wasteful side reaction of photosynthesis and is the starting point of the photorespiration pathway. We detected the same response for all C₃ plants analyzed and confirmed our interpretation by control experiments.

We then used this isotopomer method to reconstruct how the oxygenation / carboxylation ratio in plants has been affected by increasing atmospheric [CO₂] over the past century. In historic beet sugar samples that grew between ~1900 and 2012, the D₆⁸/D₆⁵ response to increasing atmospheric [CO₂] was indistinguishable from the response observed in experimental plants for an equivalent [CO₂] increase, showing that rising atmospheric [CO₂] has suppressed the oxygenation reaction. The oxygenation / carboxylation ratio increased by 35% due to the historic [CO₂] rise, seemingly unaffected by changes in cultivars, agricultural practices or by plant breeding. We further observed that herbarium samples of spinach, fireweed and a peat moss species dating back 60 to 120 years each showed a highly significant shift in the oxygenation / carboxylation ratio relative to modern samples, indicating that the flux shift is generally occurring in C₃ plants.
Conclusion: This study demonstrates that isotopomer abundances and especially isotopomer ratios, which are independent of source isotope ratios, can be used to extract metabolic information from historic plant material and to reconstruct long-term metabolic changes. Rising atmospheric [CO₂] has suppressed photorespiration in C₃ crops and in natural vegetation. The manuscript describes the first method to detect shifts in carbon metabolism over centuries, and presents the first observational data showing and quantifying the CO₂-driven suppression of photorespiration over the past century.
Manuscript V

**Limited suppression of photorespiration in trees worldwide by increasing atmospheric [CO2]**

**Aim:** Biosphere – atmosphere feedbacks are decisive for future climate change. Currently, forests account for 50% of the terrestrial net primary production, making a significant contribution to the terrestrial carbon sink, and therefore even small changes in forest carbon assimilation have large effects on the global carbon cycle and need to be considered in climate models. In Free-Air CO₂ Enrichment (FACE) experiments, transient responses are often observed, highlighting the need, especially for trees as long-lived species, to study CO₂ responses on long timescales. Here, we study metabolic changes of trees from all continents to rising atmospheric [CO₂] over the past two centuries.

**Results and Discussion:** To test whether the D6\(^8\)/D6\(^5\) isotopomer ratio of tree ring cellulose reflects the oxygenation / carboxylation flux ratio at Rubisco, as observed for annual plants in manuscript IV, we first analyzed samples from the Oak Ridge FACE experiment on sweetgum trees. In leaf starch the D6\(^8\)/D6\(^5\) dependence on [CO₂] was indistinguishable from the response previously observed in annual plants and the signal was transferred from the leaf level to the tree ring cellulose. We then analyzed 12 different tree species from globally distributed sites covering the past 60-700 years. In all tree samples - conifers and broad-leaved trees, temperate and tropical species - the D6\(^8\)/D6\(^5\) ratio decreased in response to the atmospheric [CO₂] increase, showing that photorespiration was suppressed in trees globally. A quantitative comparison of the D6\(^8\)/D6\(^5\) shifts observed in the trees to the average shift observed in CO₂ manipulation experiments revealed that the tree response was on average 50% smaller than expected for the historic [CO₂] increase, indicating that the reduction of the oxygenation/carboxylation ratio was smaller than expected. The D6\(^8\)/D6\(^5\) ratio is an integrated measure of the oxygenation / carboxylation flux ratio at Rubisco and should therefore be sensitive to all factors influencing the flux ratio, in particular the CO₂ concentration at Rubisco (Cₜ) and temperature. To test this, we analyzed samples from a factorial temperature / [CO₂] manipulation experiment on soybean. The results showed that the D6\(^8\)/D6\(^5\) ratio is sensitive to [CO₂] and temperature, and hence it integrates the influence of Cₜ and leaf temperature on the oxygenation / carboxylation ratio. Therefore the smaller reduction of the isotopomer ratio in trees can be explained as combined effect of rising Cₜ and rising leaf temperatures.
Conclusion: Rising atmospheric [CO₂] has suppressed photorespiration in trees globally over the past 150 years but the suppression is smaller than expected, indicating that the “CO₂ fertilization” effect has been diminished by opposing effects, likely by the increase in global temperature, with a possible contribution from leaf warming due to stomatal closure. This could explain why increases in tree biomass production are generally not observed and needs to be considered in predictions of future “CO₂ fertilization”.
Conclusions and Perspectives

The presented projects highlight the power of NMR to quantitatively analyze complex mixtures and to determine isotopomer distributions. In publication I, we use a simple analytical technique, $^{31}$P NMR, for phospholipid analysis but largely increase the resolution by performing two-dimensional experiments. This has the conceptual advantage that PL identifications is not only based on $^{31}$P shifts, which are known to shift depending on matrix effects, but that $^{31}$P shifts are used to suggest identities, which are tested by $^1$H shifts and coupling patterns. As a result, we achieve a more reliable PL identification and higher resolution, and can even identify unknown compounds. The method may be used in food analysis in general and in other fields where PL analysis is of interest.

By using isotopomer analysis, compounds can be linked to their precursors and mechanistic information can be provided to obtain insight into transformation pathways. We have illustrated this for the pollutants DDD and NDMA (publications II and III), demonstrating the great potential of isotopomer analysis in studying the turnover of environmental pollutants.

Studying isotopes in metabolites or xenobiotics at natural abundance has the decisive advantage that isotopes are present everywhere, and get incorporated into all systems. Thus, natural abundance studies can overcome fundamental limitations of manipulation experiments; namely the need to perturb the system of interest and to extrapolate observed results in time and space (Leuzinger et al. 2011). For pollutant turnover, this means that pollutant sources, environmental distribution and turnover can be studied in large contamination areas and over long distances.

The isotope composition of long-lived metabolites stores information about past states, therefore biological systems can be studied retrospectively over long time spans, e.g. in biogeochemistry. This enables us to extract metabolic information from historic plant material and thereby bridge the gap between plant physiological experiments and paleo studies. For the question how plants respond to climate change, this means that tree responses covering the time since industrialization and trees anywhere on Earth can be studied. In contrast, there are for example no FACE studies on mature tropical trees even though tropical forest are a major terrestrial carbon sink as they are estimated to account for up to 50 % of terrestrial net primary production (Körner 2009).

In manuscripts IV and V we present observational data on the effect of increasing atmospheric [CO$_2$] on plants since industrialization. We introduce the D$^6_{D}$/D$^6_{S}$ isotopomer ratio as a measure of the flux ratio of oxygenation to carboxylation in C$_3$ plants. Therefore trends in the isotopomer ratio in archives of plant materials reflect long-term changes in the oxygenation / carboxylation ratio. While these changes on the enzyme level do not allow direct conclusions about absolute metabolic rates or biomass production, they are the basis for all responses on higher levels. In a
sphagnum moss species and annual plants including crops, the oxygenation / carboxylation ratio and hence photorespiration was reduced during the 20th century to the degree expected from CO2 manipulation experiments. This allows quantifying a major contribution to the “CO2 fertilization effect”, and shows that these species did not show strong acclimation to increasing [CO2]. Strikingly, the suppression of photorespiration by the historic [CO2] increase is attenuated in trees, and we provide a mechanistic explanation for this observation, namely that the attenuation may be caused by increasing leaf temperatures, via global warming and additionally through reduced transpiration. This lower-than-expected suppression of photorespiration partly explains the recognized discrepancy between expected “CO2 fertilization” and biomass production. With lower sensitivity of plants to further [CO2] increases, and an increasing temperature rise expected for the next decades, the counteracting effects of CO2 and temperature have large implications for the role of the terrestrial vegetation as CO2 sink in the global carbon cycle, for future climate and crop productivity.

It is increasingly being recognized that isotopomer studies reveal information not accessible by stable isotope ratios of whole molecules, but to date applications are still limited. At this time NMR is the only practicable method for measuring isotopomer distributions for metabolite-size compounds. The biggest limitation in the application of isotopomer analysis is the large sample amount of at least 0.1 mmol needed for NMR. But with the development of specialized cryo probes and of magnets with higher fields, the required amounts have decreased significantly in recent years and will likely continue to do so. Furthermore, the preparation of samples can be labor-intensive as compounds have to be highly purified and sometimes derivatives have to be synthesized - such as for glucose, but the unique information that can be gained makes it worth the work!

While analysis of the D6°/D6 operatives ratio nicely illustrates how metabolic information can be obtained, the information content obtained from isotopomer analysis can be multiplied by analysis of the whole isotopomer pattern of glucose. Such an analysis may allow reconstruction of several linked metabolic changes, or of links between environmental drivers and metabolic changes. Furthermore, many metabolites besides glucose may be studied by isotopomer analysis and because CH groups are stable for up to 10^6 years, the accessible time scale may be extended at least to glacial cycles.

In this thesis we focused on applications of D isotopomers. In recent years 13C isotopomer analysis by NMR has become available (Chaintreau et al. 2013) and the application of 13C isotopomer analysis to pollutants will increase the understanding of pollutant turnover. Applied to plant metabolism, 13C isotopomers will be especially valuable because they may allow to directly couple plant processes to the δ13C of CO2 fluxes and hence to the carbon cycle. Because the concepts underlying interpretations of isopotomers are general, isotopomer analysis may also be applied in several other research fields.
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References


References


